Intranasal administration of alpha-synuclein aggregates: a Parkinson's disease model with behavioral and neurochemical correlates

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Short title: α-Synuclein species, behaviour and neurochemistry

Keywords**:** rodent model**,** α-synuclein oligomers, fibrils, behavior, dopamine neurochemistry

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder in which both alpha-synuclein (α-syn) and dopamine (DA) have a critical role. Our previous studies instigated a novel PD model based on nasal inoculation with α-syn aggregates which expressed parkinsonian-like behavioral and immunological features. The current study in mice substantiated the robustness of the amyloid nasal vector model by examining behavioral consequences with respect to DA-ergic neurochemical corollaries. *In vitro* generated α-syn oligomers and fibrils were characterized using atomic force microscopy and the thioflavin T binding assay. These toxic oligomers or fibrils administered alone (0.48 mg/kg) or their 50:50 combination (total dose of 0.48 mg/kg) were given intranasally for 14 days and "open-field" behavior was tested on days 0, 15 and 28 of the protocol. Behavioral deficits at the end of the 14-day dosing regime and on day 28 (i.e. 14 days after treatment completion) induced rigidity, hypokinesia and immobility. This was accompanied by elevated nigral but not striatal DA, DOPAC and HVA concentrations in response to dual administration of α-syn oligomers plus fibrils but not the oligomers by themselves. α-Syn fibrils intensified not only the hypokinesia and immobility 14 days post treatment, but also reduced vertical rearing and enhanced DA levels in the substantia nigra. Only nigral DA turnover (DOPAC/DA but not HVA/DA ratio) was augmented in response to fibril treatment but there were no changes in the striatum. Compilation of these novel behavioral and neurochemical findings substantiate the validity of the α-syn nasal vector model for investigating parkinsonian-like symptoms.

1. Introduction

Parkinson's disease is a neurodegenerative disorder in which both alpha-synuclein (α-syn) and dopamine (DA) have a critical role. α-Syn itself is known to be natively unstructured but it is in equilibrium with subpopulations of more compact structures and it is these aggregates that are thought to be linked to amyloid formation [1]. In the presence of DA, α-syn yields a diverse range of SDS-resistant, non-amyloid oligomers whose precursor conformation has not been established. However, it is known that dopamine binding to α-syn is mediated by specific conformational states [2].

Current thinking suggests that an interaction between α-syn and DA leads to the selective death of neuronal cells and further accumulation of misfolded α-syn [3]. The exact mechanism by which this occurs is not fully defined though DA oxidation could play a key role in the pathogenesis of PD by causing oxidative stress, mitochondrial dysfunction and impairment of protein metabolism [4]

Oxidative stress has been shown to be one of the crucial mechanisms involved in neurotoxicity, providing a possible explanation as to why dopaminergic neurons are highly vulnerable to apoptosis [5]. In the cytoplasm, DA can undergo oxidation via its labile quinone ring in the presence of molecular oxygen producing dopamine quinones and other reactive oxygen species. These products can account for damage in cellular elements, for example in the mitochondria via association of α-syn with oxidized lipids [6]. In this context, α-syn causes a reduction in DA release in the mesencephalon [7] and it can disrupt DA homeostasis leading to neuronal degeneration [8]. The initial neurodegenerative process is also instigated by protein aggregation in Lewy neurites in the substantia nigra [9] together with a decreased density of striatal DA-ergic vesicles and synaptic contacts. The consequent reduction in DA release leads to extensive motor impairment [7] generally associated with PD pathology. Interestingly, DA may bind to α-syn in a 3:1 ratio forming higher molecular weight species which are different from amyloid fibrils and may be critical in triggering further oligomerisation [10] which is suggestive of a

co-facilitatory outcome between these two biological molecules. However, biphasic concentration-dependent protective and neurotoxic effects of DA have been reported on neuronal survival and α-syn oligomer accumulation may occur through preservation of autophagic-lysosomal function at low concentrations and augmentation of toxicity at higher levels [11].

In order to investigate possible protective mechanisms against DA toxicity, we have described a specific profile of autoantibodies (Abs) generated simultaneously to DA, natively folded α-syn and its amyloidogenic oligomeric plus fibrillar aggregates associated with various clinical stages of the disease [5,12,13]. Consequently, we have proposed a protective role for humoral immunity against not only α-syn amyloid species but also DA in early stage PD and specific immunological targeting of toxic α-syn oligomers is of therapeutic interest [5,14]. To substantiate our clinical observations, we next developed a PD animal model recently whereby the nasal vector was used for brain delivery of α-syn oligomers either alone or in combination with fibrils [15]. In the study, PD-like behavioural outcomes, as well as autoimmune responses to DA and α-syn, were determined as potential disease biomarkers. Since it was found that intranasally administered α-syn amyloidogenic species induced both motor deficits and incited humoral immune protection, the model was thought to be a realistic mimic of the disease [15]. Our current aim is to establish further the robustness of the α-syn species nasal vector model by examining its behavioural consequences with respect to any DA-ergic neurochemical corollary.

2. Materials and Methods

Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA) were obtained from Sigma, St. Louis, MO, USA.

2.1. Subjects

Adult male C57Bl/6 mice aged 12-months and weighing 31.1±1.0g were used throughout. The animals were group housed on a 12:12 light-dark cycle at a constant temperature of 21^{o}C and 50% humidity with access to food and water *ad libitum.* All experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996); the UK Animals Scientific Procedures Act 1986 and associated guidelines; the European Communities Council Directive of 24 November 1986 (86/609/EEC) for care and use of laboratory animals. They were also approved by the Animal Care and Use Committee of the P.K. Anokhin Institute of Normal Physiology, Russian Academy of Medical Science.

2.2. Procedures and dosing protocol

Experiments were performed between 10.00-15.00 hours and animals were divided into seven groups (n = 10 per group). Group (1; naïve control) was administered saline vehicle intranasally (i.n) bilaterally in a total volume of 8 µL/animal daily (i.e. 4 µL/nostril using a Hamilton syringe) over a total dosing period of 14-days. Group (2) was administered a solution of α-syn oligomeric aggregates (15.0 µg in 8 µL = 0.48 mg/kg) bilaterally using the same 14-day dosing schedule. Group (3) was co-administered aggregates of α-syn oligomers plus fibrils simultaneously each in a 50%:50% concentration of 7.5µg in 4 µL/animal bilaterally (i.e. total α-syn equivalent dose 15 µg = 0.48 mg/kg)

i.n. over the 14-day protocol. Group (4) received α-syn fibrillar aggregates (15.0 μg in 8 μL = 0.48 mg/kg bilaterally) for the 14-day schedule. At the end of the 14-day protocol (i.e. on day 15), animal groups 1-4, underwent behavioral testing. They were then killed, both nigral as well as striatal neurochemical analysis subsequently being performed. The 14-day treatment schedule was also carried out for animal groups (5; oligomeric aggregates), (6; oligomers plus fibrils 50%:50%) and (7; fibrillar aggregates) then 14-days post treatment (i.e. day 28), they were tested behaviorally then killed and nigral along with striatal neurochemical analysis was completed. All behavioral tests and neurochemical analyses were performed under blind conditions.

2.3. Behavioural tests

Animal behavioral analysis was performed in all groups before (day 0), one day after the end of the α-syn amyloidogenic species dosing protocol (i.e. day 15), or 14 days post-treatment (i.e. day 29) and a total of eight behavioral indicators of PD-mimetic symptoms (collectively assessing hypokinesia, muscle rigidity and tremor) were evaluated [15,16]. Firstly, hypokinesia was assessed by quantifying "open field" spontaneous locomotor activity based on previous methodologies but in response to MPTP treatment as a prototypic drug [17,18] for a period of 0-6 min after 5 min acclimatization in an animal activity meter (Opto-Variomex-3 Auto-Track system, Columbus Instruments, Columbus, Ohio, USA). The 0-6 min recording time was chosen since it represented an optimal period for detecting neurotoxin-induced locomotor hypokinesia in 3-minute intervals up to a total of 30 minutes in C57Bl/6 mice [15,19] Additionally, total locomotor distance, cumulative ambulation time as well as speed (horizontal activity measures) and vertical rearing (vertical activity measure) as well as immobility time were recorded. Secondly, muscle rigidity was gauged using a "gibbosity" test manifested by the shortening of the neck to the tailbase measurement and scored by the following scoring scale: (0) = no rigidity; (1) = 1.0cm decrease; (2) = 2.0cm decrease; (3) = >2.0cm decrease [15]. Thirdly, the presence

or absence of tremor was checked behaviorally [20] using the following scoring scale: (0) = no tremor; (1) = head tremor; (2) = head and forepaw tremor, (3) = whole body tremor.

2.4. Production of α-synuclein

Escherichia coli BL21 (DE3) cells transformed with pRK173 plasmid harboring the α-synuclein gene were used for the production of the recombinant protein [21]. The recombinant protein was purified as previously described [22] with some modifications outlined below. Plated cultures were used to inoculate Nutrient Broth medium (Oxoid Ltd, UK) containing ampicillin. Cultures were grown until the late log-phase ($A_{600\ nm}$, 0.8) at 30°C and protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were cultured at 30°C overnight, harvested by centrifugation (3000 g, 20 min), washed, re-suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA, 0.2 mM PMSF and disrupted by sonication. The cell homogenate was boiled for 10 min, the cell-free extract was loaded onto a HiPrep™ Q FF 16/10 Column (GE Healthcare) in 20 mM Tris-HCl, pH 7.5, and eluted by a linear 0-1 M NaCl gradient. Fractions containing α-synuclein were analyzed by a Coomassie stained SDS-PAGE and dialyzed against 20 mM Tris, pH 7.5. Collected fractions were loaded onto a HiTrap ANX FF (high sub) column and eluted by a linear 0-1 M NaCl gradient. Fractions containing α-synuclein were combined, dialyzed against 10 mM NH_4HCO_3 and lyophilized.

2.5. Production of α-synuclein amyloidogenic species

The α-syn concentration was determined by optical absorbance measurements at 280 nm (ND-1000 spectrophotometer, Nano-drop, Sweden), using an extinction coefficient $E_{1\ mg/ml}$= 0.354 [23]. In order to produce amyloid oligomers and fibrils of α-synuclein, protein was incubated at 0.21 mM and 0.71 mM concentrations in 10 mM sodium phosphate buffer, pH 7.4 and 37°C, using continuous agitation at 300 rpm during 7 and 14 days, respectively. The formation of oligomers and fibrils was verified as described earlier [15].

2.6. Spectroscopic amyloid assays

The thioflavin T (ThT) binding assay was performed using a modification of LeVine's method [24]. Thioflavin T fluorescence was measured by a Jasco FP-6500 spectrofluorometer (Jasco, Japan), using excitation at 440 nm and collecting emission between 450–550 nm, with excitation and emission slits set at 3 nm width. Congo red assay was performed using a ND-1000 spectrophotometer for optical absorbance measurements [23]. UV circular dichroism (CD) measurements were carried out using a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Jasco CDF-426L thermostat, employing 0.1- and 0.5-cm path length cuvettes. At least three scans were averaged for each spectrum.

2.7. Atomic force microscopy (AFM)

AFM measurements were performed on a PICO PLUS microscope (Agilent, USA) in a tapping mode as outlined previously [25]. A scanner with a 100 mm scan size and acoustically driven cantilevers carrying etched silicon probes of the TESP model of 10 nm diameter (Veeco, Netherlands) were used. Typically, we applied a resonance frequency in the 312–340 kHz range, scan rate of 1 Hz and a resolution of 5126512 pixels. Height, amplitude and phase data were collected simultaneously. Images were flattened and plane adjusted. The scanning of samples was performed in trace and retrace to avoid scan artifacts. The scanner was calibrated by measuring atomic steps on highly orientated pyrolytic graphite in the z-axis and using a standard 1-mm calibration grid (Agilent, USA) in the xy-plane. Amyloid samples were deposited on the surface of freshly cleaved mica (Goodfellow, UK) for 30 min, washed three times with 100 ml of MilliQ water, and dried at room temperature. To determine the dimensions of amyloid species cross-section analysis in the height images was carried out using PICO PLUS software (Agilent, USA).

2.8. Neurochemical determination of tissue content of dopamine (DA), and its metabolites (DOPAC, HVA and 3-MT) in mouse brain structures by high performance liquid chromatography with electrochemical detection (HPLC/ED)

Substantia nigra (SN) and striatum mouse brain structures (n=10 per group) were dissected on ice (+4^{0}C) then weighed and immediately stored in liquid nitrogen for subsequent analysis. Tissue samples were homogenized in 0.1 N perchloric acid (1:20) with 0.5 μM 3,4-dihydroxybenzoic acid as internal standard and centrifuged (10,000g x 10 min, 4^{0}C; Eppendorf 5415 R, Germany). The supernatant was analyzed by high performance liquid chromatography with electrochemical detection (HPLC/ED) [26]. DA and it's metabolites, DOPAC, 3-MT and HVA were detected using a glassy carbon electrode set at +0.85 V compared with an Ag/AgCl reference electrode using an electrochemical detector LC-4B (Bioanalytical Systems, West Lafayette, Indiana, USA). The mobile phase contained 0.1 M citrate-phosphate buffer (pH 2.9), 1.85 mM 1-octanesulfonic acid, 0.27 mM ethylenediaminetetra-acetate (EDTA) and 8% acetonitrile and pH was adjusted to 3.0 with 6M KOH. All reagents used for the mobile phase were of analytical grade (Sigma-Aldrich, USA). The mobile phase was filtered through a 0.22 μm nylon filter (Merck Millipore, Merck KGaA, Germany). DA and its metabolites were separated by an analytical reverse-phase column on reprosil C18, pore size 4 μm, 100 x 4 mm. (Dr. Maisch GMBH) at a flow rate of 1.0 ml/min. The experimental sample monoamine levels were quantified by external standard curve calibration using peak area for quantification. Sample analysis was performed using MULTICHROM 1.5 (Ampersand, Russia) software. Turnover of monoamines was expressed as the ratio of metabolite tissue concentrations (DOPAC, HVA and 3-MT) to the parent monoamine (DA). Samples from all the animals were processed in parallel on the same day for each brain structure. Data was calculated as nM /g wet brain tissue.

2.9. Statistical analysis

Data were expressed as means ± s.e.m. Statistical analysis was performed using the Statistica 6 package and homogeneity of variance was checked by Levene's test. Since dispersion was not homogeneous within groups, non-parametric criteria were applied using the Mann-Whitney U-test for two populations and the Kruskal-Wallis test for multiple comparisons. Statistical significance was assumed at $p < 0.05$, for all measurements.

3. Results

3.1. Characterization of α-synuclein oligomeric and fibrillar aggregates

Oligomeric species of α-synuclein were produced at pH 7.4 with agitation and characterized by the thioflavin-T binding assay then AFM analysis prior to intranasal administration, which was particularly important since amyloid species display an inherent diversity of structures dependent on solution conditions. The samples containing α-synuclein oligomers were collected at the end of the lag-phase (7 days), at which time a detectable fluorescence increase was observed**,** indicative of cross β-sheet formation. The oligomers and fibrils of α-syn also bind the amyloid specific dye - Congo red, which is reflected in a long-wavelength shift and increase of the dye absorbance spectra compared to the control measurement of the Congo red spectrum in the presence of monomeric α-syn. The oligomers of α-syn were characterized by a round-shaped morphology assessed by AFM imaging (Fig. 1A). The distribution of oligomeric particle heights measured in AFM cross-sections was performed. They were represented by a wide range of species with heights from 1.2 nm to 3.9 nm. Their maximal population was centered around species with ca. 1.8-2.0 nm heights, which corresponded to 20-mers as estimated

previously and the oligomeric nature of these species has been verified by interaction with generic A11 antibodies reactive towards amyloid oligomers [15].

Mature fibrils were developed after 14 days of incubation and were characterized by a 10 fold increase in thioflavin-T fluorescence intensity which displayed typical fibrillar morphology with up to a micron length (Fig. 1B). They were constituted by a few single stranded protofilaments intertwined around each other and resulting in structures of 8 to 10 nm height measured by AFM cross-section analysis. In order to exclude the presence of some spontaneously formed fibrils in the oligomeric fractions, the oligomers produced at the lower concentration of protein (0.21 mM) were taken for further behavioral and neurochemical experiments.

3.2. Behavioral analysis of PD-like activity on completion of 14-day intranasal dosing with α-synuclein aggregates

In pilot studies, the duration of sampling time following 5 min open field habituation for behavioural parameters was examined over three epochs, namely 0-3 min, 3-6 min and 0-6 min in all animal groups before dosing, after dosing (ie. day 15) and 14 days (i.e. day 29) after treatment completion. Statistical analysis showed no significant difference in behavioral parameter values (P>0.05) in any of the sampling times. Consequently, in order to capture the widest prospective behavioral profile, 0-6 minutes was selected for all behavioral study measurements.

Animal behavioral analysis at the end of 14-day treatment with intranasal saline vehicle (control) revealed no significant change (P>0.05) in open field horizontal activity as reflected by mean total locomotor distance, cumulative ambulation time, ambulation speed, immobility time or vertical rearing behavior. Neither was there any inherent evidence of muscle rigidity or tremor in any of these control vehicle-treated mice (Table1).

3.2.1. α-Synuclein oligomer treatment

Similar to the controls, there was no change (P>0.05) in any of the above parameters following 14-day i.n. treatment with α-syn oligomers (15µg/day i.e. 0.48 mg/kg) nor any difference from corresponding control group values (Table 1).

3.2.2. α-Synuclein oligomer and fibril combined treatment

Combined treatment with α-syn oligomers (7.5µg/day) plus fibrils (7.5µg/day) making a total equivalent dose of 15 µg/day (0.48 mg/kg) of α-syn species evoked significant reductions in mean total locomotor distance traveled (P<0.001). Thus, there was a -38.3% reduction in mean total locomotor distance traveled compared with the value observed before commencement of dosing in this treatment group (Table 1). When compared to 14-day vehicle control, this reduction was of a comparable value (-23.3%). However, treatment with the combination of oligomeric and fibrillar α-syn aggregates versus oligomers alone resulted in a decrease of mean locomotor distance of -27.7% (Table1). Moreover, there was also a significant increase (P<0.05) in immobility time versus either the predose value (+42.1%) or the control (+18.8%) (Table 1). Most notably, there was a presence of rigidity in animals which received the aggregate combination and 70% of the group displayed Straub tail while the remaining 30% additionally manifested an arched back.

3.2.3. α-Synuclein fibril treatment

In the α-syn fibril treatment group (Table 1), there were significant reductions compared with controls (P<0.05) in total locomotor distance (-22.5%), cumulative ambulation time (-25.3%) and vertical rearing (-29.8%) but an increase immobility time (+23.6 %, P<0.05).

In the case of ambulation speed, there were no significant differences between any of the mean group values for oligomer alone, oligomer plus fibril or fibril alone treatments throughout (see Table 1) and no expression of tremor.

3.3. Behavioral analysis of PD-like activity 14 days after treatment completion of intranasal dosing with α-synuclein aggregates

An intervening post treatment washout period of fourteen days without treatment was chosen for the second behavioral session since it matched the initial 14 days of the treatment protocol.

3.3.1. 14-days after treatment completion with α-Synuclein oligomers

Statistical analysis revealed significant differences (P<0.05) between the oligomer alone post treatment group and the vehicle post treatment controls with respect to decrements in total locomotor distance (-40.9%), cumulative ambulation time (-37.1%), and vertical rearing (-54.8%) but an increase in immobility time (+28.3%) (Table 2).

3.3.2. 14-days after treatment completion with α-Synuclein oligomers plus fibrils

Fourteen days after completion of combinative treatment with two α-syn aggregates affected animal open field behavior in comparison with the controls and those parameters which were tested before dosing. Thus, significant attenuations were observed versus control in total locomotor distance (-53.4%, P<0.001), cumulative ambulation time (-55.6%, P<0.05), vertical rearing (-62.1%, P<0.05) accompanied by an elevation of immobility time values (+60.3%, P<0.05) (Table 2).

Additionally, comparison of the 14-day post combination aggregate treatment completion group with their appropriate pre-dose groups disclosed an identical behavioral pattern of significant reductions in total locomotor distance (-62.5%, P<0.001), cumulative ambulation time (-62.8%, P<0.001), vertical

rearing (-65.1%, $P<0.001$) accompanied by an elevation of immobility time values (+91.6%, $P<0.001$) (Table 2).

3.3.3. 14-days after treatment completion with α-Synuclein fibrils

Fourteen days after completion of treatment with fibrillar α-syn aggregates there were significant differences from the controls and those parameters which were tested before dosing. Consequently, significant decremental changes were exposed with respect to their controls for cumulative ambulation time (-86.3%, $P<0.001$), vertical rearing (-89.2%, $P<0.001$) accompanied by an elevation of immobility time values (+107.8%, $P<0.05$) (Table 2).

Likewise, comparison of the 14-day post fibrillar aggregate treatment completion group with their appropriate pre-dose groups disclosed an analogous behavioral profile of significant reductions in total locomotor distance (-84.2%, $P<0.001$), cumulative ambulation time (-84.5%, $P<0.001$), vertical rearing (-82.2%, $P<0.001$) accompanied by an elevation of immobility time values (+87.5%, $P<0.001$) (Table 2).

Regarding ambulation speed, there were no significant differences between any of the mean group values for oligomer alone, oligomer plus fibril or fibril alone 14-day post treatments throughout (see Table 2) and no evidence of tremor.

3.4. Neurochemical assay of DA and metabolite concentrations in the SN and striatum in control and α-synuclein aggregate treated mice.

On completion of 14 days intranasal inoculation and also 14 days after treatment termination with different amyloidogenic species of α-syn, the levels of DA and its metabolites were measured in PD pathology relevant mice midbrain structures (SN and striatum). Control levels of DA, DOPAC, HVA

and 3-MT were 4.2±1.9, 1.8±0.9, 1.8±0.3 and 0.6±0.2 nM/g of wet tissue respectively in the SN and 75.5±11.0, 5.0±0.4, 7.0±1.2 and 1.3±0.4 nM/g of wet tissue respectively in the striatum.

The control DOPAC/DA and HVA/DA ratios throughout were 0.5±0.1 and 0.6±0.2 in the SN and 0.1±0.009 and 0.1±0.001 in the striatum.

3.4.1. DA, DOPAC, HVA and 3-MT concentrations in mouse SN after 14-day α-synuclein aggregate intranasal inoculation and 14 days after treatment cessation.

It was found that after dosing with α-syn oligomers, a reduction in nigral DA levels to 42% of control occurred. This was associated with a decrease in DOPAC, HVA and 3-MT levels (71, 61 and 43% of control respectively). An opposite effect was noted in mice following administration of the oligomeric/fibrillar α-syn combination whereby there was a significant increase ($P<0.05$) in DA content along with DOPAC and 3-MT levels but not HVA (Fig 2A). Similarly, there was an increase in DA and its metabolites disclosed in the SN after administration of fibrillar α-syn. DA, DOPAC, HVA and 3-MT levels were all elevated up to 145, 398, 256 and 231% of control respectively (Fig 2A).

Observation of DA and its metabolite content 14 days after cessation of treatment in the SN revealed an augmentation ($P<0.05$) of DOPAC, HVA and 3-MT concentrations (247, 227 and 150% of control) in the case of treatment with α-syn fibrils but not with oligomers or their combination with fibrils (Fig 2B).

3.4.2. DA, DOPAC, HVA and 3-MT concentrations in mouse striatum after 14-day α-synuclein aggregate intranasal inoculation and 14 days after treatment cessation.

Scrutiny of DA and its three metabolites (DOPAC, HVA and 3-MT) in the striatum revealed no striking % changes in comparison with controls following oligomer, fibril or combination treatments. A similar pattern was observed 14 days after completion of all treatments (Fig 3A, B.)

3.4.3. DA turnover rates relative to DOPAC and HVA in the SN and striatum in control and after 14-day α-synuclein aggregate intranasal inoculation and 14 days after treatment cessation.

In the SN, on completion of 14 days treatment, the DOPAC/DA ratio in groups administered oligomeric alone or the oligomeric/fibrillar combination were not statistically modified in contrast with that shown in the group which received α-syn fibrils where a 200% increase ($P<0.05$) in this ratio was detected. Moreover, the HVA/DA turnover ratio for all three α-syn species treatments did not vary relative to control (Fig 4A). It was noteworthy that 14 days after the period of dosing with α-syn aggregates both DOPAC/DA and HVA/DA turnover rates in SN returned to control ratios (Fig 4B).

In complete accordance with the findings in the SN, the striatal DOPAC/DA and HVA/DA turnover rates were comparable with control values either at the end of 14 days administration or two weeks after α-syn amyloidogenic intranasal intervention (Fig 5A, B).

4. Discussion

Animal models of PD have been widely used to investigate the pathogenesis of this neurodegenerative disorder which is typically associated with the specific and largely disordered protein α-syn as well as a DA system misbalance [27]. However, the relationship between α-syn aggregation and dopaminergic dysregulation has not been examined in detail in vivo. In this context, a relatively novel hypothesis on the pathogenesis of PD which hinges upon the premise that functional α-syn is critical to cell survival and that a reduction in biologically functional α-syn, whether through aggregation or reduced expression, may lead to neurodegeneration [28].

In a recent study, we developed a propitious animal model of PD based on the nasal vector for delivering α-syn aggregates to the brain which was accompanied by motor deficits and activation of humoral immunity not only against α-syn monomeric and amyloidogenic species but also dopamine

[15]. Data is accumulating that sporadic PD involves non-motor symptoms in its earlier stages including a loss of olfactory function and this phenomenon has even been shown in mice which overexpress human wild type α-syn [29]. Correspondingly, it has been proposed that neurotoxins may penetrate the brain firstly through the nasal route by means of anterograde trafficking into the temporal lobe and also via ingestion of nasal secretions in saliva ultimately accessing the vagus nerve where retrograde transport would ultimately allow access to the SN. This has come to be known as the "dual-hit hypothesis [30] and this route has been employed extensively to deliver the neurotoxin MPTP [31]. Moreover, clinical studies have shown that the dorsal motor nucleus of the vagus nerve is disrupted at an early disease stage [32] by *a*-syn and Lewy body pathology in Parkinson's disease [33]. This finding was followed by the discovery that there was a close relationship between peripheral vagus nerve impairment and inhibition of the dopamine system in brain structures [34].

To gain an insight into the development and molecular basis of motor behavioral impairments initiated by α-syn misfolding with PD like outcomes, we have now extended our earlier findings concerning changes in "open field" behavior arising from α-syn oligomeric and fibrillar aggregate effects in mice. Hence, in the current study, no discernable behavioral upshot was incited at the end of two weeks α-syn oligomeric treatment. Taking into account that signs of motor deficit are produced later than protein/neurotransmitter changes, it may be postulated that α-syn toxic oligomerization only invokes brain regions relevant to motor behavior regulation. Additionally, it should be noted that autopsy-based studies with patients who died at different stages of PD also suggest that the dorsal motor nucleus of the vagus, which is connected with the gastrointestinal system, is affected very early in the disease [35].

We have postulated [15] that the initial toxicity associated with interactions between α-syn oligomers and DA-ergic neuronal membranes [36,37] may provoke cell apoptosis instigating dopamine release

which manifests the early stages of the disease and an ensuing dysregulation of motor function. Furthermore, the present results substantiate our previous observation that α-syn combinative oligomeric and fibrillar treatment evokes animal rigidity, hypokinesia and immobility. After a matching course of α-syn fibrillar dosing, mild bradykinesia and decreased vertical rearing were also recorded (Scheme 1). These data support the nasal vector hypothesis whereby introduced misfolded protein species penetrate brain structures changing structural functional interactions which then result in behavioral impairments. Such effects may persist beyond cessation of α-syn aggregate delivery [38]. Our data primarily based on behavioral testing in the open field after two-week treatment cessation revealed that the animal group treated beforehand with α-syn oligomers produced mild bradykinesia, immobility and a reduction of vertical rearing which signified the onset of behavioral impairment and continuing neurotoxicity. It is interesting that the mixture of oligomers and fibrils also produced long-term effects on motor activity in mice. Fourteen days after treatment cessation, hyperkinesias and immobility were augmented and accompanied by a reduction in vertical rearing. These data demonstrate not only the biological activity of amyloidogenic forms of α-syn, but also the sensitivity of mesencephalic structures to their toxicity which consequently influences behavior. A similar phenomenon was also noted after two weeks washout from α-syn fibril intranasal treatment with a comparable elevation of hyperkinesia and immobility status which was attended by reduced vertical rearing. Thus, in essence, the behavioral data indicated that misfolded structures of α-syn had specific, heterogeneous and heterochronic impacts on PD-like symptoms (Scheme 1). It is well known that in PD, behavioral symptoms are associated at the molecular level with DA-ergic system dysfunction [39]. Moreover, it has been demonstrated in vitro that there is a co-facilitatory activity between DA, its metabolites and α-syn [40,41] which can eventuate in α-syn aggregation [4].

In this regard, investigation of nigrostriatal interlinks between the DA-ergic system, α-syn aggregation and behavioral deficits are of a special interest [42]. Recent molecular studies have focused on the

interaction between α-syn and dopamine in the pathogenesis of PD, and fluorescent anisotropy has suggested that the C-terminal region of α-syn may be an identifiable target for modification by dopamine [43]. Moreover, dysregulation of the DA pathway emanating from the SN could act as a trigger for induction of increased toxicity in DA-ergic neurons and can explain how these neurons become more vulnerable and degenerate in the disease process [42]. However, monoamine metabolism is associated with oxidative stress conditions that may contribute to DA-α-syn interactions promoting aggregation and neuronal damage. Since only DA-ergic neurons contain significant amounts of DA, this has been hypothesized to account for the selective susceptibility of SN neurons. Consequently, DA itself may not be toxic at physiologically relevant levels, so it is probable that DA metabolites may also play a major role in α-syn aggregation [40]. It has been disclosed that the DA metabolite DOPAC, at low concentrations, can actually prevent fibrillar development through non-covalently binding α-syn [44].

Our neurochemical experiments involving nigrostriatal concentrations of DA and its metabolites DOPAC, HVA and 3-MT demonstrate a specific pattern of neurotransmitter and metabolite levels along with DA turnover. At the end of 14-day treatment with α-syn oligomers, a reduction in DA, DOPAC, HVA and 3-MT was detected only in the SN. Following a fortnight of non treatment, the concentration of DA in the SN returned to the control level. No statistical changes in DA metabolites were unveiled and this may reflect the initial stage of a DA-ergic system disbalance conceivably connected either with modulation of tyrosine hydroxylase activity [45] and/or the vesicular monoamine transporter (VMAT2) [46,47].

It should be noted that the striatum was not affected by toxic oligomeric species since there were no significant changes in DA or its metabolite levels detected following the treatment protocol. One of the conclusions derived from our data with α-syn oligomeric intervention is that the toxic effects are not evident at DA-ergic axons and terminals in the striatum but on nigral cell bodies. In the light of this

finding, it is noteworthy that not only monomeric α-syn [48] but also MPTP administration [49] are thought to target firstly the axons and terminals of the nigrostriatal system [50]. Our experimental protocol was focused on behavioral postsymptomatic parameters which are expressed later rather than earlier during a programme of substance administration. Neurochemical measurements of DA and its metabolite concentrations were determined at two time points: firstly, after recording PD-like symptoms and secondly, at the stage of a decline in rigidity. Therefore it might be proposed that primarily, toxic amyloidogenic species of α-syn attacked the terminals and axons of striatal neuronal cells but later on (as in our protocol at two weeks and beyond) the main target is directed to the SN cell bodies. Thus, in the current experimental protocol, DA changes were outside the time scale and accordingly no changes were detected in the striatum which was substantiated at the second time point after 4 weeks from start of the intranasal inoculation.

To develop this debate further, we used a combination of α-syn oligomers and the more advanced fibrillar species to evaluate the possibility of DA-ergic disbalance. The upshot was that the aggregate combination only elevated nigral DA and DOPAC concentrations after treatment, with a recovery to normal control levels after 14 days. In contrast, α-syn fibrils did not change DA levels but significantly increased DOPAC, HVA and 3-MT concentrations after the treatment period and this action was maintained during non treatment for the ensuing two weeks.

Striatal measurement of DA and its metabolites in all treatment groups either at the end of treatment or two weeks later did not reveal any significant neurochemical changes in comparison with control. Thus, it may be concluded that both species of α-syn aggregates or their combination did not affect striatal DA-ergic terminals in the confines of our experimental protocol.

DA turnover in the SN (DOPAC/DA and HVA/DA) increased two fold in the animal group at the end of α-syn fibril treatment but returned to the control level after 14-days washout and this reflects an

upregulation of DA-ergic activity. By way of contrast, nigral DA turnover, after administration of α-syn oligomers alone or in combination with fibrils, remained at the control level throughout treatment or upon cessation. Analysis of DA turnover in the striatum confirmed our conclusion that the effects of α-syn aggregates mainly occur in SN cells but not in striatal terminals under the experimental conditions which modified behavior. Moreover, neurochemical events which underlie the modulation of behavior after nasal delivery of protein toxins reflect temporal neurotoxic failure of the DA-ergic system but this is not crucial for restoring metabolism of DA in the SN.

Findings from this co-administration study with α-syn oligomeric plus fibrillar species are not only in accordance with the olfactory vector hypothesis of Parkinson's disease [49] but also the results of cell culture experiments. Thus, the data support the concept that intracellular α-syn aggregation is normally limited by the number of active nucleation sites present in the cytoplasm and that small quantities of α-syn fibrils can alter this balance by acting as seeds for aggregation [51]. Additionally, it can be proposed that degradation of protein fibrils may increase the concentration of the toxic α-syn oligomeric fraction and initiate further apoptosis of DA-ergic cells in the SN.

It is important to note, that chronic intranasal dosing with α-syn aggregates may modulate additional expression of native α-syn and its overexpression in nigral dopamine neurons along with deficient vesicular storage of dopamine leads to a significant increase in dopaminergic neurodegeneration. Significantly, silencing tyrosine hydroxylase enzyme to reduce DA levels in nigral neurons reverses their increased vulnerability to the baseline level, but fails to eliminate it completely [42]. Although we have not yet demonstrated anatomical damage to PD relevant brain structures in response to chronic misfolded α-syn exposure, the longer term aspect of this process is currently under investigation.

In conclusion, progress in modelling Parkinson's disease not only promotes better understanding of the pathological processes underlying the human illness, but also contributes to the development of

potentially new therapeutic strategies [52]. Compilation of our behavioral and neurochemical findings substantiates the validity of the α-syn nasal vector model for investigating parkinsonian-like symptoms. In this vein, there are distinct routes for the transfer of substances by means of this vector into the central nervous system [53]. These include the olfactory epithelial pathway (via sustentacular cells, their intercellular tight junctions and/or their clefts with olfactory neurons), the olfactory nerve pathway, the trigeminal pathway and also through the systemic circulation directly from the nasal mucosa [54,15].

Additionally, α-syn aggregates delivered intranasally, induced changes in DA-ergic perturbations which underlie motor deficits emphasizing the possibility that both toxic endogenous and exogenous substances [49,55] may cause neurodegenerative processes reflective of PD. Furthermore, the animals used in this study were aged 12 months and this is an important variable in an age-related model which is germane to the human neurodegenerative condition. Hence this particular model informs our knowledge concerning PD degeneration because it involves endogenous protein toxins (α-syn oligomeric and fibrillic amyloidogenic species) which are actually generated during the disease process.

Conflict of interest

The authors declare no conflict of interest

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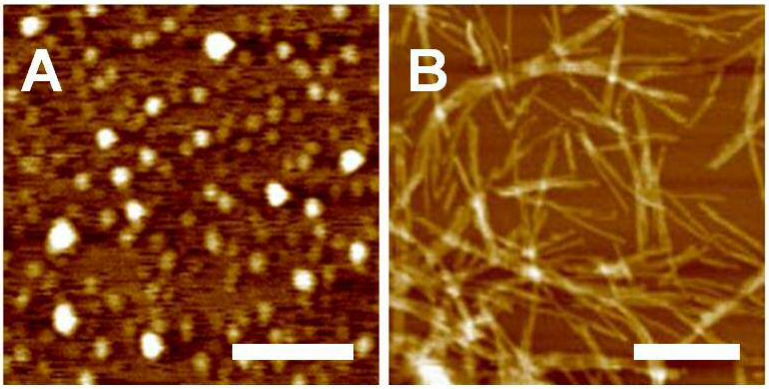

Fig. 1. Characterisation of α-synuclein amyloidogenic species

AFM images of α-synuclein amyloid species produced in sodium phosphate buffer pH 7.4 at 22^{o}C with agitation (In each case, scale bars represent 500 nm).

(A) AFM height image of amyloid oligomers formed after 7 days of incubation; (B) AFM height image of amyloid fibrils of α-synuclein produced under the same conditions after 14 days of incubation.

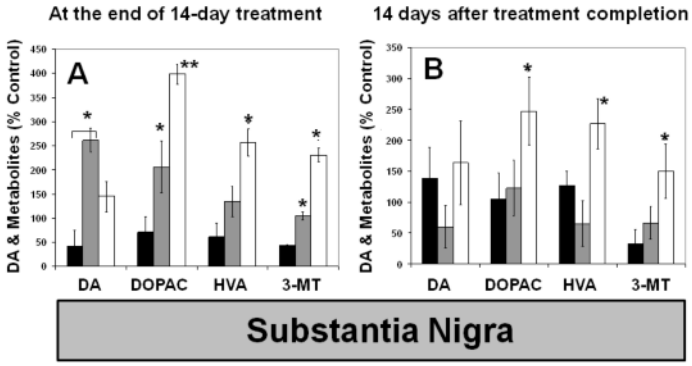

Fig. 2. DA, DOPAC, HVA or 3-MT content in mouse substantia nigra (SN) following 14-day treatment with amyloidogenic α-synuclein species either alone or in combination.

All values (mean ± sem, n = 10 per group) were expressed as % of the following control concentrations (nmol/g tissue): DA = 4.2 ± 1.9, DOPAC = 1.8 ± 0.9, HVA = 1.8 ± 0.3, 3-MT = 0.6 ± 0.3.

(A) DA, DOPAC, HVA or 3-MT content in SN (% control) at the end of 14-day treatment with α-syn oligomers (black bars), α-syn oligomers + α-syn fibrils (grey bars) or α-syn fibrils (open bars).

(B) DA, DOPAC, HVA or 3-MT content in SN (% control) 14 days after completion of treatment with α-syn oligomers (black bars), α-syn oligomers + α-syn fibrils (grey bars) or α-syn fibrils (open bars).

*p≤ 0.05 in comparison with combinative α-syn oligomers + α-syn fibrils or single α-syn fibrillar treatment. ** p≤ 0.05 in comparison with α-syn oligomers or combinative α-syn oligomers + α-syn fibrilar treatment.

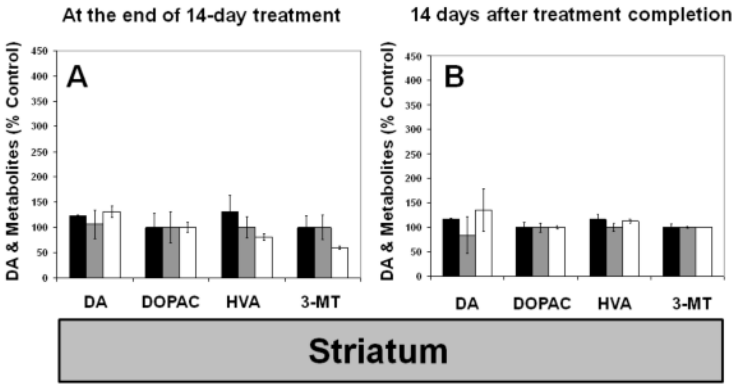

Fig. 3. DA, DOPAC, HVA, or 3-MTcontent in mouse striatum following 14-day treatment with amyloidogenic α-synuclein species either alone or in combination.

All values (mean ± sem, n = 10 per group) were expressed as % of the following control concentrations (nmol/g tissue): DA = 75.4 ± 11.0, DOPAC = 5.0 ± 0.4, HVA = 7.0 ± 1.2, 3-MT = 1.3 ± 0.4.

(A) DA, DOPAC, HVA or 3-MT content in striatum (% control) at the end of 14-day treatment with α-syn oligomers (black bars), α-syn oligomers + α-syn fibrils (grey bars) or α-syn fibrils (open bars).

(B) DA, DOPAC, HVA or 3-MT content in striatum (% control) 14 days after completion of treatment with α-syn oligomers (black bars), α-syn oligomers + α-syn fibrils (grey bars) or α-syn fibrils (open bars).

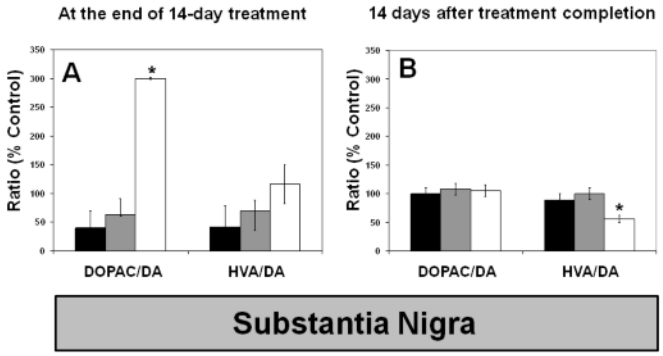

Fig. 4. Turnover of DA in mouse substantia nigra (SN) after treatment with amyloidogenic α-synuclein species either alone or in combination.

All values (mean ± sem, n = 10 per group) were expressed as % of the following control ratios DOPAC/DA = 0.5 ± 0.07, HVA/DA = 0.6 ± 0.02.

(A) Ratio of DOPAC/DA and HVA/DA (% control) in the SN at the end of 14-day treatment with α-syn oligomers (black bars), α-syn oligomers + α-syn fibrils (grey bars) or α-syn fibrils (open bars).

(B) Ratio of DOPAC/DA and HVA/DA (% from control) in the SN 14 days after completion of treatment with α-syn oligomers (black bars), α-syn oligomers + α-syn fibrils (grey bars) or α-syn fibrils (open bars).

*p≤ 0.05 in comparison with α-syn oligomers alone or α-syn oligomers + α-syn fibrils combined treatment.

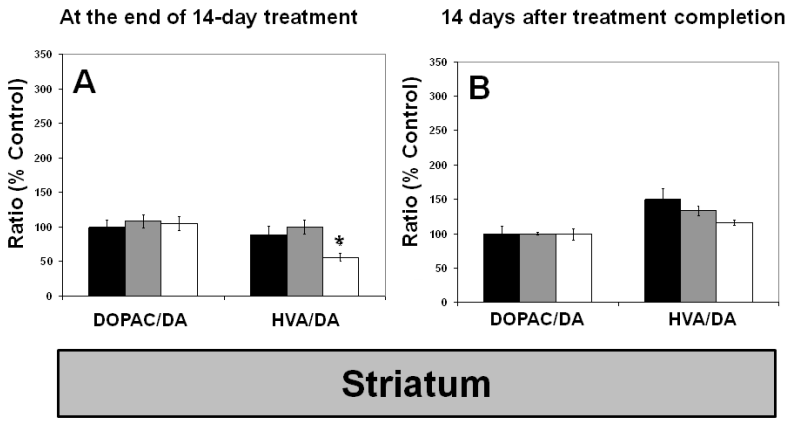

Fig. 5. Turnover of DA in mouse striatum after treatment with amyloidogenic α-synuclein species either alone or in combination.

All values (mean ± sem, n = 10 per group) were expressed as % of the following control ratios DOPAC/DA = 0.07 ± 0.01, HVA/DA = 0.1 ± 0.01.

(A) Ratio of DOPAC/DA and HVA/DA (% control) in the striatum at the end of 14-day treatment with α-syn oligomers (black bars), α-syn oligomers + α-syn fibrils (grey bars) or α-syn fibrils (open bars).

(B) Ratio of DOPAC/DA and HVA/DA (% control) in the striatum 14 days after completion of treatment with α-syn oligomers (black bars), α-syn oligomers + α-syn fibrils (grey bars) or α-syn fibrils (open bars).

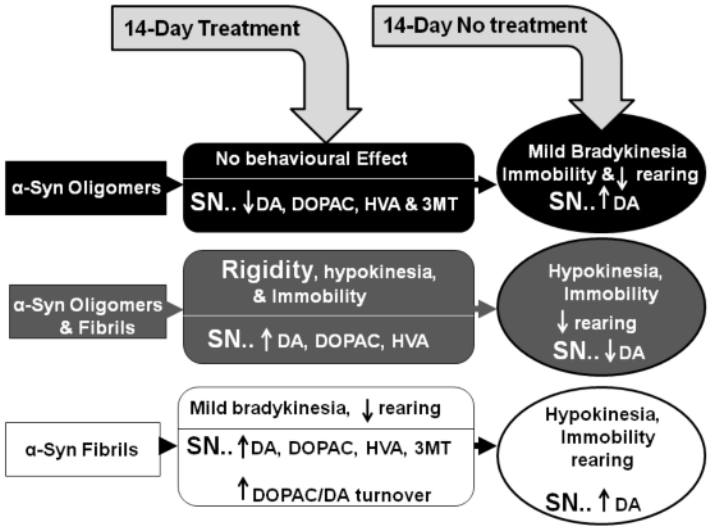

Fig. 6. Scheme providing an overview of behavioural and neurochemical outcomes following 14-day treatment then 14 days after treatment with α-synuclein oligomers, fibrils or oligomers plus fibrils.

Table 1. Behavioral parameters recorded for 0-6 mins at the end of 14-day intranasal daily dosing with α-synuclein aggregates

Animal Treatment Groups (n=10)	Total Locomotor Distance (cm) (mean±sem)		Cumulative Ambulation Time (sec) (mean±sem)		Immobility Time (sec) (mean±sem)		Ambulation Speed (cm/sec) (mean±sem)		Vertical Rearing (incidence) (mean±sem)		Rigidity Score (mean±sem)	
	Before dosing	After dosing	Before dosing	After dosing	Before dosing	After dosing	Before dosing	After dosing	Before dosing	After dosing	Before dosing	After dosing
Vehicle Control	1042.8 ± 121.3	1055.6 ± 156.3	84.4 ± 9.6	87.4 ± 8.3	134.0 ± 14.8	128.4 ± 8.3	12.4 ± 0.2	12.2 ± 1.2	17.3 ± 4. 8	23.2 ± 8.1	0	0
α-Synuclein oligomeric aggregates (15µg)	993.8 ± 64.7	947.2 ± 78.0	80.2 ± 4.9	74.1 ± 6.5	137.9 ± 10.0	136.3 ± 12.7	12.3 ± 0.1	12.8 ± 0.2	17.8 ± 4.4	26.6 ± 3.1	0	0
α-Synuclein oligomeric (7.5µg) & fibrillar (7.5µg) aggregates	1312.0 ± 71.7	#** 809.8 ± 96.0	104.4 ± 5.1	89.0 ± 17.2	107.4 ± 6.3	#* 152.6 ± 13.5	12.4 ± 0.2	10.8 ± 0.7	25.2 ± 4.6	20.0 ± 3.5	0	##** 1.3 ± 0.2 7 Straub tail 3 Arched back
α-Synuclein fibrillar aggregates (15µg)	938.3 ± 128.2	# 817.8 ± 47.5	77.3 ± 9.6	# 65.3 ± 4.2	142.3 ± 17.7	# 158.8 ± 5.0	12.0 ± 0.3	12.6 ± 0.2	13.3 ± 4.6	# 16.3 ± 4.6	0	0

Group mean after dosing significance of difference from group mean before dosing: * $P < 0.05$, ** $P < 0.001$
Group mean significance of difference from vehicle control group # $P < 0.05$, ## $P < 0.001$.

Table 2. Behavioral parameters recorded for 0-6 mins 14-days post intranasal dosing with α-synuclein aggregates

Animal Treatment Groups (n=10)	Total Locomotor Distance (cm) (mean±sem)		Cumulative Ambulation Time (sec) (mean±sem)		Immobility Time (sec) (mean±sem)		Ambulation Speed (cm/sec) (mean±sem)		Vertical Rearing (incidence) (mean±sem)		Rigidity Score (mean±sem)	
	Before dosing	After dosing	Before dosing	After dosing	Before dosing	After dosing	Before dosing	After dosing	Before dosing	After dosing	Before dosing	After dosing
Vehicle control	1042.8 ± 121.3	1055.4 ± 155.1	84.4 ± 9.6	87.4 ± 10.6	134.0 ± 14. 8	128.4 ± 8.3	12.4 ± 0.2	12.2 ± 1.2	17.3 ± 4.8	23.2 ± 8.1	0	0
α-Synuclein oligomeric aggregates (15µg)	993.8 ± 64.7	# 624.0 ± 187.0	80.2 ± 4.9	# 55.0 ± 11.1	137.9 ± 10.0	# 164.8 ± 16.7	12.3 ± 0.1	10.2 ± 2.2	17.8 ± 4.4	# 10.5 ± 3.7	0	0
α-Synuclein oligomeric (7.5µg) & fibrillar (7.5µg) aggregates	1312.0 ± 71.7	##** 492.0 ± 114.6	104.4 ± 5.1	#** 38.8 ± 9.5	107.4 ± 6.3	#** 205.8 ± 15.1	12.4 ± 0.2	12.8 ± 0.5	25.2 ± 4.6	#** 8.8 ± 3.0	0	0
α-Synuclein fibrillar aggregates (15µg)	938.3 ± 128.2	** 148.5 ± 49.5	77.3 ± 9.6	##** 12.0 ± 4.0	142.3 ± 17.7	#** 266.8 ± 30.8	12.0 ± 0.3	9.2 ± 3.1	13.3 ± 4.6	##** 2.5 ± 0.5	0	0

Group mean after dosing significance of difference from group mean before dosing: * $P < 0.05$, ** $P < 0.001$
Group mean significance of difference from vehicle control group # $P < 0.05$, ## $P < 0.001$