Application of Patient-Derived Induced Pluripotent Stem Cells (iPSCs) to Study the Role of Neurite Inhibition and Mechanisms of Recovery in Alzheimer's Disease

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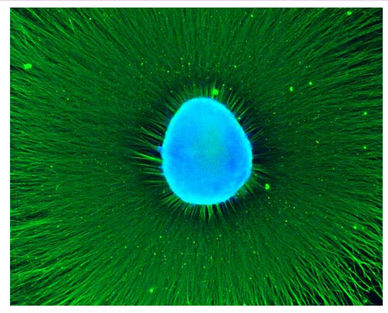


Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disease that leads to a clinical decline in neurological function due to a **loss of neuronal connectivity** in the brain[1-3]. The aberrant processing and accumulation of proteins is a hallmark of AD, particularly the extracellular **deposition of β-amyloid** (Aβ) peptides that leads **to senile plaque** formation and **morphological abnormalities** in developing neurites[4]. An increase in the ratio of the 42 amino acid Aβ species ($A\beta_{42}$) , that is longer and more insoluble, compared with the 40 amino acid long Aβ species ($A\beta_{40}$) is a feature of the disease[5,6].

This study utilises **iPSC-derived AD-model cell** lines to study the process of Aβ induced neurite inhibition and the molecular signalling pathways that drive the process. Aβ is known to bind to the **Nogo receptor** (NgR)[7], which is involved in the inhibitory response that results in the inability of neurons to regenerate following **spinal cord injury** (SCI)[8,9]. NgR is known to activate **Rho A and ROCK** following SCI[8,9], impacting actin dynamics and resulting in **neurite retraction**. Evidence also suggests that Aβ induction of neurite inhibition involves Rho A and ROCK[10] activation.

The principal aim of this study was to further elucidate the molecular signalling events involved in Aβ-mediated neurite inhibition through adding **inhibitors** of **Rho A** (**ibuprofen**) and **ROCK** (**Y-27632**) signalling to **two distinct models** of **Aβ-induced neurite inhibition**. The first of which involves the **exogenous** addition of Aβ species to neuronal cultures, and the second involves the use of **iPSC-derived** patient and transfected **AD-model cells.**

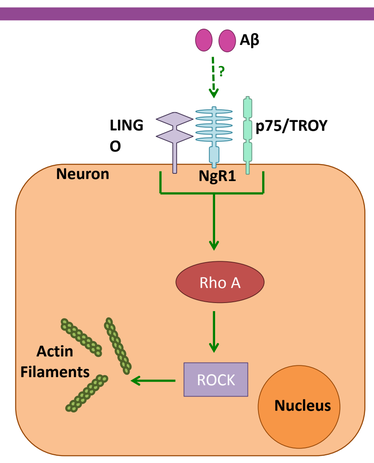


Figure 1: Proposed molecular signalling pathways involved in Aβ-mediated neurite inhibition. *Aβ is known to bind to the NgR involved in neurite inhibition following SCI. NgR activates Rho A and ROCK, which ultimately results in actin stabalisation. This effect upon cellular actin dynamics results in growth cone collapse and neurite retraction. Aβ is thought to activate Rho A and ROCK, however it is not yet known if this is through activation of NgR.*

Methodology

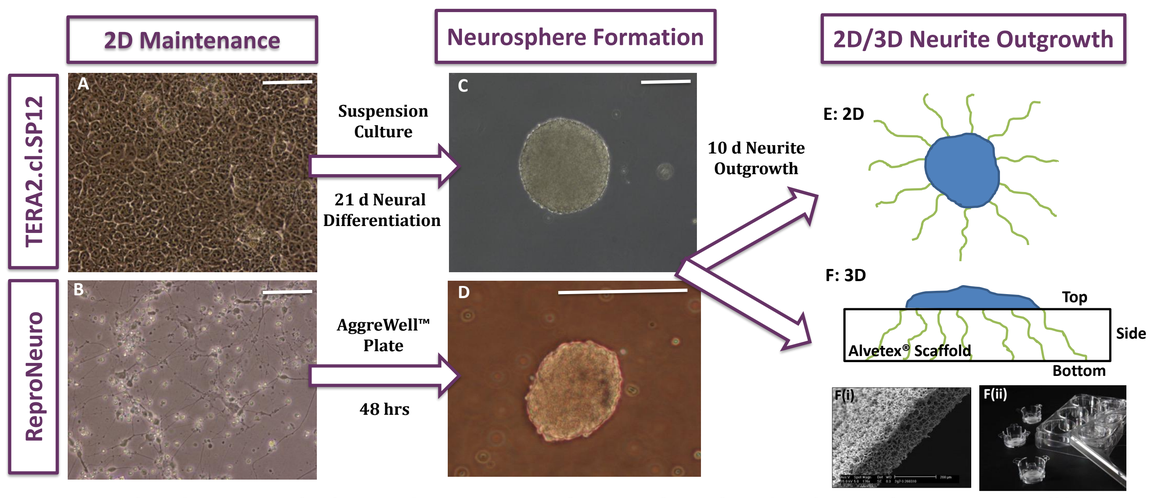
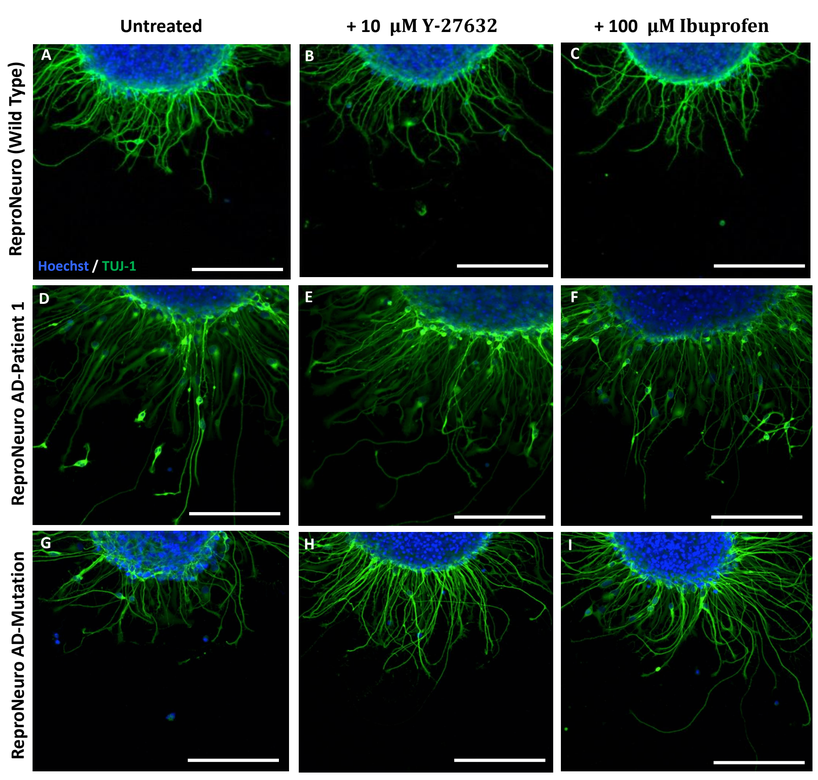


Fig 2: Neurosphere formation and induction of neurite outgrowth in 2D and 3D culture from human pluripotent stem cell lines. *Neurospheres were formed from both embryonal carcinoma (EC) stem cell and induced pluripotent stem cell (iPSC) derived cell lines. The EC cell line, TERA2.cl.SP12 was first maintained in 2D monolayer culture (A) before being seeded in suspension and differentiated for 21 days with the synthetic retinoid EC23. This promoted the formation of mature neurospheres (C) for use in neurite outgrowth studies. Neurospheres were also formed from the iPSC-derived neuroprogenitor line, ReproNeuro, which were initially maintained as monolayer cultures (B). AggreWell™ plates were used to form spheroid structures (D). Neurospheres from both cell lines were subsequently seeded onto 2D (E) or 3D (F) ECM coated growth substrates for a further 10 days during which time neurites are formed. In 2D culture neurites radiate from the central neurosphere, whereas in 3D culture neurite penetrate the 200 µm depth of the scaffold and are visible from the underside of the scaffold. To study the effect of Aβ species on neurite outgrowth, exogenous Aβ was added to cultures of EC-derived neurospheres and ReproNeuro AD model cell types were used to further study this mechanism.*

ReproNeuro Patient-1 cells are neuroprogenitor cells derived from an AD patient with a mutation in presenilin 2 (PS2) and ReproNeuro Mutation are neuroprogenitor cells derived from a healthy individual transfected with a mutation in presenilin 1 (PS1). Presinilins form the basis of the γ-secretase complex involved in generation of Aβ species and both cell types can be used to model the aberrant generation of Aβ associated with AD. Scale Bars: A,B and D: 200 µm C: 500µm.

Induction of Neurite Outgrowth from Healthy and AD-Model Cells



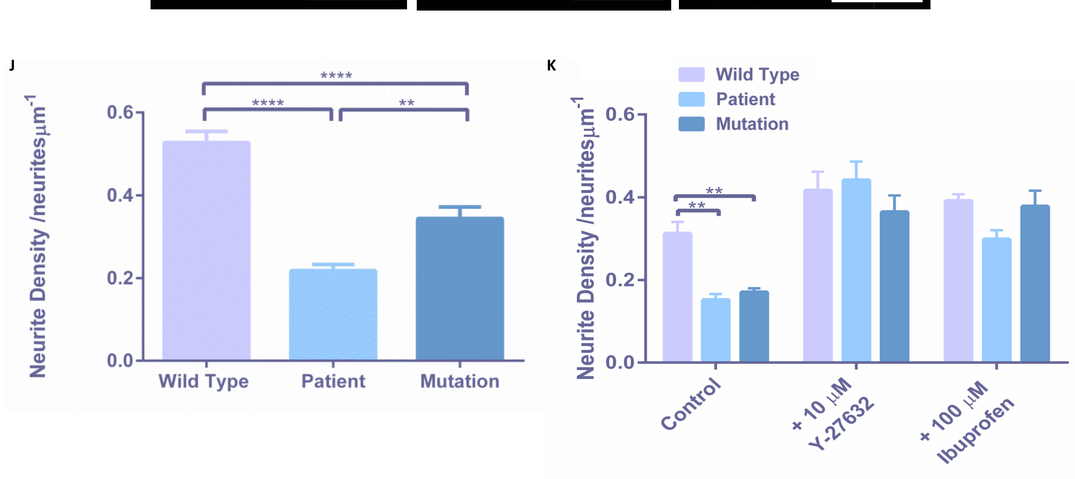
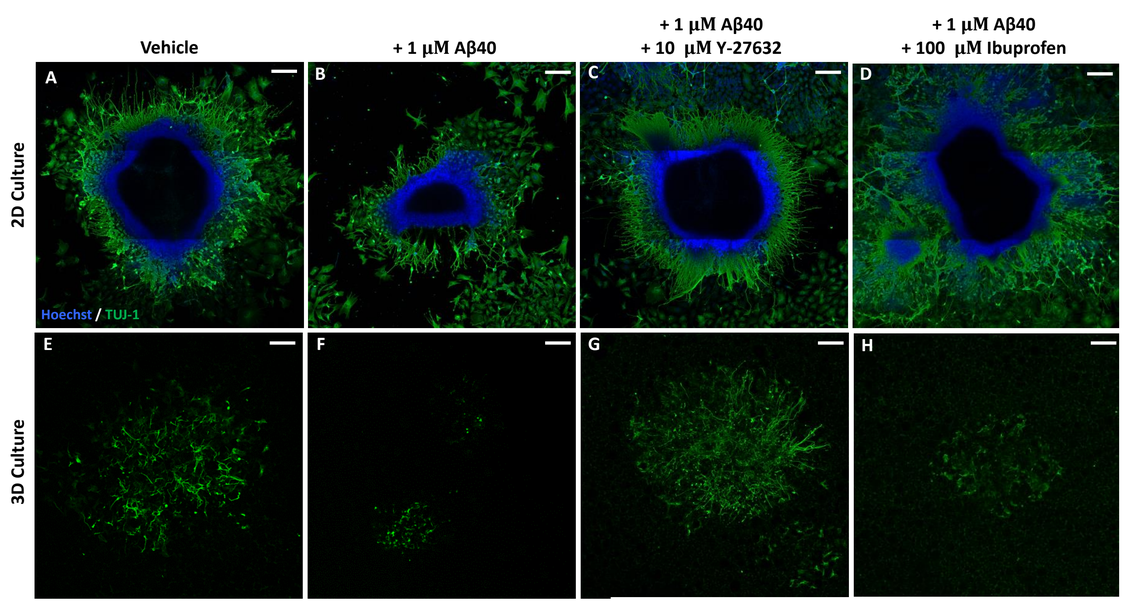


Fig 4: Neurite outgrowth is inhibited from Alzheimer's disease model cells in 2D culture and recovered by inhibition of Rho A and ROCK.
*ReproNeuro iPSC-derived wild type (A-C) and AD-model cells (D-I) were cultured in 2D with the selective ROCK inhibitor Y-27632 and inhibitor of Rho A ibuprofen, to identify any differences in their ability to form neurites. Patient derived neurospheres produce significantly less dense neurite outgrowth than any other cell type tested (J) and both patient and mutation AD-model cell lines produce significantly less dense neurite outgrowth than wild type cells. Medium supplementation with Y-27632 enhanced neurite density to a level greater than the control and reduced any differences between the cell types (K). Similarly, ibuprofen treatment also reduced any differences in neurite density between the cell types tested. Scale bars: 100 µm, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001*

Neurite Inhibition Induced by Exogenous Aβ



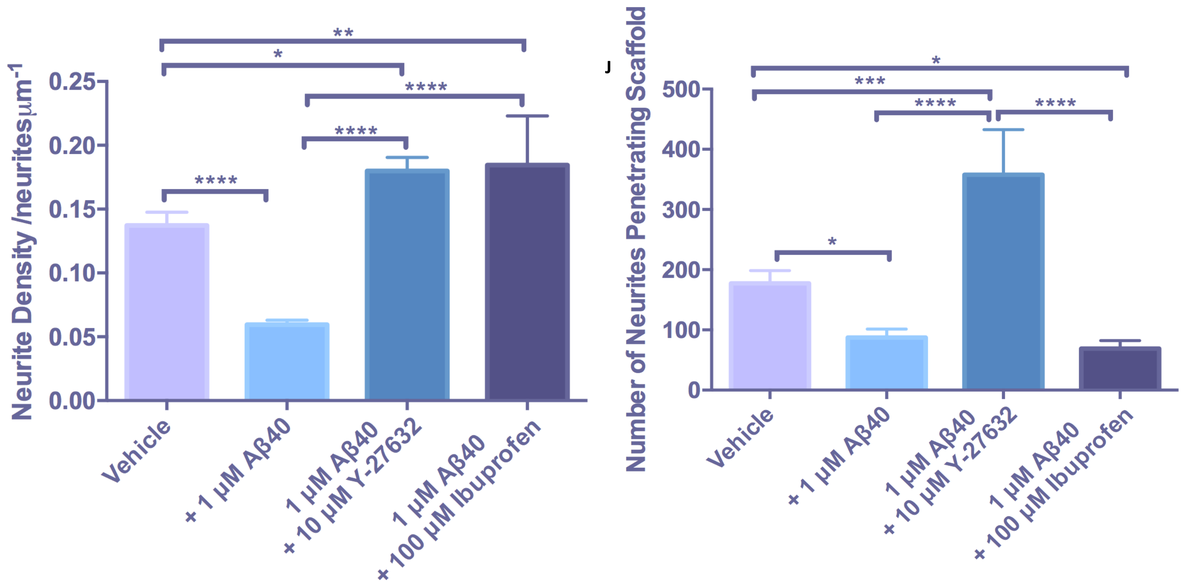


Fig 3: Exogenous Aβ species inhibit neurite outgrowth and is recovered through inhibition of Rho A and ROCK.
*Neurospheres derived from EC cells were cultured in 2D (A-D) and 3D (E-H) conditions with the exogenous addition of $A\beta_{40}$ to the culture medium (B,F). In addition to this treatment, neurospheres were also treated with the selective ROCK inhibitor Y-27632 (C,G) and inhibitor of Rho A, ibuprofen (D,H). This approach was used to determine if $A\beta_{40}$ supplementation had an inhibitory effect on neurite growth and if inhibition of ROCK and Rho A known to be involved in downstream NgR and Aβ signalling was capable of recovering such an effect. Both quantification of neurite density from neurospheres cultured in 2D (I) and neurite penetration in 3D cultures (J) revealed that $A\beta_{40}$ significantly reduced neurite outgrowth, whilst Y-27632 treatment restored neurite outgrowth despite the presence of $A\beta_{40,}$ enhancing levels of neuritogenesis compared with the vehicle matched control. Ibuprofen had a similar effect in 2D culture enhancing neurite outgrowth to a level that surpassed the control, however ibuprofen treatment had no effect upon on neurite outgrowth in 3D culture. Scale bars: 200 µm, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001*

Conclusions

- **Exogenous** addition of **$A\beta_{40}$** to culture medium significantly **inhibits neurite outgrowth** in both 2D and 3D culture.
- **Inhibition** of **Rho A** and **ROCK** by ibuprofen and Y-27632 respectively can **restore neurite outgrowth** in the presence of inhibitory $A\beta_{40}$.
- Alzheimer's disease **model cells** with common AD-associated **mutations** provide a **novel system** to study the role of $A\beta_{40}$ in neurite inhibition.
- **Neurite density** in 2D culture is **reduced** in **AD-model cells** compared with their wild type counterpart.
- **Inhibition** of **Rho A** by ibuprofen and **ROCK** by Y-27632 **restores neurite density** in AD-model cells.
- **Rho A** and **ROCK** signalling potentially involved in **Aβ-mediated neurite inhibition**.
- **Neurite outgrowth assay** utilising AD-model cells can be used as a potential **screening tool** and has implications in the field **of personalised medicine**.

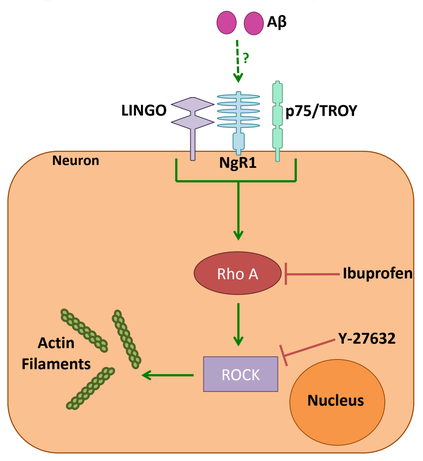


Fig 5: Inhibition of Aβ induced signalling.
It is not known whether Aβ activates NgR itself, however, Aβ is known to activate downstream Rho A and ROCK. This is thought to be the major signalling mechanism responsible for Aβ-induced neurite inhibition. For this reason inhibition of Rho A and ROCK through modulators such as ibuprofen and Y-27632, is a potential method of intervention that could restore neurite outgrowth and neural connectivity in the context of AD.

References

1. La Ferla et *al*, Intracellular amyloid-beta in Alzheimer's disease, Nature Reviews Neuroscience. (2007) 8(7)499-509 2. Cummings *et al*, Alzheimer's disease drug-development pipeline: few candidates, frequent failures, Alzheimer's Research & Therapy. (2014) 6(4):37 3. Hardy *et al*, Pathways to Alzheimer's disease, Journal of Internal Medicine. (2014) 275(3):296-303 4. Grutzendler *et al*, Various dendritic abnormalities are associated with fibrillar amyloid deposits in Alzheimer's disease, Annals of the New York Academy of Sciences. (2007) 1097(1):30-9 5. Sauvee *et al*, Additional use of Aβ42/Aβ40 ratio with cerebrospinal fluid biomarkers P-tau and Aβ42 increases the level of evidenace of Alzheimer's disease pathophysiological process in routine practice, J. Alzheimer's Disease. (2014) 41 6. Dumurgier *et al*, Cerebrospinal fluid amyloid-β 42/40 ratio in clinical setting of memory centers: a multicentric study, Alzheimer's Research and Therapy. (2015) 7(1):1-9 7. Park *et al*, Nogo Receptor Interacts with Brain APP and Aβ to Reduce Pathologic Changes in Alzheimer's Transgenic Mice, Current Alzheimer Research. (2007) 4(5):568-570 8. Yiu & He, Glial inhibition of CNS axon regeneration, Nature Reviews Neuroscience (2006) 7(8):617-626 9. Fawcett & Asher, The glial scar and central nervous system repair, Brain Research Bulletin (1999) 49 (6):377-391 10. Petratos *et al*, The beta-amyloid protein of Alzheimer's disease increases neuronal CRMP-2 phosphorylation by a Rho-GTP mechanism, Brain. (2008) 131:90:108