Glial-Neuronal Interaction Prompted by Recombinant Fibrillar Protein Constructs of Alpha-Synuclein – an in vitro Characterisation

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Irish Research Council An Chomhairle um Thaighde in Éirinn

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Introduction

Alpha-synuclein (αSyn) is a 14 kDa soluble cytosolic protein expressed abundantly at neuronal synaptic terminals[1]. Intracellular aggregation of this misfolded protein represents a hallmark of synucleinopathy-related neurodegenerative disorders – Lewy bodies[2,3]. Of the many conformational states in which endogenous αSyn can exist, the fibrillar configuration is increasingly understood to be the primary toxic species which triggers the neuroinflammation, neurodegeneration, motor deficits, and cognitive decline seen in patients with such diseases[4,5,6]. Recombinant fibrillar protein constructs of αSyn have recently been developed in efforts to establish new model systems in which synucleinopathy-related pathology may be generated.

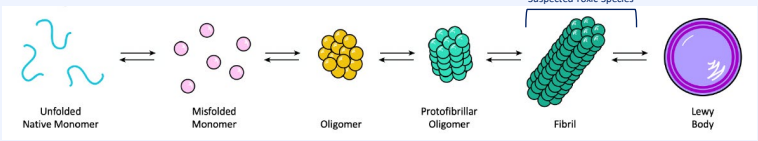


Figure 1: Schematic of αSyn fibril-formation pathway and conformation of suspected toxic species[9].

Experimental Aims

The present investigation explores the potential of αSyn preformed fibrils (PFFs) to induce glial activation, neuroinflammation, and subsequent neurodegeneration. Here, we explore in primary mixed glial (MxG) and neuronal cultures glial-neuronal interactions triggered by administration of fibrillar αSyn preparations provided by StressMarq Biosciences Inc.

Research Questions

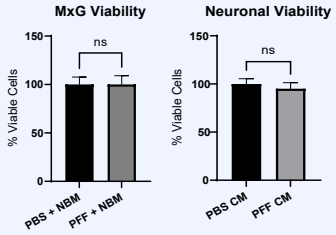
- Do αSyn PFFs affect cell viability in primary MxG or neuronal cultures? **(Results 1)**
- Do αSyn PFFs induce modifications to astrocytic morphology - determined by immunoreactivity of the astrocytic marker glial fibrillary acid protein (GFAP) - in MxG cultures? **(Results 2, 4)**
- Do αSyn PFFs induce modifications to microglial morphology - determined by immunoreactivity of microglial marker ionised calcium binding adaptor molecule 1 (Iba1) - in MxG cultures? **(Results 3, 5)**
- Do αSyn PFFs, when administered via CM from MxG cultures, induce modifications to neuronal complexity or synaptic integrity of primary neuronal cultures? **(Results 6)**

Method of PFF Preparation and Treatment

Type 1 αSyn PFFs (Stressmarq Biosciences Ltd.) are thawed at room temperature and diluted in sterile Phosphate-Buffered Saline (PBS) to a concentration of 100µg/mL. In solution, PFFs are sonicated for 6 sets of 10 x 0.5 second pulses, with a 10 second pause between each set to obviate heat generation and possible protein denaturation. Sonicated PFFs are further diluted to a working concentration of 4µg/mL in sterile Neural Basal Medium (NBM), which is administered directly to primary MxG cultures at 14-days in vitro (DIV)[5,7]. Control MxG cultures are treated with NBM with equivalent volumes of PBS. After 24 or 48 hours, CM from both treatment groups is lifted, filter-sterilised, and used for treatment of mature primary neurons in DIV14 cultures. The results presented are derived from a single experiment with 100-200 cells analysed per treatment group per time point.

Results 1: Cell viability is not affected by treatment of mixed glial (direct) or neuronal cultures (CM) with αSyn PFFs for 24hours (4µg/mL).

Cell Counting Kit 8 (CCK8) colorimetric assay confirmed no significant difference in viability of mixed glia treated with PFF for 24 hours compared to PBS-treated controls, or of neuronal cultures treated with respective PFF and PBS-control CMs. Data represent mean ± SEM (ns = P > 0.05; Mann-Whitney U test).

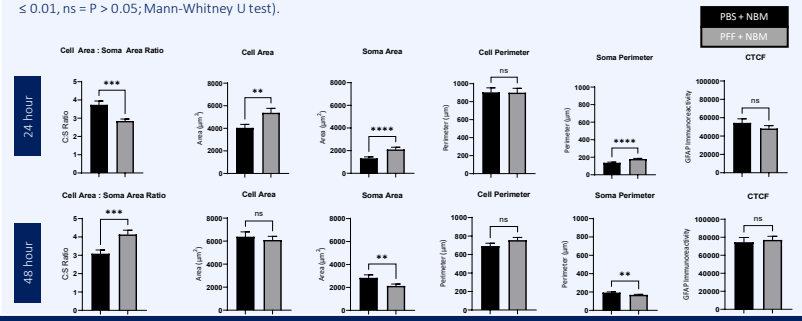


References

[1]Burré et a., 2010, [2]Bendor et al., 2013, [3]Spillantini *et al.* 1997, [4]Volpicelli-Daley et al., 2011, [5]Volpicelli-Daley et al., 2014, [6]Gribaudo et al., 2019, [7]McNamee et al, 2010, [8]Wong and Krainc, 2017, [9]Illustration by Broder-Rodgers, 2020.

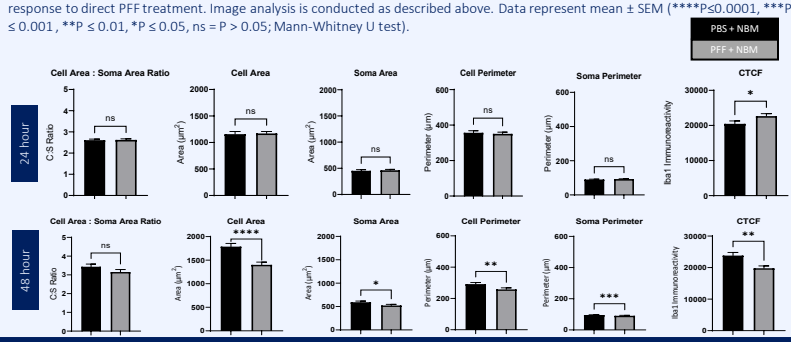
Results 2: αSyn PFFs induce morphological changes to astrocytes in primary mixed glial cultures at 24- and 48-hours post-treatment.

GFAP immunostaining is conducted to examine the morphology, complexity, and immunoreactivity of astrocytes in response to direct PFF treatment. Cell area and perimeter are quantified using the particle measurement feature on ImageJ, while GFAP immunoreactivity is quantified using the integrated density feature. Corrected total cell fluorescence (CTCF) = [integrated density – (area of selected cell x mean fluorescence of background)]. Data represent mean ± SEM (****P≤0.0001, ***P ≤ 0.001, **P ≤ 0.01, ns = P > 0.05; Mann-Whitney U test).



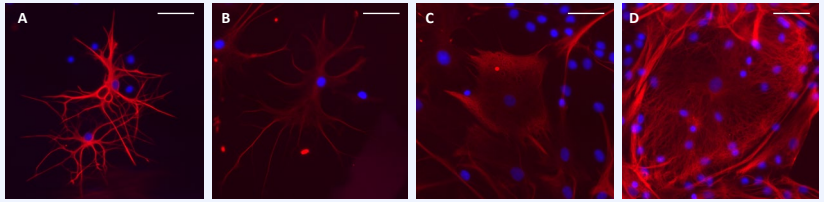
Results 3: αSyn PFFs induce morphological changes to microglia in primary mixed glial cultures at 48-hours post-treatment.

Iba1 immunostaining is conducted to examine the morphology, complexity and immunoreactivity of microglia in response to direct PFF treatment. Image analysis is conducted as described above. Data represent mean ± SEM (****P≤0.0001, ***P ≤ 0.001 , **P ≤ 0.01, *P ≤ 0.05, ns = P > 0.05; Mann-Whitney U test).



Results 4: Stage-based differences in GFAP-expressing immunoreactive astrocytes show cells on an activation-based morphological trajectory.

While uniformity of astrocytic morphology was not observed within treatment groups in mixed glial cultures, four astrocytic phenotypes - which may represent distinct activation stages in cell transformation - were identified. Representative images of astrocytic morphology describe cell types with a) a small cell body with extensive ramifications, b) a larger cell body with retracting ramifications c) a large cell body with short extending processes, and d) a large, hypertrophic cell with few to no processes. Immunoreactivity for GFAP (red) and DAPI (blue). Scalebar represents 50 µM.



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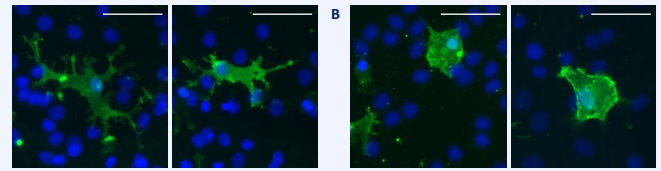
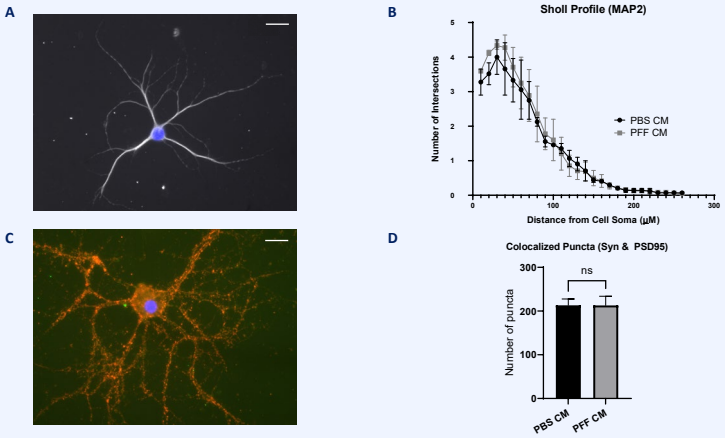
Results 5: Iba1-expressing immunoreactive microglia exist in two distinct morphologies.

Two well-described microglial phenotypes are identified within these MxG cultures, representative images of which are presented below. A) Extensive and ramified phenotypes characterise resting microglia, which serve to maintain a healthy inflammatory state in the CNS by surveying the surrounding environment. B) Rounded ameboid phenotypes characterise activated microglia, typically stimulated by inflammatory insults, foreign particles, or damaged cellular components. Uniformity of microglial morphology was not observed within distinct treatment groups in mixed glial cultures. Immunoreactivity for Iba1 (green) and DAPI (blue). Scalebar represents 50 µM.



Results 6: Conditioned media from mixed glial cultures treated with PFFs have no effect on mature neuronal complexity or synaptic integrity.

A) MAP2 immunostaining and B) Scholl analysis confirmed no changes in neurite morphology or complexity in response to PFF. C, D) Synaptophysin (Syn) and postsynaptic density protein 95 (PDS95) immunostaining demonstrated uniform expression and co-localisation of pre- and post-synaptic markers within primary neurons across both treatment groups, representative of synapse integrity. Synaptic marker co-localisation was analysed with the ImageJ Puncta Analyser Plugin. Data represent mean ± SEM (ns = P > 0.05; Mann-Whitney U test). Immunoreactivity for MAP2 (white), DAPI (blue), Syn (green), PSD95 (red), and Syn+/PSD95+ (orange). Scalebar represents 20 µM.



Conclusions

Astrocytic response to PFF: At 24 hours, astrocytes transition from star-like resting state to hypertrophied activated state in response to application of PFF. This effect is reversed after 48 hours, at which the increased cell to soma ratio indicates a transition from activated to resting state. While no apparent changes in GFAP expression are identified through immunostaining, changes in GFAP expression at mRNA level within this time frame may underpin protein-level changes observed at more distal time points.

Microglial response to PFF: At 24 hours, no morphological differences between PFF- and PBS-treated cultures are observed. The subsequent decrease in cell area, soma area, cell perimeter, and soma perimeter in response to PFF at 48 hours indicates retraction of microglial processes and shift to an activated state upon treatment administration. Decreased Iba1 immunoreactivity in response to PFF could suggest reduced protein expression, but is likely a result of reduced surface area upon which Iba1 can be identified.

Neuronal response to PFF: Neuronal complexity and synapse formation may be affected by CM containing PFF at higher concentrations, or CM from MxG exposed to PFF for longer. A difference in complexity and/or integrity between neurons treated directly with PFF and neurons treated with CM drawn from PFF-treated glia may unveil mechanisms by which αSyn induces neurodegeneration via inflammatory processes.

Follow-Up Research Questions

- Do αSyn PFFs induce modifications to GFAP or Iba1 expression at the mRNA level within MxG cultures at these time points? **(PCR)**
- What are the optimal treatment dose and time conditions required to observe representative synucleinopathy-related cellular pathologies with Type 1 αSyn PFFs ? **(Dose-response)**