Assessing the immunogenicity of alpha-synuclein oligomers, filaments, and fibrils in a clinically relevant model of synucleinopathy in vitro

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Introduction

α-Synuclein (αSyn) is a protein implicated in the etiopathogenesis of Synucleinopathies[1]. Aggregation of αSyn is a hallmark pathogenic process that spearheads the formation of Lewy bodies – small intracytoplasmic inclusions which lead to neuronal damage and death[1,2]. Prior to Lewy Body formation and neurodegeneration, αSyn aggregates interact directly with glial cells, inducing their activation, and initiating an inflammatory response with widespread consequences for neuronal health [3]. Of the many conformational states in which endogenous αSyn can exist, the fibrillar configuration is increasingly understood to be the species responsible for driving the initiation of pro-inflammatory pathways which trigger downstream neurodegeneration, motor deficits, and cognitive decline seen in patients with such diseases [4,5,6]. Recombinant oligomeric, filamentous, and fibrillar protein constructs of αSyn have recently been developed in efforts to establish new model systems in which synucleinopathy-related pathology may be generated, and glial-neuronal interactions triggered by αSyn can be assessed.

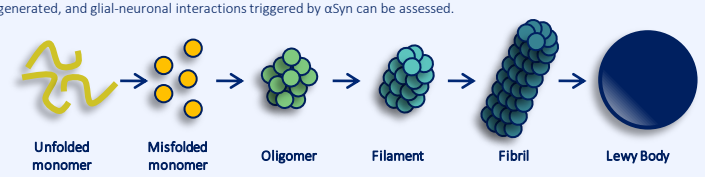


Figure 1: Schematic of αSyn fibril-formation pathway and conformation of suspected toxic species. Original image.

Experimental Aims

The present investigation explores in primary mixed glial and neuronal cultures the potential of αSyn oligomers, filaments, and fibrils, to induce glial activation, neuroinflammation, and subsequent neurodegeneration. We assess a putative role for glial cells as mediators of αSyn-induced neuronal atrophy, and specifically assess variant forms of α-syn in producing these effects.

Experimental Questions

Do αSyn oligomers, filaments, or fibrils induce morphological changes to microglia after 24-hour incubation in primary mixed glial cultures? Determined by immunoreactivity of microglial marker ionised-calcium binding adaptor molecule 1 (Iba1) (**Results 1**)

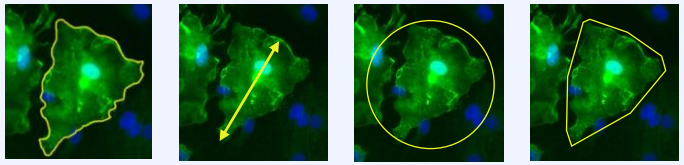
Do αSyn oligomers, filaments, or fibrils induce differential secretory profiles of pro-inflammatory cytokines from glial cells in primary mixed glia in cultures after 24-hour incubation? Determined by multiplex immunoassay of cell supernatant **(Results 2)**

Do αSyn oligomers, filaments, or fibrils induce morphological changes to microglia in primary mixed glial cultures overtime? Determined by immunoreactivity of microglial marker Iba1 **(Results 3)**

Does glial-derived conditioned media (CM) from αSyn oligomer-, filament-, or fibril-treated mixed glial cultures affect complexity of primary neurons in culture? Determined by immunoreactivity of neuronal marker microtubule-associated protein 2 (MAP2) **(Results 4)**

Glial Image Analysis

An outline of each glial cell is manually fitted using the freehand selection tool on ImageJ to generate the following parameters of interest[7,8,9]:



Area and perimeter

Feret's Diameter
Distance between two furthest points

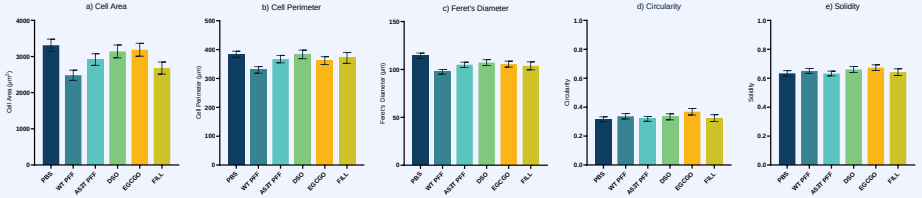
Circularity
$(4\pi*(Area)/(Perimeter)^2)$

Solidity
(Convex area / cell area)

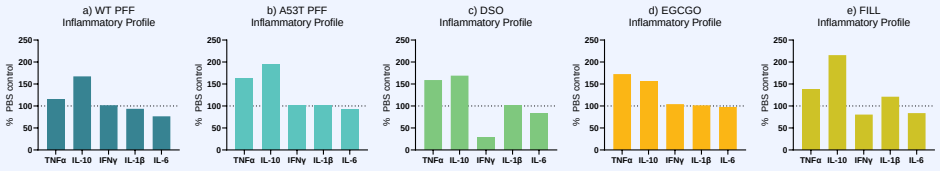
αSyn preparations, provided by Stressmarq Biosciences ltd.

PBS	WT PFF	A53T PFF	DSO	EGCGO	FILL
Phosphate Buffered Saline	Wild-Type Preformed Fibril	A53T Mutated Preformed Fibril	Dopamine-Stabalised Oligomer	EGCG-stabalised Oligomer	Filament

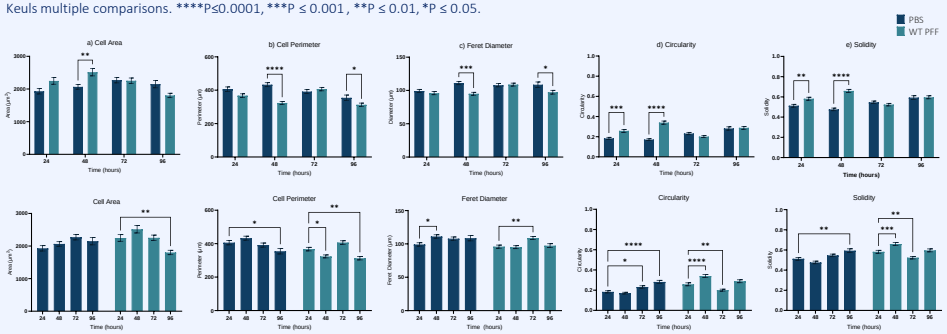
Results 1: Neither αSyn oligomers, filaments, nor fibrils induce morphological changes to microglia in primary cortical mixed glial cultures at 24 hours. Mature primary cortical mixed glia (DIV14) are treated with 4μg/ml WT PFF, A53T PFF, DSO, EGCGO, FILL, or PBS for 24 hours. Cells are fixed and fluorescently stained for Iba1. Image analysis is performed on ImageJ to quantity a) cell area, b) cell perimeter, c) Feret's diameter, d) circularity, and e) solidity. Data are expressed as mean ± SEM. n = 15-63 cells per treatment group per experiment, from 3 independent experiments. One-way ANOVA.



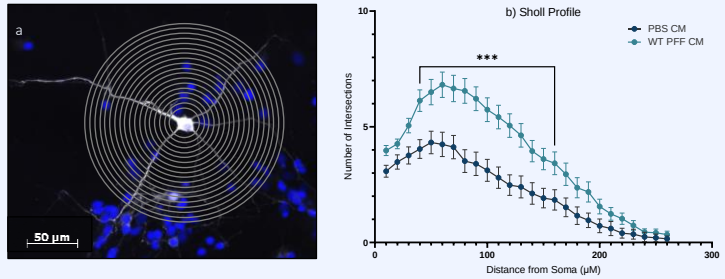
Results 2: αSyn oligomers, filaments, and fibrils promote microglial release of proinflammatory cytokine TNFα and anti-inflammatory cytokine IL-10. Mature primary cortical mixed glia (DIV14) are treated with 4μg/ml a) WT PFF, b) A53T PFF, c) DSO, d) EGCGO, e) FILL or PBS for 24 hours. Concentration of cytokines IFNγ, TNFα, IL-1β, IL-10, and IL-6 in the resulting conditioned media is quantified using Mesoscale Diagnostic's multiplex immunoassay kit. Data represent mean of 2 replicate values, from 1 independent experiment, expressed as % of cytokine levels in PBS-treated control cultures.

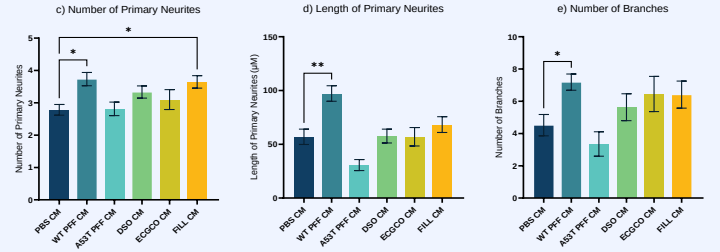


Results 3: WT PFFs enhance the time-dependant activation of microglia in primary cortical mixed glial cultures at 48 hours. Mature primary cortical mixed glia (DIV14) are treated with WT PFF (4ug/ml) or PBS for 24, 48, 72 or 96 hours. Cells are fixed and fluorescently stained for Iba1. Image analysis is performed on ImageJ to quantity a) cell area, b) cell perimeter, c) Feret's diameter, d) circularity, and e) solidity. Graphs in top row indicate significant changes between treatment groups within time points, graphs in bottom row indicate significant changes between 24-hours and other time points within treatment groups. Data are expressed as mean ± SEM. n = 45-60 cells per treatment group per experiment, from 3 independent experiments. Two-way ANOVA with Newman-Keuls multiple comparisons. ****P≤0.0001, ***P ≤ 0.001 , **P ≤ 0.01, *P ≤ 0.05.



Results 4: Neither αSyn oligomer-, filament-, nor fibril-treated conditioned media from 24-hour-stimulated mixed glial cultures affects complexity of mature primary neurons. Changes in complexity likely result from trophic effects of conditioned media on neuronal cultures. Mature primary cortical mixed glia (DIV14) are treated with 4μg/ml WT PFF, A53T PFF, DSO, EGCGO, FILL, or PBS for 24 hours. After 24 hours, the conditioned media is removed and used to treat mature primary cortical neurons (DIV14) for 24 hours. Cells are fixed and fluorescently stained for MAP2. a) Sholl analysis is performed to quantify b) Sholl profile, c) number of primary neurites, d) length of primary neurites, and e) number of secondary branches. Data are expressed as mean ± SEM. n = 45-50 cells per treatment group per experiment, from one independent experiment. b) Two-way ANOVA with Newman-Keuls multiple comparisons, c-e) One-way ANOVA. **P ≤ 0.01, *P ≤ 0.05.





Conclusions

Although not apparent through morphological analysis at 24 hours, mixed glial cultures treated with oligomers, filaments, and fibrils exhibit an early-stage pro-inflammatory phenotype through increased release of TNFα, likely from microglia. IL-10 is likely released concomitantly with TNFα to combat potentially deleterious effects of αSyn-induced inflammation. While no changes in IL-1β or IL-6 are observed after 24 hours, their release may be triggered at a later time point, as indicated by glial morphological analysis. After 48-hour incubation, microglia adopt an ameboid-like morphology, indicative of activation. Subsequent application of pro-inflammatory-enriched conditioned media to primary cortical neuronal cultures may drive a reduction in neuronal complexity. With the development of this in vitro model, we continue to explore the cellular and molecular mechanisms underpinning the process by which αSyn mediates reactive-glial-associated neurodegeneration, and generate a platform upon which to develop neuroprotective treatment strategies which target these mechanisms. Clinically, immunomodulatory agents may prove useful in the treatment of neurodegenerative conditions associated with brain inflammation.

References

[1]Spillantini *et al.* 1997, [2]Bendor et al., 2013, [3]Sanchez-guajardo et al., 2015, [4]Volpicelli-Daley et al., 2011, [5]Volpicelli-Daley et al., 2014, [6]Gribaudo et al., 2019, [7]Heppner et al., 1998, [8]Zanier et al., 2015, [9]Msibi et al. 2019

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