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Research report

Nasal inoculation with α-synuclein aggregates evokes rigidity, locomotor deficits and immunity to such misfolded species as well as dopamine

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HIGHLIGHTS

- α-Synuclein fibrils and/or oligomers were given intranasally for 10 days in mice.
- Oligomers + fibrils but not oligomers only caused rigidity and locomotor impairment.
- Autoantibodies to α-synuclein species and dopamine were increased to all treatments.
- Intranasal α-synuclein species treatment may be a potential model for Parkinson's disease.

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ABSTRACT

Animal models of Parkinson's disease (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-synuclein (α-syn). In the current study, the nasal vector was used to deliver α-syn aggregates to the brain. Both α-syn oligomers and its fibrils were firstly characterized using atomic force microscopy and the thioflavin T binding assay. The toxic oligomers alone (0.48 mg/kg) or their 50:50 combination with fibrils (in a total dose of 0.48 mg/kg) were then given intranasally for ten days in mice and PD-mimetic symptoms as well as humoral immunity to these species and dopamine (DA) were evaluated simultaneously. Open-field behavioral deficits indicated by rigidity and reduced locomotor activity were induced by the dual administration of α-syn oligomers plus fibrils but not the oligomers by themselves under the 10-day dosing regimen. In contrast, using ELISA, high levels of serum autoantibodies to α-syn monomeric, oligomeric and fibrillar conformers as well as DA were observed in both treatment groups reflecting immune system activation and this substantiates previous clinical studies in Parkinson's disease patients. Thus, nasal administration of α-syn amyloidogenic species may be a potential experimental PD model which results not only in motor deficits but also incitement of humoral protection to mimic the disease. Such a paradigm may be exploitable in the quest for potential therapeutic strategies and further studies are warranted.

1. Introduction

Amyloidogenic neurodegenerative diseases are incurable conditions that are typically caused by specific, largely disordered proteins [1,2]. Protein aggregation during amyloidogenesis of α-synuclein (α-syn) is manifested in Parkinson's disease (PD), dementia with Lewy bodies and multiple system atrophy [3–5]. The molecular mechanisms underlying α-syn aggregation into filamentous inclusions [6–8] and their contribution to parkinsonian behavioral impairments [9,10] are still under scrutiny. However, the consequential activity of amyloidogenic aggregates directly on behavior requires not only additional cautious analysis but innovative animal models of parkinsonian-like neurodegeneration which adequately mimic the disease [11,12]. Pathogenic modeling of PD has centered on the actions of a range of toxins such as 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA) and more recently, the agricultural chemicals paraquat and rotenone [13,14] to deplete nigrostriatal dopamine (DA). Other

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paradigms have been based on the use of transgenic models of gene defects in familial PD and mutant rodent strains [14–16]. These neurotoxins elicit motor deficits in different animal species although MPTP fails to incite significant dopaminergic neurodegeneration in rats when the drug is administered systemically [12,17–20]. More recently, this contention has been re-evaluated following the finding that the infusion of MPTP directly into the rat substantia nigra pars compacta (SNc) [21–24] or by the intranasal route [25–28] causes a partial loss of dopaminergic neurons and depletion of striatal DA. This results in sensorial and memory deficits with no major motor impairments thus modeling the early phase of PD. Induction of DA depletion and overexpression of mesencephalic α-syn may also serve as a PD animal model which emulates the pathological, neurochemical, and behavioral features of the human disease. [29].

Common routes of MPTP administration are by intravenous or subcutaneous injection in order to evoke degeneration of mesencephalic dopaminergic neurons [30]. However, less invasive techniques such as oral dosing with MPTP have been documented in mice resulting in a sub-effective brain accumulation of the neurotoxin [31]. More recently, a rodent model of PD induced overexpression of α-syn using adeno-associated viral vectors (AAV) has been established [32,33] and a subsequent commentary has examined differences with the 6-OHDA model [34]. However, a relatively unexplored route of brain penetration is via the nasal cavity where the mucosal lining possesses a large surface area, porous endothelial membrane, high total blood flow avoiding first-pass metabolism, and a low barrier to absorption [35]. In the last few years an increasing number of studies have investigated the effects of intranasal administration of several neurotoxicants (e.g., viruses, metals, pesticides MPTP and 6-OHDA) in an attempt to induce behavioral or neurochemical changes similar to those seen in PD [36]. The symptomatic loss of smell and the pathological involvement of olfactory pathways in the early stages of PD are in accord with the olfactory vector hypothesis. This postulates that some variants of PD may be caused or catalyzed by environmental agents that enter the brain via the olfactory mucosa [36]. Moreover, there is no evidence in the literature concerning the use of α-syn aggregates as exogenously administered toxins to model PD-like pathological conditions in order to probe their mode(s) of action. Such a potential animal model may serve as a platform from which to investigate intersystemic PD regulatory mechanisms. It has been documented that there is an interaction between the CNS and immune system during progression of PD pathology [37]. Accordingly, a possible link between α-syn humorally mediated mechanisms and the pathological events in PD has been postulated [38].

Recently, we have shown that PD clinical deficits are accompanied by a specific profile of autoantibodies (Abs) generated to natively folded α-syn and its amyloidogenic oligomeric and fibrillar aggregates associated with various stages of the disease [39–41]. Consequently, we have proposed a protective role for humoral immunity against α-syn amyloid species in early stage PD and specific immunological targeting of toxic α-syn oligomers is of therapeutic interest [8,39]. In the current study, we focused on murine parkinsonian-like behavioral outcomes as well as autoimmune responses to DA and α-syn species as PD biomarkers following intranasal administration of α-syn oligomers or their combination with fibrils. Consequently, this study was intended to substantiate our previous clinical observations in PD patients [39–41].

2. Materials and methods

Dopamine (DA) was purchased from Sigma, St. Louis, MO, USA.

2.1. Subjects

Adult male C57Bl/6 mice aged 12-months and weighing 31.1 ± 1.0 g were used. Animals were group housed on a 12:12 light–dark cycle at a constant temperature of 21 °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 h and 15.00 h and animals were divided into three groups. The control group ($n = 10$) was administered saline vehicle intranasally (i.n.) in a volume of 10 μL/animal daily over a total dosing period of 10-days. The first experimental group ($n = 10$) was administered a solution of α-syn oligomeric aggregates (15.0 μg in 10 μL or 0.48 mg/kg) intranasally using the same dosing schedule. The second experimental group ($n = 10$) was co-administered aggregates of α-syn oligomers plus fibrils simultaneously each in a 50:50 concentration of 7.5 μg in 10 μL/animal (i.e. total α-syn equivalent dose = 0.48 mg/kg) i.n. in the 10-day protocol.

2.3. Behavioral tests

Animal behavioral analysis was performed in all groups before, and one day after the α-syn amyloidogenic species dosing protocol (i.e. day 11) and behavioral indicators of PD-mimetic symptoms (hypokinesia, muscle rigidity and tremor) were evaluated [42]. 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 [43,44] for a period of 3 min after 5 min acclimatization in an animal activity meter (Opto-Variomex-3 Auto-Track system, Columbus Instruments, Columbus, Ohio, USA). The 3-min recording time was chosen since it represented an optimal period for detecting MPTP-locomotor hypokinesia in 3-min intervals up to a total of 30 min in C57Bl/6 mice [45,46] Additionally, total locomotor distance, cumulative ambulation time as well as speed (horizontal activity measures) and vertical rearing (vertical activity measure) 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) = 1.0 cm decrease; (2) = 2.0 cm decrease; (3) = > 2.0 cm decrease [34]. Thirdly, the presence or absence of tremor was checked behaviorally [47] using the following scoring scale: (0) = no tremor; (1) = head tremor; (2) = head and forepaw tremor, (3) = whole body tremor.

2.4. Source and preparation of blood samples

Animal blood samples (300 μL) were collected without additives and 1 h after coagulation, samples were centrifuged for 15 min at 3500 × *g* (4 °C). Sera were collected, aliquoted into Eppendorf tubes, immediately frozen and stored at −80 °C before being defrosted and subjected to immunological analysis.

2.5. Production of α-synuclein

The *Escherichia coli* BL21 (DE3) cells transformed with pRK173 plasmid harboring the α-synuclein gene were used for the production of the recombinant protein [48]. The recombinant protein was purified as previously described [49] 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.

2.6. 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 $E_{1\ mg/ml} = 0.354$ [50]. 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.