Noradrenergic and serotonergic neurochemistry arising from intranasal inoculation with α-synuclein aggregates which incite parkinsonian-like symptoms

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Abstract

Alpha-synuclein (α-syn) toxic aggregates delivered by the nasal vector have been shown to modify the neurochemistry of dopamine (DA) which is associated with parkinsonian-like motor symptoms. The aim was therefore to study the intranasal effects of α-syn oligomers, fibrils or their combination on the motor behavior of aged mice in relation to possible noradrenergic and serotonergic correlates. *In vitro* generated α-syn oligomers and fibrils were verified using atomic force microscopy and the thioflavin T binding assay. Levels of noradrenaline (NA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were detected using HPLC with electrochemical detection in the substantia nigra (SN) and striatum. The oligomers or fibrils administered alone or in a 50:50 combination (total dose of 0.48 mg/kg) were given intranasally for 14 days and "open-field" behaviour was tested on days 0, 15 and 28 of the protocol, at which time brain structures were sampled. Behavioral deficits at the end of the 14-day dosing regime and on day 28 (i.e. 14 days after treatment completion) induced hypokinesia and immobility whilst the aggregate combination additionally produced rigidity. The α-Syn oligomer/fibril mixture also instigated PD-like motor symptoms which correlated heterochronically with elevated NA levels in the striatum but then later in the SN while intranasal fibrils alone augmented 5-HT and 5-HIAA nigral concentrations throughout the protocol. In contrast, α-syn oligomers displayed a delayed serotonin upsurge in the SN. Neurodegenerative and/or actions on neurotransmitter transporters (such as NET, SERT and VMAT2) are discussed as being implicated in these α-syn amyloid induced neurochemical and motoric disturbances.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder largely characterized by behavioural abnormalities which arise from aberrant brain neurochemistry [1]. PD motor signs (akinesia, rigidity, resting tremor and postural instability) have been ascribed primarily to progressive degeneration of dopaminergic neurons in the substantia nigra (SN) [2]. Current thinking suggests that neurotransmitters other than dopamine (DA) exhibit modulatory roles within the basal ganglia to directly and/or indirectly influence the dopaminergic system. Hence, various non-dopaminergic neurotransmitter systems have been implicated in the mechanisms which contribute to the motor features of PD. Accordingly, it is now well established that noradrenergic, serotonergic, glutamatergic, GABAergic, cholinergic, opioidergic, histaminergic, and adenosinergic systems, are implicated in the pathogenesis of PD [3]. Noradrenaline (NA), for example, is hypothesized to play a role in PD balance and gait dysfunction chiefly because of the disease-associated neurodegeneration connected with the locus coeruleus [4]. Similarly, it has been shown that the protein α-synuclein (α-syn) regulates the homeostasis not only of dopaminergic [5] and [6] but also serotonergic and noradrenergic synapses [7], [8] and [9] in addition to being a prion-like protein that can adopt a self-propagating conformation that causes neurodegeneration [10]. Moreover, α-syn can directly modulate the activity of monoamine transporters (MAT) including the dopamine transporter (DAT), serotonin transporter (SERT) and NET, leading to modified trafficking to the cell surface, in a manner that is dependent on α-syn expression levels [11], [12], [13], [14] and [15].

Therefore, it has been established that PD involves a neuropathology in which α-syn and its amyloidogenic species, along with neurotransmitter systems (including DA, NA, serotonin (5-HT) and glutamate) are interrelated [16], [17] and [18]. In this respect, it is noteworthy that α-syn is co-localized in terminals of noradrenergic (locus coeruleus) or serotonergic (raphe nuclei) neurons. 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 [19], [20] and [21]. Both α-syn misfolding and intracellular aggregation processes are thought to be crucial factors in the pathogenesis of Lewy body PD [22], [23] and [24] but precisely how these phenomena are linked to brain neurochemistry is still under debate.

In addition, the pathology of PD entails not only the loss of dopaminergic neurons in the SN but also degeneration of noradrenergic neurons in the locus coeruleus and deposition of misfolded α-syn aggregates in Lewy bodies. α-Syn also modulates noradrenergic activity by its interaction with the NET via the non-Aβ component of Alzheimers disease amyloid (NAC) domain, the region directly responsible for self-aggregation [13]. Moreover, α-syn also regulates the function of serotonergic synapses, through trafficking of the serotonin transporter (SERT) [14]. The serotonergic system is also affected by the neurodegenerative process underlying PD and neuronal loss in the dorsal raphe nucleus leads to reduced serotonin levels in a variety of brain areas, including the striatum, globus pallidus, SN and some cortical areas [25] and [26]. Thus, α-syn has an important central role in the homeostasis of both serotonergic and noradrenergic neurons.

In an earlier study, we instigated a novel PD model based on nasal inoculation with α-syn aggregates expressing parkinsonian-like behavioural and immunological features in mice [17]. More recently, we verified the robustness of the amyloid nasal vector model by examining behavioral consequences with respect to DA-ergic neurochemical corollaries [18]. Behavioral deficits at the end of a 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 combined administration of α-syn oligomers plus fibrils but not the oligomers alone. 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 substantiated the validity of the α-syn nasal vector model for investigating parkinsonian-like symptoms and this has prompted inquiry into the relationship between aberrant misfolded α-syn and other neurotransmitter systems with respect to PD-like motor activity. The aim of the current study was therefore to further validate the intranasal effects of α-syn oligomers, fibrils or their combination on the motor behavior of aged mice in relation to possible noradrenergic and serotonergic correlates.

2. Materials and Methods

Noradrenaline (NA) 5-hydroxytryptamine (serotonin, 5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) 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) received α-syn fibrillar aggregates (15.0 µg in 8 µL = 0.48 mg/kg bilaterally) for the 14-day schedule. Group (4) 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. At the end of the 14-day protocol (i.e. on day 15), animal groups 1-4, underwent behavioural 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; fibrillar aggregates) and (7; oligomers plus fibrils 50%:50%) then 14-days post treatment (i.e. day 28), they were tested behaviorally then killed and nigral along with striatal neurochemical analyses were completed. All behavioral tests and neurochemical analyses were performed under blind conditions.

2.3. Behavioural tests

Animal behavioural 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 behavioural indicators of PD-mimetic symptoms (collectively assessing hypokinesia, muscle rigidity and tremor) were evaluated [27] and [28]. 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 [29] and [30] 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 [27] and [31]. 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 [27]. Thirdly, the presence or absence of tremor was checked behaviourally [32] 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 harbouring the α-synuclein gene were used for the production of the recombinant protein [33]. The recombinant protein was purified as previously described [34] 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 dialized 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, dialized 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 [35]. 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 [27].

2.6. Spectroscopic amyloid assays

The thioflavin T (ThT) binding assay was performed using a modification of LeVine's method [36]. 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 [35]. 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 [37]. 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 noradrenaline (NA), serotonin (5-HT) and it metabolite 5-hydroxyindole acetic acid 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) [38]. NA, 5-HT and 5-HIAA 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 serotonin was expressed as the ratio of metabolite tissue concentration (5-HIAA) to the parent monoamine (5-HT). Samples from all the animals were processed in parallel on the same day for each brain structure. Data were 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. Characterisation 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 was performed 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 an increase of the dye absorbance spectra compared to the control measurement of the Congo red spectrum in the presence of monomeric α-syn. The generated 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 carried out and 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 [27] and [39].

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 behavioural 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 (i.e. day 15) and 14 days (i.e. day 29) after treatment completion. Statistical analysis showed no significant difference in behavioural 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 behaviour. Neither was there any inherent evidence of muscle rigidity or tremor in any of these control vehicle-treated mice.

3.2.1. α-Synuclein oligomer treatment

Similar to controls, there was no change (P>0.05) in any of the above behavioural 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 (Fig 2). In addition, there was no evidence of tremor or rigidity.

3.2.2 .α-Synuclein fibril treatment

In the α-syn fibril treatment group (Fig 2), there were significant reductions compared with controls (P<0.05) in total locomotor distance (77.5%), cumulative ambulation time (74.7%) and vertical rearing (70.3%) but an increase immobility time (123.75%, P<0.05) .

3.2.3. 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 a significant reduction in mean total locomotor distance traveled (P<0.001, Fig 2). When compared to the 14-day vehicle treatment control, this reduction was of a comparable magnitude (i.e. 76.7% of the after dosing control). Moreover, there was also a concomitant increase (P<0.05) in immobility time versus either the pre-dose value or the control (118.8%, Fig 2). Most notably, there was a presence of rigidity in animals which received the aggregate combination (P<0.001) and 70% of the group displayed Straub tail while the remaining 30% additionally manifested an arched back.

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 at the end of the 14-day treatment schedule (see Fig 2).

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 behavioural 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 (59.1% of control), cumulative ambulation time (62.9%), and vertical rearing (45.3%) with a corresponding increase in immobility time (128.3%, Fig 3) and no expression of tremor or rigidity.

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

Fourteen days after completion of combinative treatment with two species of α-syn aggregate there were changes in animal open field behaviour in comparison with the controls and those parameters which were tested before dosing. Thus, significant attenuations were observed versus control in total locomotor distance (46.6%, $P<0.001$), cumulative ambulation time (44.4%, $P<0.05$), vertical rearing (37.9%, $P<0.05$) and this was accompanied by an elevation of immobility time values (160.3%, $P<0.05$, Fig 3).

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 ($P<0.001$), cumulative ambulation time ($P<0.001$), vertical rearing ($P<0.001$) accompanied by an elevation of immobility time values ($P<0.001$, Fig 3).

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 (13.7%%, $P<0.001$) and vertical rearing (10.8%, $P<0.001$) which were accompanied by an elevation of immobility time values (207.8%, $P<0.05$, Fig 3).

Likewise, comparison of the 14-day post fibrillar aggregate treatment completion group with the appropriate pre-dose group disclosed an analogous behavioral profile of significant reductions in total locomotor distance ($P<0.001$), cumulative ambulation time ($P<0.001$) and vertical rearing ($P<0.001$) which were accompanied by an elevation of immobility time values ($P<0.001$).

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 Fig 3) and there was no evidence of tremor or rigidity.

3.4. Neurochemical assay of NA, 5-HT and 5-HIAA 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 NA, 5-HT and 5-HIAA were measured in PD pathology relevant mice midbrain structures (SN and striatum). Control levels of NA on commencement of the protocol were 1.60±0.35 nM/g of wet tissue in the SN and 0.38±0.05 nM/g of wet tissue in the striatum. Control levels of 5-HT and 5-HIAA were respectively 7.62±2.71 nM/g and 3.76±0.59 nM/g in the SN and 0.08±0.01 nM/g and 0.04±0.001 nM/g in the striatum. The control 5-HIAA/5-HT ratios at the start of the protocol were 0.49±0.21 in the SN and 0.50±0.10 in the striatum.

All NA, 5-HT and 5-HIAA levels following intranasal α-syn aggregate treatments were expressed as a percentage of these controls.

3.4.1. NA concentrations in mouse SN after 14-day α-synuclein aggregate intranasal inoculation and 14 days after treatment cessation.

After dosing with α-syn oligomers, there was no change in nigral NA levels (Fig 4A). In contrast, at the end of 14-day administration with α-syn fibrils, there was a significant increase (P<0.05) in NA concentration (2.96±0.04 nM/g of wet tissue, +81%) in the SN. Furthermore, analysis of the

NA content after combinative treatment with the mixture of α-syn oligomeric/fibrillar species did not induce any change in nigral NA content.

Fourteen days after cessation of treatment with a mixture of α-syn species exposed an augmentation *(P*<0.05) of the NA concentration to 10.2±1.5 nM/g of wet tissue, +537% (*P*<0.001) of control in the SN. However, no change occurred in the groups formerly treated with α-syn oligomers or fibrils (Fig 4B).

3.4.2. NA concentrations in mouse striatum after 14-day α-synuclein aggregate intranasal inoculation and 14 days after treatment cessation.

Scrutiny of NA striatal concentrations at the end of 14-day α-syn species treatment divulged no striking changes in levels compared with controls following oligomeric or fibrilar administration. Conversely, the aggregate combination treatment produced an extensive elevation (18.23±3.60 nM/g of wet tissue, +4660%, *P*<0.001) of NA in the striatum (Fig 3A). A reversal of this effect back to the control levels was detected to the combination treatment along with no alterations in striatal NA to oligomers and fibrils given separately 14 days after protocol completion. (Fig 5A, B)

3.4.3. 5-HT and 5-HIAA concentrations plus 5-HIAA/5-HT ratios in mouse SN after 14-day α-synuclein aggregate intranasal inoculation and 14 days after treatment cessation.

On completion of α-syn intranasal administration (14-days) there was no change in nigral 5-HT concentration following either oligomer or aggregate combinative (oligomer + fibril) treatment but (25.5±3.9 nM/g of wet tissue, i.e. +235.5 %, *P*<0.05) an increase in the level of this neurotransmitter after fibril alone exposure (Fig 6A). Simultaneously, the SN concentration of the

5-HT metabolite 5-HIAA was decreased (1.61±0.09 nM/g of wet tissue, -57.2%, P<0.05) by oligomer treatment, but increased (6.13±1.2 nM/g of wet tissue, +63%, P<0.05) after fibril administration while there was no change following the oligomer plus fibril combination. In addition, the 5-HIAA/5-HT ratio remained unchanged by any of the α-syn species 14-day treatments.

Fourteen days after α-syn species treatment completion, 5-HT levels in the SN were elevated by oligomer alone dosing (16.1±2.99 nM/g of wet tissue, +111.81%, P<0.05) and fibril alone administration (12.6±2.0 nM/g of wet tissue, +65.7 %, P<0.05) but there was no change with the combinative species treated group (Fig 6B). However, there was a significant increase in the 5-HIAA/5-HT ratio caused 14 days after the cessation of fibril treatment 0.75±0,15 +53 %, P<0.05, Fig 6B).

3.5. 5-HT and 5-HIAA concentrations plus 5-HIAA/5-HT ratios in mouse striatum after 14-day α-synuclein aggregate intranasal inoculation and 14 days after treatment cessation.

There were no significant alterations in 5-HT, 5-HIAA or 5-HIAA/5-HT ratios induced by 14-day i.n. treatment or 14 days after treatment cessation with α-synuclein oligomers, fibrils or their combination (Fig 7A, B).

4. Discussion

Parkinson's disease is a degenerative condition of the central nervous system, whose neuropathology warrants further elucidation. The cardinal motor manifestations of PD which include rigidity, tremor and bradykinesia, are mostly regarded as the sequalae of a deficiency of dopamine in the SN and striatum [1] and [40]. Normal motor behavior involves the creation of

appropriate activity patterns across motor networks, enabling firing synchrony, synaptic integration, and normal functioning of these networks. The defective striatal signaling in PD could therefore lead to abnormal oscillatory activity and aberrant plasticity at multiple levels within the interlinked motor networks [41]. In the overall neuropathological symptom profile of PD, motor behavioural abnormalities are thought to originate not only from degeneration and loss of dopaminergic, but also noradrenergic and serotonergic neurons [26] and [42]. A considerable body of neuropathological, biochemical and genetic evidence suggests that the cerebral accumulation of amyloid fibrils is the core event in the pathogenesis of PD [43] and [44]. Oligomeric intermediates of α-syn are non-fibrillar polypeptide assemblies that occur during amyloid fibril formation and they are thought to underlie the aetiology of amyloid diseases, such as PD. Similar to the other amyloids [45], α-syn misfolding in the context of PD generates toxic amyloidogenic species [10] and [46] associated primarily with a perturbation of the DA system. In this connection, the carboxy terminal of the DA transporter (DAT) physically interacts with native α-syn [47] and it was subsequently shown that the α-syn NAC domain is implicated [11] and [12] (Fig 9).

In a recent study, [18] we showed that α-syn aggregates delivered intranasally, induced changes in DA-ergic neurochemistry which correlated with motor deficits emphasizing the possibility that both toxic endogenous and exogenous substances [48] and [49] may cause neurodegenerative processes reflective of PD. Since DA and not its metabolites were affected by oligomeric α-syn, it has been suggested that in the initial stage of a DA-ergic system disbalance, the vesicular monoamine transporter (VMAT2) is a probable target for α-syn amyloid [18], (Fig 9).

Furthermore, in the current protocol, the animals used were 12 months old 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 fibrillar amyloidogenic species) which are incited during the *de facto* disease process. [18]. The current data confirmed the alterations in

motor behavior, in the open-field mouse model after 14-days of intranasal inoculation with the α-syn oligomer/fibril combination.

These data therefore 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 [50].

In the striatum there was an extensive increase in NA levels on completion of treatment with the oligomer/fibril combination and this was associated with rigidity, hypokinesia and immobility. Along with elevated nigral DA [18] it appears that raised striatal NA also coincides with PD-like motor deficits. This may suggest that the NA terminals in this region were affected by α-syn aggregates thereby releasing their neurotransmitter. In relation to this tenet, it has been shown that an increasing level of α-syn expression in a cell model negatively modulates noradrenaline transporter (NET) reuptake by reducing the expression of this transporter at the neuronal cell surface [9], [13] and [15].

Intranasal delivery of the α-syn aggregate combination and conceivable access to the nigrostriatal area may change the normal interactions of α-syn with NET and also influence the expression of α-syn itself. An increasing level of α-syn expression negatively modulates NET uptake activity by decreasing NET expression at the cell surface [13] and [15]. Moderating NET function and changing membrane permeability will therefore perturb NA release and critically enhance its concentrations in the extracellular milieu.

Fourteen days after cessation of treatment with the α-syn aggregate combination, the levels of striatal NA had returned to those of the controls and there was a delayed, but substantial, elevation of NA in the SN. This concurs with the hypothesis that aberrant α-syn toxic effects expressed in

the SN, preferentially degenerate NA terminals which are more vulnerable than the DA system and this accords with the major NA-ergic damage seen in PD patients [51].

The data emphasises the part played by the striatal α-syn amyloid disrupted NA-ergic system in tandem with dysregulated nigral DA function underlying PD motor deficits [18] (Fig 8). Thus, neurochemical heterochrony was operative since α-syn aggregates initially targeted DA cell bodies (SN) whilst simultaneously damaging striatal NA terminals. However, 14 days after cessation of aggregate treatment, nigral NA levels were boosted and DA concentrations were reduced but the motor deficits were maintained throughout.

The toxic specificity of α-syn aggregates [46] was identified during analysis of their upshot on the serotonergic system which is known to be involved in PD pathogenesis [52]. It was interesting to note, that in the case of the serotonergic system, α-syn oligomers, fibrils or their combination did not evoke any effect in the striatum at the end of treatment or 14 days later. Hence, this protocol with aggregates of α-syn had no neurochemical activity with respect to serotonin or 5-HIAA in the striatum and this corresponds with previously reported results concerning striatal DA metabolism [18]. These findings are in sharp contrast with the NA augmentation seen currently at the end of dosing with the α-syn oligomer/fibril combination at which juncture marked rigidity was also present.

A key brain area relevant to PD pathology (SN) expressed an upsurge in 5-HT concentrations at the end of α-syn fibril treatment and 14 days later. Both of these chronological phenomena were attended by mild bradykinesia and reduced rearing indicating that there may be a causal link. It is notable that native α-syn physically and functionally interacts with the serotonin transporter (SERT) in a negative modulatory fashion which is NAC dependent [14]. Thus, brain regional dispersion of α-syn fibrillar aggregates changes the functional interactions of native α-syn not only

regarding NET but also SERT and this is reflected by the monoamine neurochemistry presently observed (Figs 8 and 9).

In addition, raised levels of 5-HIAA in the SN following fibrillar treatment and after treatment washout support the notion that 5-HT release is markedly increased from nigral serotonergic terminals. Moreover, the lagged fibril-induced raised ratio of 5-HIAA/5-HT was further evidence of an increase in 5-HT release in this brain area. In the light of this, in the oligomeric post-treatment period, the concentration of 5-HT was augmented. This finding demonstrates the delayed toxic effects of α-syn oligomers on serotonergic terminals as a source of 5-HT release which was associated with motor poverty (Fig 8). Surprisingly, using the current protocol, there was no effect of combinative oligomer plus fibril treatment on the nigral serotonin concentration which could be attributed to an insufficient fibril concentration in the mixture. The central event leading to synaptic and neuronal loss in PD is not completely clear yet. However, recent advances in the field suggest that nerve damage might chiefly result from the conversion of nontoxic monomers to toxic oligomers and protofibrils. The mechanisms by which misfolded α-syn species may lead to synapse loss are currently under investigation. In the case of intranasally administered misfolded α-syn aggregates, there are inevitable differences from native α-syn regarding their interactions with the above processes. α-Syn amyloidogenic species may contribute by increasing soluble α-syn in the extracellular space thereby seeding additional aggregation [23], [53] and [54]. Moreover, a contemporary view posits that oligomeric α-syn is the misfolded form of the protein most likely to cause neuronal death [55]. Several lines of evidence support the possibility that α-syn might interact to cause mitochondrial and plasma membrane damage upon translocation of its protofibrils to the membranes [56]. However the possibility cannot be excluded that α-syn aggregates may directly disrupt cell membranes or vesicles via initiation of amyloid apoptotic pores [57] and [58] (Fig 9) and initiate an apoptotic cascade leading to cell death [59]. Finally, there is increasing evidence that α-syn binds to various cellular proteins, for instance, p-21-

activated kinase 4 (PAK4) and because of this property, toxic α-syn oligomers may affect physiological function, [60]. Signaling deficits arising from such a mechanism may therefore be a notable contributory element in the behavioral defects in PD.

In summary, nasal inoculation with different types of α-syn amyloidogenic species instigated PD-like motor symptoms which correlated not only with DA [18] but also NA and serotonin disordered neurochemistry. These findings substantiate the involvement NA and serotonin pathways in α-syn amyloid intervention in motor function. A distinct possibility is that α-syn aggregates interact with monoamine transporters (Fig 9) in mediating the deviant motor behavior.

Novel data concerning α-syn amyloid intervention in monoaminergic transmission provides an insight into future possibilities for PD therapy targeting not only DA-ergic but also NA-ergic and serotonergic systems.

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Figure legends

Fig. 1. Characterisation of generated α-synuclein amyloidogenic species

Atomic force microscopy (AFM) images of generated α-synuclein amyloid species produced in sodium phosphate buffer pH 7.4 at 22ºC with agitation.

(A) AFM height images of amyloid oligomers formed after 7 days of incubation (scale bar = 180 nm);

(B) AFM height images of amyloid fibrils of α-synuclein produced under the same conditions after 14 days of incubation (scale bar = 800 nm).

Fig. 2. Behavioral parameters recorded for 0-6 mins at the end of 14-day intranasal daily dosing with α-synuclein aggregates.

Behavioral components including total locomotor distance, cumulative ambulation time, immobility time, ambulation speed, vertical rearing and rigidity were recorded before and at the end of the dosing schedule (after dosing). During the schedule, groups of mice were treated daily for 14 days with: α-synuclein oligomeric (7.5µg) plus fibrillar (7.5µg) aggregates, α-synuclein oligomeric aggregates (15µg) or α-synuclein fibrillar aggregates (15µg) and the data are presented as radar plots. In the case of the combined oligomer plus fibril treated group the rigidity score is shown in the highlighted star.

*P<0.05; ** P<0.001 for the after dosing groups in comparison with their chronologically simultaneous controls.

Fig. 3. Behavioral parameters recorded for 0-6 mins 14 days after treatment completion with daily intranasal α-synuclein aggregates.

Behavioral components including total locomotor distance, cumulative ambulation time, immobility time, ambulation speed, vertical rearing and rigidity were recorded before and 14 days after the dosing schedule. During the schedule, groups of mice were treated daily for 14 days with:

α-synuclein oligomeric (7.5µg) plus fibrillar (7.5µg) aggregates, α-synuclein oligomeric aggregates (15µg) or α-synuclein fibrillar aggregates (15µg) and the data are presented as radar plots.

*P<0.05; ** P<0.001 for the groups 14 days after treatment completion (after dosing) in comparison with their chronologically simultaneous controls.

Fig. 4. NA content in mouse substantia nigra (SN) following 14-day treatment or 14 days after the treatment completion with amyloidogenic α-synuclein species either alone or in combination.

All values (mean ± sem, n = 10 per group) were expressed as % of the following nigral control concentration: NA = 1.60± 0.35 nM/g of tissue.

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

(B) NA content in SN (% control) 14 days after the treatment completion with α-syn oligomers (black bars), α-syn fibrils (grey bars) or α-syn oligomers + α-syn fibrils (open bars).

*P<0.05, in comparison with control, α-syn oligomers or α-syn oligomers + α-syn fibril treatment ; ** P<0.001 in comparison with control, α-syn oligomers, or single α-syn fibrillar treatment.

Fig. 5. NA content in mouse striatum following 14-day treatment or 14 days after the treatment completion with amyloidogenic α-synuclein species either alone or in combination.

All values (mean ± sem, n = 10 per group) were expressed as % of the following striatal control concentration: NA = 0.38±0.05 nM/g of tissue.

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

(B) NA content in striatum (% control) 14 days after the treatment completion with α-syn oligomers (black bars), α-syn fibrils (grey bars) or α-syn oligomers + α-syn fibrils (open bars).

** P<0.01 in comparison with control and α-syn oligomer or α-syn fibrilar treatments alone.

Fig. 6. 5-HT and 5-HIAA content as well as the 5-HT/5-HIAA ratio in mouse substantia nigra (SN) following 14-day treatment or 14 days after the treatment completion with amyloidogenic α-synuclein species either alone or in combination.

All values (mean ± sem, n = 10 per group) were expressed as % of the following nigral control concentrations: 5-HT = 7.62±2.11 nmol/g tissue, 5-HIAA = 3.76±0.59 nM/g tissue or the 5-HT/5-HIAA control ratio = 0.49±0.21 nmol/g tissue.

(A) 5-HT (black bars) and 5-HIAA (grey bars) content or the 5-HIAA/5-HT ratio (open bars) in the SN (% control) at the end of 14-day treatment with α-syn oligomers , α-syn fibrils or α-syn oligomers + α-syn fibrils.

(B) 5-HT (black bars) and 5-HIAA (grey bars) content or the 5-HIAA/5-HT ratio (open bars) in the SN (% control) 14 days after the treatment completion with α-syn oligomers , α-syn fibrils or α-syn oligomers + α-syn fibrils.

*P<0.05, in comparison with control 5-HT, 5-HIAA nigral concentrations or the 5-HIAA/5-HT ratio

Fig. 7. 5-HT and 5-HIAA content as well as the 5-HT/5-HIAA ratio in mouse striatum following 14-day treatment or 14 days after the treatment completion with amyloidogenic α-synuclein species either alone or in combination.

All values (mean ± sem, n = 10 per group) were expressed as % of the following striatal control concentrations: 5-HT = 0.08±0.01 nM/g tissue and 5-HIAA = 0.04±0.001 nM/g tissue nmol/g tissue or the 5-HT/5-HIAA control ratio = 0.50±0.10 nM/g tissue.

(A) 5-HT (black bars) and 5-HIAA (grey bars) content or the 5-HIAA/5-HT ratio in the striatum (% control) at the end of 14-day treatment with α-syn oligomers, α-syn fibrils or α-syn oligomers + α-syn fibrils.

(B) 5-HT (black bars) and 5-HIAA (grey bars) content or the 5-HIAA/5-HT ratio in the striatum (% control) 14 days after the treatment completion with α-syn oligomers, α-syn fibrils or α-syn oligomers + α-syn fibrils.

Fig. 8. Scheme providing an overview of behavioural and neurochemical outcomes (striatal or nigral NA, 5-HT and 5-HIAA levels or 5-HIAA/5-HT ratios) at the end of 14-day treatment or 14 days after the treatment completion with α-synuclein oligomers, fibrils or oligomers plus fibrils.

Fig. 9. Prospective α-synuclein aggregate (oligomer and fibril) influence on monoamine homeostasis and transporter trafficking in central neuronal terminals.

Generated wild-type α-Synuclein (α-syn) oligomers and fibrils have been proposed (1) to mix and seed aggregates inter- and intra-neuronally [23] and [54]. Once located intracellularly, (**1a**) the oligomers may (**1b**) propagate fibrils and have the potential (**2**) to influence monoamine biosynthesis by modifying/overactivating not only tyrosine hydroxylase, the rate limiting enzyme in catecholamine (DA and NA) synthesis [61], but also aromatic amino acid decarboxylase activity in dopaminergic cells [62]. In this regard, a loss of neuronal nuclei containing tryptophan hydroxylase, the rate limiting serotonin synthesizing enzyme, has also been associated with neurodegenerative synucleinopathies [63]. (**3**) There is evidence that native α-syn is implicated in the storage and release of monoaminergic transmitters via loading [9] and refilling of vesicles [64] and [65], vesicular monoamine transporter 2 (VMAT2) expression [66] as well as vesicular distribution and translocation [13] and [67]. Consequently, these monoamine vesicular mechanisms are inevitably modified by the formation of α-syn misfolded species with an ensuing impact on monoamine function. Similarly, native α-syn also (**4**) modulates monoamine neuronal reuptake by means of the monoamine transporters (MAT) which include DAT [68], NET [15] and [69] and SERT [14] so α-syn aggregation will also impinge on presynaptic monoamine resorption processes. (**5**) Finally, α-syn oligomers have been shown to evoke inappropriate permeabilisation of cell membranes [57] and amyloid pore formation [54] and [70] and these actions are detrimental to neuronal integrity and overall survival.