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Title of the article: Modulation of the Nogo signaling pathway to overcome β-amyloid mediated neurite inhibition in human pluripotent stem cell derived neurites

Contributors

1. Kirsty Goncalves [1]
2. Stefan Przyborski [1,2]
3.
4.

Department(s) and institution(s)

[1] Department of Biosciences, Durham University, Durham, UK

[2] Reprocell Europe Ltd, Glasgow, UK

Corresponding Author:

Prof. Stefan Przyborski, Department of Biosciences, Durham University, Durham, DH1 3LE, UK, +44(0) 191 3343988, stefan.przyborski@durham.ac.uk

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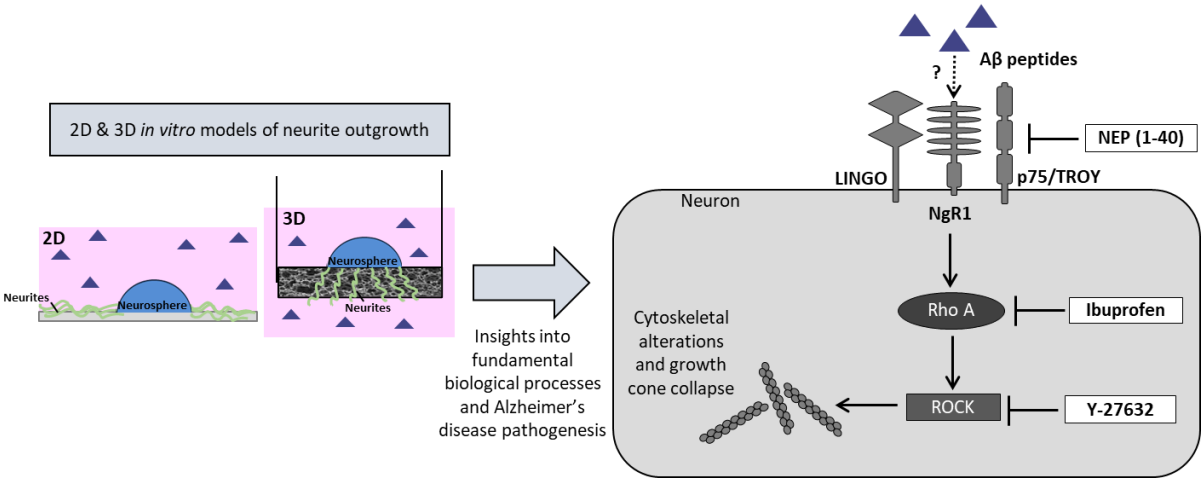
Abstract: Neuronal cell death and loss of connectivity is one of the main pathological mechanisms of Alzheimer's disease (AD). Accumulation of β-amyloid (Aβ) peptides, a major hallmark of AD is thought to induce neuritic abnormalities such as reduced growth, extension, and abnormal growth cone morphology, all of which contribute to reduced connectivity. In this study, we use an innovative approach to demonstrate the impact of Aβ on neurite dynamics in both 2D and 3D culture systems, in order to provide a more physiologically relevant culture geometry. We utilize a number of methodologies including addition of exogenous Aβ peptides to culture medium, growth substrate coating and use of induced pluripotent stem cell (iPSC) technology to explore the role of endogenous Aβ secretion on neurite outgrowth, and thus provide scope for future use within personalized medicine. In addition to this, we also explore the involvement of the Nogo signalling cascade within Aβ-induced neurite inhibition. We evidence that inhibition of downstream Rho A and ROCK through modulation with Y-2732 and Ibuprofen respectively can restore and even enhance neuronal connectivity in the presence of Aβ. In summary, this study provides both an application for a novel culture approach with the possibility to provide insight into the biological process of neurite growth and inhibition, but also proposes a specific mechanism for reduced neural connectivity in the presence of Aβ-peptides with points of intervention that restore neurite growth. Thereby, creating a culture system with potential as an assay to measure pre-clinical, predictive outcomes of drugs and their ability to enhance neurite outgrowth both in a broad sense and also in a patient-specific manner.

Key-words: neurite outgrowth, Alzheimer's disease, Nogo, ROCK, Rho A, iPSC, stem cell, neuron, 3D culture, actin, neuroregeneration, neurodegenerative

Graphical abstract:



Introduction:

Alzheimer's disease (AD), a common neurodegenerative disease, leads to progressive memory loss and functional decline (Kumar et al., 2015). AD symptoms reduce life expectancy, quality of life, and result in physical disability. This places a burden not only on those affected and their families, but also economically due to an ageing population (Sadigh-Eteghad et al., 2015). One of the most widely recognised pathological hallmarks of AD is the extracellular accumulation of aggregated β-amyloid (Aβ) in senile plaques (Murphy and LeVine, 2010; Kumar et al., 2015).

Aberrant processing of amyloid precursor protein (APP) gives rise to two major Aβ peptides: $A\beta_{40}$ and $A\beta_{42}$ (Zhang et al., 2011). The longer $A\beta_{42}$ readily forms insoluble fibrils, and is more closely linked to AD pathogenesis (Zhang et al., 2011). Elevated levels of $A\beta_{42}$ relative to $A\beta_{40}$ is critical for disease pathogenesis and even considered diagnostic (Spies et al., 2010; Dumurgier et al., 2015; Lewczuk et al., 2015). As neurites pass through senile plaques, they lose their characteristic morphology and retract, disrupting signal transduction, which contributes to memory loss (Jin et al., 2011; Serrano-Pozo et al., 2011). Dendritic abnormalities have been identified in the brains of AD-patients, including spine loss, shaft atrophy, bending, branch breaking and sprouting (Grutzendler et al., 2007), which have been recapitulated *in vitro* through application of Aβ-species to cell based model systems (Jin et al., 2011). This reduction in connectivity as a consequence of Aβ-deposition and neurite inhibition, is thought to contribute to a reduction in plasticity, signal transduction and even cell-cell communication via synapses (Knowles et al., 1999). This, in combination with other pathogenic mechanisms, is thought to contribute to memory loss, functional decline and disease progression, despite the underlying molecular mechanisms remaining relatively unknown.

In this study, we combine several advanced *in vitro* techniques to provide insights into the biological mechanism that governs Aβ-mediated neurite inhibition. These include methods of exogenous Aβ peptide presentation, a 3D culture system and multiple stem cell technologies to demonstrate the extent of Aβ's impact on neurite extension. We utilise both a well-established neurite outgrowth methodology based on a robust model of neural differentiation from a embryonal carcinoma cells, and induced pluripotent stem cell neuroprogenitor cells that allow for patient or disease-specific studies. Through this combination of innovative technologies we aim to understand the cellular impact of Aβ on neurite dynamics by both a simplistic, reductionist approach and through increasing complexity and physiological relevance. This has allowed us to demonstrate the inhibitory capacity of both $A\beta_{40}$ and $A\beta_{42}$ alone and in combination.

The platform upon which this investigation is built, is a well-characterised model system involving differentiation and neurite outgrowth from a well-established embryonal carcinoma cell line (Clarke et al., 2017). This robust, reproducible and quantifiable model of neurite outgrowth has been applied to many other investigations such as glial scar-mediated neurite inhibition (Clarke et al., 2017) and the role of extracellular matrix (ECM) motifs in neurite development (Goncalves et al., 2023). To advance this study and re-create a more physiologically relevant system capable of endogenous Aβ production, we also applied this methodology to induced pluripotent stem cell (iPSC) derived neuroprogenitor cells that express a mutation in Presenilin-1 (PSEN1), an enzyme involved in amyloidgenic processing (Cai et al., 2015).

These two, novel platforms have allowed us to probe the molecular pathways that govern Aβ-mediated neurite inhibition and establish a link between Nogo-receptor activation (a formidable step in glial scar-mediated inhibition), activation of Rho A/ROCK and neurite retraction. We demonstrate the use of ibuprofen (a non-steroidal anti-inflammatory drug, NSAID and Rho A inhibitor) and Y-2372 (selective ROCK inhibitor) to enhance neurite outgrowth despite the presence of inhibitory Aβ. This validates the use of this *in vitro* model system as an assay to measure neurotoxicity and subsequently screen methods of recovery, acting as a pre-clinical tool that can help to streamline the drug development pipeline, in a robust and quantifiable manner.

Materials and Methods:

Cell Culture: Induction of neurite outgrowth from human pluripotent stem cells

Neurite outgrowth was induced from the embryonal carcinoma (EC) cell line, TERA2.cl.SP12, as previously described (Clarke et al., 2017). ReproNeuro, iPSC derived neuroprogenitor cells (ReproCELL Europe Ltd.) were revived directly into an AggreWell™ 800 plate (STEMCELL Technologies, Cambridge, UK) to induce cellular aggregation. Neurospheres were then placed on 2D (48-well tissue culture plate (Greiner Bio-One, Stonehouse, UK)) or 3D (Alvetex® Scaffold (ReproCELL Europe Ltd.)) surfaces coated overnight in 10 $\mu g.mL^{-1}$ poly-D-lysine (Sigma-Aldrich, Irvine, UK) and laminin (Sigma-Aldrich) and cultured in the presence of mitotic inhibitors for 10 days, during which time, neurites extended from the neurosphere mass. Throughout this 10 day neurite outgrowth phase, additives were included in the culture medium: 1 μM $A\beta_{40}$ (Sigma-Aldrich), 1 μM $A\beta_{42}$ (Tocris Bioscience, Bristol, UK), 1 μM NEP (1-40) (Tocris), 10 μM Y-27632 (Tocris) and 100 μM ibuprofen (Sigma-Aldrich). Following the 10 day neurite outgrowth period, cultures were fixed in 4 % PFA for immunofluorescent analysis.

Alternatively 2D growth substrates (48-well tissue culture plates) were coated with Aβ peptides prior to seeding of neurospheres at a concentration range of 0.05-50 $\mu g.mL^{-1}$ overnight and were washed twice PBS prior to neurosphere seeding.

Test compounds were added to mature cultures during the neurite outgrowth phase of growth and not during the differentiation process, therefore their impact on neurite growth specifically has been tested in this study, as opposed to their role in neuronal differentiation.

Peptide Aggregation Assay

The PROTEOSTAT® Protein Aggregation Assay (Enzo, Exeter, UK) was used to measure aggregation of Aβ peptides as per manufacturer's instructions and analysed using the Biotek Synergy H4 fluorescent plate reader with excitation of 550 nm and emission of 600 nm.

Immunofluorescence analysis of 2D and 3D cultures

Both 2D and 3D neurite outgrowth models were immunostained for TUJ-1 (anti-β-III-tubulin, TUJ-1, ARG62683 (Cambridge bioscience, Cambridge, UK) and counter stained with Hoechst 33342 (ThermoFisher Scientific) as previously described (Goncalves et al., 2023). Samples were imaged using the Zeiss 880 confocal microscope with Zen software.

Image analysis

Image J software was used to quantify neurite outgrowth by tracing TUJ-1 positive neurites from 2D cultures and to measure neurite penetration through 3D scaffolds as previously described (Clarke et al., 2017; Goncalves et al., 2023). Briefly, for 2D cultures a grid was overlay was applied to the image, and three squares were selected at random for quantification. Neurites that made contact with the neurosphere in these squares were quantified, neurites were traced using the freehand line tool to produce measurements of length and number. For 3D cultures, the multi-point tool was used to identify neurites from the underside of the scaffold, the total number of neurites that penetrated the scaffold was recorded in each case.(Clarke et al., 2017)

Statistical analysis

GraphPad Prism was used to determine statistical significance and either a one-way or two-way ANOVA with Tukey's post-hoc analysis was conducted as appropriate. Significance is depicted graphically * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. In all cases neurites from 3 neurospheres were measured across 3 independent replicates, in total 9 neurospheres for each experimental condition were quantified, with over 100 neurites measured from each neurosphere (Clarke et al., 2017; Goncalves et al., 2023). This sample size is consistent with previous studies, and has been evaluated for statistical significance previously (Clarke et al., 2017).

Results:

Exogenous Aβ inhibits neurite outgrowth in vitro

Previous studies have demonstrated reduced neurite growth across Aβ-bound surfaces (Postuma et al., 2000). To test this in our EC cell-derived neuronal model system, we coated 2D culture substrates with a range of concentrations (0.05 – 50 $\mu g.mL^{-1}$) $A\beta_{40}$ or $A\beta_{42}$ and measured neurite outgrowth following 10 days (Figure 1A). TUJ-1 positive (green) neurites radiated from the central neurosphere mass (blue) in all conditions (Figure 1B), with neurites visibly shorter and sparse at the highest concentration (50 $\mu g.mL^{-1}$) for both peptides.

Quantification of neurite density (Figure 1C) confirms a dose dependent decrease in neurite outgrowth with increasing concentration of both $A\beta_{40}$ and $A\beta_{42}$, with less neurites present on $A\beta_{42}$ coated substrates. Neurite length (Figure 1D) was particularly reduced at the highest concentration of peptide coating (50 $\mu g.mL^{-1}$) and with $A\beta_{42}$ coating as opposed to $A\beta_{40}$.

Although this data supports that of other studies described in the literature, in the brain of AD patients, neurites are not exposed to Aβ peptides in isolation but in combination, the ratio of which is indicative of disease progression (Hansson et al., 2019). We, therefore progressed to addition of exogenous Aβ to the culture medium of neurospheres, either alone, or in combination at a variety of ratios (Figure 2A). Similarly, neurite growth was identified (Figure 2B) as TUJ-1 positive protrusions (green) radiating from the central neurosphere mass (blue).

The enhanced capacity of $A\beta_{42}$ to aggregate in this environment was confirmed through a protein aggregation assay (Figure 2C). Neurite density (Figure 2D) was significantly reduced both in the case of $A\beta_{40}$ ($p<0.0001$) and $A\beta_{42}$ ($p<0.0001$) in isolation compared with the vehicle control, but not significantly distinguishable from one another. Neurite length (Figure 2E) was also significantly reduced for both $A\beta_{40}$ ($p<0.0001$) and $A\beta_{42}$ ($p<0.0001$) treatments, but in this case $A\beta_{42}$ treatment resulted in significantly shorter neurites than $A\beta_{40}$ treatment alone ($p<0.0001$).

When applied in combination, all Aβ conditions resulted in significantly reduced neurite density compared with vehicle matched controls (Figure 2F), however the extent of the reduction varied. Whereas neurite length (Figure 2G) was significantly reduced in all Aβ conditions compared with vehicle control and further significantly reduced in all combination treatments compared with either $A\beta_{40}$ or $A\beta_{42}$ alone. An $A\beta_{42/40}$ of 1:10 is typical of healthy brain (Näslund et al., 1994) whereas increasing $A\beta_{42/40}$ of 5:1 and 10:1 is indicative of an AD phenotype, and resulted in the shortest neurite measured.

Modulation of downstream Nogo signalling promotes neurite regrowth in 2D and 3D cultures

The inhibitory environment that arises post-spinal cord injury (SCI): the glial scar has been widely studied. It involves to activation of receptors including the Nogo receptor (NgR) by inhibitory molecules released from damaged neurons, resulting in perturbation of actin

dynamics via Rho A and ROCK, ultimately leading to growth cone collapse and neurite retraction (Schwab, 2010). It has been speculated that Aβ-mediated neurite inhibition may share commonalities with this pathway through interaction of Aβ with NgR (Park and Strittmatter, 2007). To investigate this in 2D culture, we added a combination of 1 μM $A\beta_{40}$ with NgR pathway inhibitors to the culture medium of neurospheres during the 10 day neurite outgrowth period (Figure 3A). NEP (1-40), a competitive inhibitor of the NgR, Y-27632 a ROCK inhibitor and ibuprofen, which inhibits Rho A, were all used to elucidate the molecular mechanisms that underpin Aβ-mediated neurite inhibition.

Immunofluorescence analysis of TUJ-1 (green) and nuclei (blue) reveals significant neurite outgrowth from samples (Figure 3B). Neurite density measurements (Figure 3C) reveal significantly reduced neurite outgrowth due to Aβ treatment (*p<0.0001*), restoration of neurite growth to levels that did not significantly differ from vehicle matched control through NEP (1-40) and Y-27632 treatments and enhancement of neurite growth that significantly surpassed that of the vehicle control by ibuprofen treatment (*p<0.0001*). Neurite length (Figure 3D), however, was significantly reduced in all conditions compared with the vehicle matched control (*p<0.0001*).

A 3D culture environment was also adopted, to provide a more physiologically relevant geometry for developing neurites. This well-characterised model of neurite outgrowth has been applied to many previous studies, providing a robust, quantifiable assay (Clarke et al., 2017; Goncalves et al., 2023). Neurospheres were placed onto the 3D scaffold for the 10 days neurite outgrowth phase of culture with the same combination of molecules added to the culture medium: Aβ-alone or in combination with NEP (1-40), ibuprofen or Y-27632 (Figure 4A). Neurites were visualised as TUJ-1 positive protrusions visible from the underside of the scaffold (Figure 4B) having completed penetrated the material.

Neurite penetration (Figure 4C) was significantly reduced in the presence of Aβ-alone (*p=0.0010*), confirming 2D findings. Neurite penetration, however was not restored by the addition of NEP (1-40) and was still significantly reduced compared with controls (*p=0.0366*). Ibuprofen treatment restored neurite penetration to a level that did not significantly differ from the vehicle control, whereas Y-27632 enhanced neurite penetration to a level that surpassed that of the control (*p<0.0001*).

Endogenous production of Aβ reduces neurite outgrowth which is restored by Rho A/ROCK inhibition

To further increase the complexity and physiological relevance of this assay system, we incorporated iPSC-derived neuroprogenitor cells that express an AD-associated mutation (*PSEN1*) and endogenously secrete an elevated $A\beta_{42}/_{40}$. The same method was applied, whereby neurospheres were seeded onto 2D growth surfaces in the presence of an array of

small molecules or drugs and cultured for 10 days to allow for neurite outgrowth (Figure 5A). Neurite growth from wild type (WT) cells with (Figure 5Bd-f) and without (Figure 5Ba-c) exogenous $A\beta_{40}$ was observed and in combination with ibuprofen (Figure 5Be) or Y-27632 (Figure 5Bf). Similarly, mutation cells were cultured without supplementation (Figure 5Bg) or supplemented with ibuprofen (Figure 5Bh) or Y-27632 (Figure 5Bi).

Neurite density (Figure 5C) was significantly reduced in WT cells exposed to exogenous $A\beta_{40}$ (*p<0.0001*) and mutation cells. Ibuprofen (*p=0.0038*) and Y-27362 (*p=0.0046*) significantly enhanced neurite outgrowth to a level comparable to WT in the presence of exogenous $A\beta_{40}$. Similarly, Ibuprofen (*p=0.0038*) and Y-27632 (*p=0.0046*) treatment significantly enhanced neurite outgrowth from mutation cells to a similar level as WT. However, although neurite length (Figure 5D) was significantly reduced in WT with exogenous $A\beta_{40}$ (*p=0.0055*), there was no significant difference between mutation and WT. Ibuprofen reduced neurite length in all conditions, significantly in the case of WT (*p<0.0001*). However, Y-27632 had little impact on neurite length in WT ± exogenous $A\beta_{40}$, but did significantly increase length in the case of mutation cells (*p=0.0377*).

This study was then translated into a 3D environment more reminiscent of *in vivo* neurite development, by adding neurospheres-derived from WT or mutation phenotype iPSCs onto a 3D culture substrate in the presence or absence of test compounds (Figure 6A). Neurite penetration from the underside of the scaffold was visualised as TUJ-1 positive (green) protrusions (Figure 6B). Quantification (Figure 6C) revealed significantly reduced neurite penetration in WT cells supplemented with $A\beta_{40}$ (*p=0.0018*) and mutation containing cells (*p=0.0030*). Ibuprofen (*p=0.0101*) and Y-27632 (*p=0.0404*) significantly reduced neurite penetration in WT cells, but increased neurite penetration to a small degree in cells supplemented with $A\beta_{40}$ and mutation samples.

Discussion:

Loss of neuronal connectivity is one of the pathogenic mechanisms responsible for memory loss in AD (Citron, 2002; Rapoport et al., 2002; Selkoe, 2002; Mattson, 2004; Ittner and Gotz, 2011; Mokhtar et al., 2013; Cummings et al., 2014; Mangialasche et al., 2017). Many factors contribute toward this loss of connectivity, one of which is the deposition of Aβ as senile plaques (Zheng et al., 2002; LaFerla et al., 2007; Gouras et al., 2015) leading to neuritic abnormalities that impede neuronal signal transmission (Knowles et al., 1999; Grutzendler et al., 2007). The ability of Aβ to induce neurite inhibition in simple culture systems has previously been demonstrated (Postuma et al., 2000; Petratos et al., 2008). However, the molecular intricacies that govern neurite inhibition in this context, remain relatively unknown. Here, we describe that exogenous $A\beta_{40}$ and $A\beta_{42}$ are inhibitory to neurite outgrowth in a variety of ratios representative of disease progression, and attribute a reduction in neurite

length to a rise in the ratio of $A\beta_{42}$ relative to $A\beta_{40}$, a hallmark of AD progression. Therefore indicating a potential mechanism linking a rise in $A\beta_{42}$ species in the brain of AD-patients and reduced neuronal transmissions.

Aβ interactions with the NgR, have been speculated in the literature (Park et al., 2006; Zhou et al., 2011). NgR is one of the receptors that mediates neurite inhibition in the glial scar that arises post-SCI, and acts through downstream Rho A and ROCK activation leading to growth cone collapse. Here, we found partial but incomplete recovery of neurite inhibition through deactivation of NgR, a restoration of neurite density but still, a reduction in length in 2D samples. No effect was observed with 3D cultured samples, suggesting that although Aβ may induce neurite inhibition through NgR it may be one of multiple pathways involved. This is also the case in molecular signaling post-SCI, as NgR is one of many receptors implicated in neurite inhibition in this context, with NgR2, NgR3, LAR and PTPσ receptor complexes also involved in this response. Aβ governed neurite inhibition may in-part be mediated by NgR, but also involve additional receptor complexes such as those involved in glial scar signaling, or even a novel receptor. However, in this study we do provide evidence as to partial involvement of NgR, along with strong evidence that the molecular pathways involved in Aβ-mediated inhibition are governed by Rho A/ROCK activation.

The ability of the selective ROCK inhibitor, Y-27632, to overcome Aβ-mediated inhibition has previously been documented in a simple SH-SY5Y model of neurite outgrowth (Postuma et al., 2000). This suggests that the Rho A/ROCK signalling pathway is involved Aβ-induction of neurite inhibition or potently induces neurite growth to a point that can overcome the deleterious effects of Aβ, similar to the signalling events that underpin inhibition following SCI (Fawcett and Asher, 1999; Monnier et al., 2003; Chan et al., 2005; Yiu and He, 2006; Lingor et al., 2007; Gopalakrishnan et al., 2008). In this study, we have further evidenced this, by demonstrating the ability of Y-27632 and ibuprofen, an inhibitor of Rho A known to enhance neurite outgrowth (Fu et al., 2007; Dill et al., 2010; Kopp et al., 2012; Roloff et al., 2015), to overcome neurite inhibition induced by exogenous and endogenously secreted Aβ in two model systems. Besides being an inhibitor of Rho A, ibuprofen is also a non-steroidal anti-inflammatory drug (NSAID) that inhibits prostaglandin synthesis, therefore its mechanism of neurotrophic action may also be a consequence of its anti-inflammatory properties, which is an avenue worthy of further investigation.

These data suggest that Aβ inhibition is mediated by Rho A/ROCK activation or that deactivation of Rho A/ROCK potently drives neurite initiation and extension that it is able to overcome inhibitory stimuli such as Aβ. It also highlights the potential neural regeneration properties of Rho A/ROCK inhibitors, particularly as ibuprofen enhanced neurite outgrowth to greater than control levels in some of the conditions outlined within this study. The disparity

observed between neurite density and length in several of the experimental conditions described, suggests that neurite initiation and extension may be controlled by independent mechanisms, and warrants follow up investigation.

We also describe a novel AD-specific model of neurite outgrowth that has many potential applications including drug discovery and personalised medicine. Building on the development of several iPSC-derived AD-model neuroprogenitor cell lines such as those described by Yagi *et al* in 2011 (Yagi et al., 2011), we have developed a novel neurosphere-based neurite outgrowth assay. We have demonstrated an application of this model to study neurite induction from cells transfected with an AD-associated mutation in presenilin 1 (PSEN1). The presenilins form the γ-secretase complex, which is involved in the proteolytic cleavage of APP and the formation of Aβ species (Nelson et al., n.d.; Citron et al., 1997; Selkoe, 1998; Campion et al., 1999; Haapasalo and Kovacs, 2011; Yagi et al., 2011; Sproul et al., 2014). Due to this mutation, cells express a higher $A\beta_{42}$:$A\beta_{40}$ ratio, providing a more complex *in vitro* model that better recapitulates aspects of the *in vivo* diseased state.

We found that neurospheres derived from AD-model cells, generated less dense neurite outgrowth than WT neurospheres, indicating impaired neurite generation. This effect was recovered by medium supplementation with Y-27632 and ibuprofen, further evidencing the role of Rho A and ROCK-mediated signalling in driving neurite growth.

In this study, we not only present a novel application for an established neurite outgrowth assay, we also introduce the concept of disease-specific modifications to the existing methodology. Through incorporation of iPSC-derived neuroprogenitor cells containing an AD-associated mutation, we have been able to demonstrate the ability to tailor this system to meet the needs of specific diseases where impaired neural connectivity is implicated as a pathogenic mechanism such as Parkinson's disease (Takenouchi et al., 2001), schizophrenia (Miyoshi et al., n.d.) and Down's syndrome (Murtomäki et al., 1992). This methodology also lends itself to other neurological diseases and through iPSC technology and proof-of-concept data described in this study, our model system offers a new assay to measure changes in neurite growth, development and connectivity in a wide range of conditions. Incorporation of iPSC derived neuroprogenitor cells also has implications in terms of personalized medicine, as patient derived differentiated iPSCs could be incorporated into this model system, which in turn acts as a quantifiable assay to screen the efficacy and regenerative capacity of drugs in a patient-specific manner. Therefore, the data described in this study and the technology utilized, has far reaching impact on many aspects of the scientific community including medical, industrial and academic applications.

Furthermore, it also provides a screening tool with the capacity to test a range of molecules for their ability to promote neurite regeneration. These proof-of-concept data demonstrate

that neurite induction is robust and quantifiable from iPSC-neurospheres and can be applied to both personalized medicine modelling and disease-specific investigations.

In conclusion, our findings suggest that both Y-27632 and ibuprofen are potent inducers of neuritogenesis that can be used *in vitro* to overcome an inhibitory stimulus. We have also demonstrated the detrimental effects of exogenous Aβ species on neurite growth in human model neurites achieved through different methodologies, validating and strengthening the outcomes of the study. Moreover, we have demonstrated the ability of small molecule inhibition of Rho A and ROCK to overcome this inhibitory phenotype and promote neurite growth. Understanding the molecular mechanisms that underpin neurite dynamics in complex neurodegenerative diseases, can help provide novel therapeutic targets that restore neural connectivity and provide a valuable *in vitro* pre-clinical platform. Furthermore, we also describe the development of a novel iPSC-based neurite outgrowth assay that has potential applications in the field of personalised medicine and drug discovery.

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Figures and Tables:

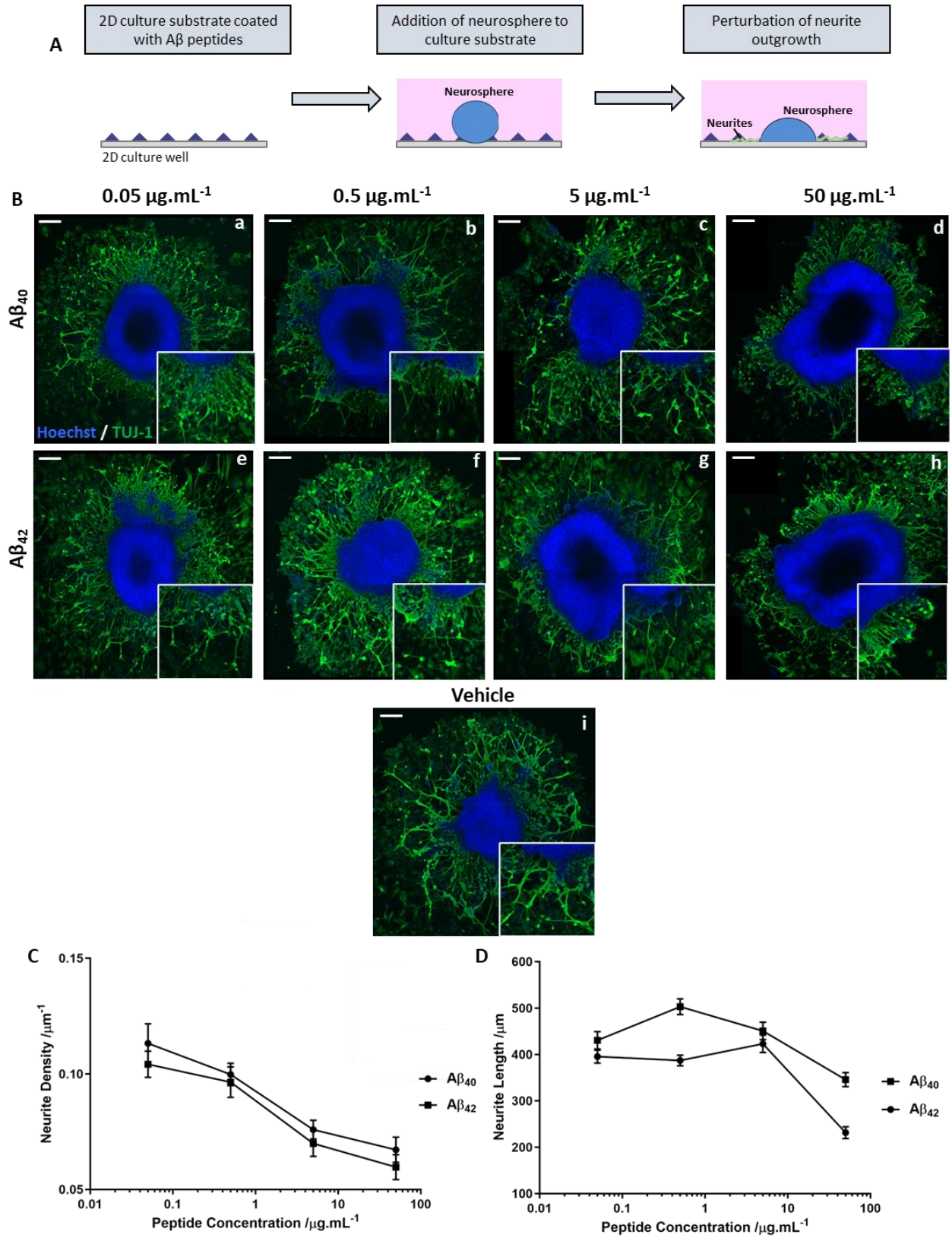


Figure 1: Substrate bound β-amyloid peptides inhibit neurite growth in a dose dependent manner

Differentiated embryonal carcinoma (EC) cell derived neurospheres were placed on 2D growth substrates coated with poly-D-lysine and laminin supplemented with range of concentrations (0.05 – 50 µg.mL^{-1}) β-amyloid peptides for 10 days prior to neurite observation (A). Immunofluorescence images (B) highlighting the pan-neuronal marker TUJ-1 (green) and cell

nuclei (blue) demonstrate radiating neurite growth from the central neurosphere mass. A dose dependent reduction in neurite density (C) (data represent mean ± SEM, n=9) was observed for both $A\beta_{40}$ and $A\beta_{42}$ peptides. Similarly, a reduction in neurite length (D) (data represent mean ± SEM, n=9) was observed at the highest concentrations tested. Scale bar: 100 μm.

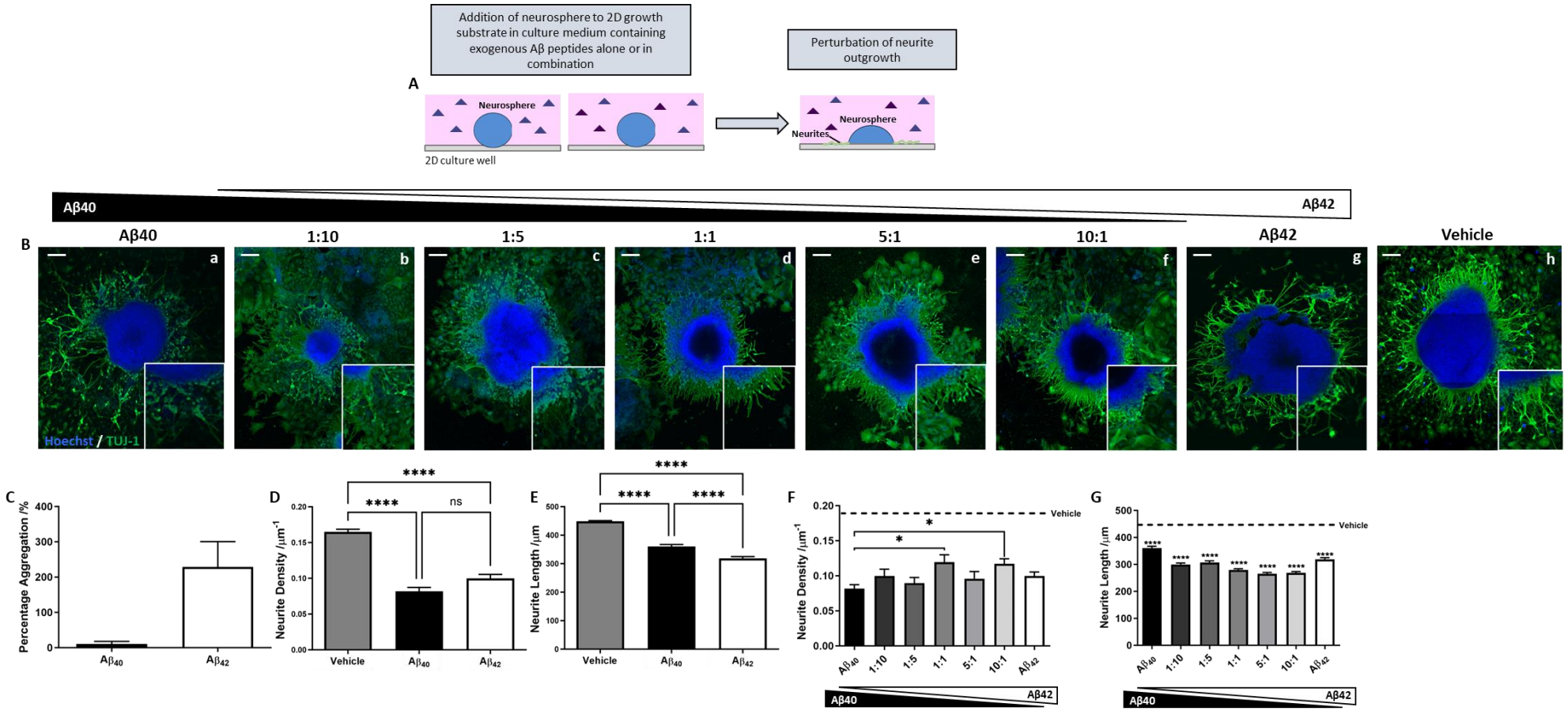


Figure 2: Exogenous β-amyloid reduces neurite outgrowth in physiologically relevant ratios

Embryonal carcinoma (EC) derived neurospheres were placed on 2D growth substrates in culture medium containing 1 μM β-amyloid peptides for 10 days prior to visualisation of neurite outgrowth (A). $A\beta_{40}$ and $A\beta_{42}$ were added alone or in combination, totalling to a final concentration of 1 μM**.** Immunofluorescence analysis (B) reveals TUJ-1 positive (green) neurites radiating from a central neurosphere mass (blue), the extent of which varies with treatment condition. The ability of $A\beta_{42}$ to more readily aggregate was confirmed through a protein aggregation assay, and expressed as percentage aggregation (C) (data represent mean ± SEM, n=3). Neurite density (D) in the presence

of $A\beta_{40}$ and $A\beta_{42}$ supplementation was significantly reduced compared with controls, but did not significantly differ from one another (data represent mean ± SEM, n=9, one-way ANOVA with Tukey's multiple comparisons, $p<0.0001$). Neurite length (E) was significantly reduced with both $A\beta_{40}$ and $A\beta_{42}$ treatments (data represent mean ± SEM, n=9, one-way ANOVA with Tukey's multiple comparisons, $p<0.0001$). Neurite density (F) was significantly reduced in the presence of Aβ combination treatments compared with vehicle matched control (dashed line) (data represent mean ± SEM, n=9, one-way ANOVA with Tukey's multiple comparisons, $p<0.0001$). Neurite length (G) was significantly reduced by all Aβ combination treatments compared with vehicle matched control or either $A\beta_{40}$ and $A\beta_{42}$ alone (data represent mean ± SEM, n=9, one-way ANOVA with Tukey's multiple comparisons, $p<0.0001$). * = $p < 0.05$, **** = $p < 0.0001$. Scale bar: 100 μm

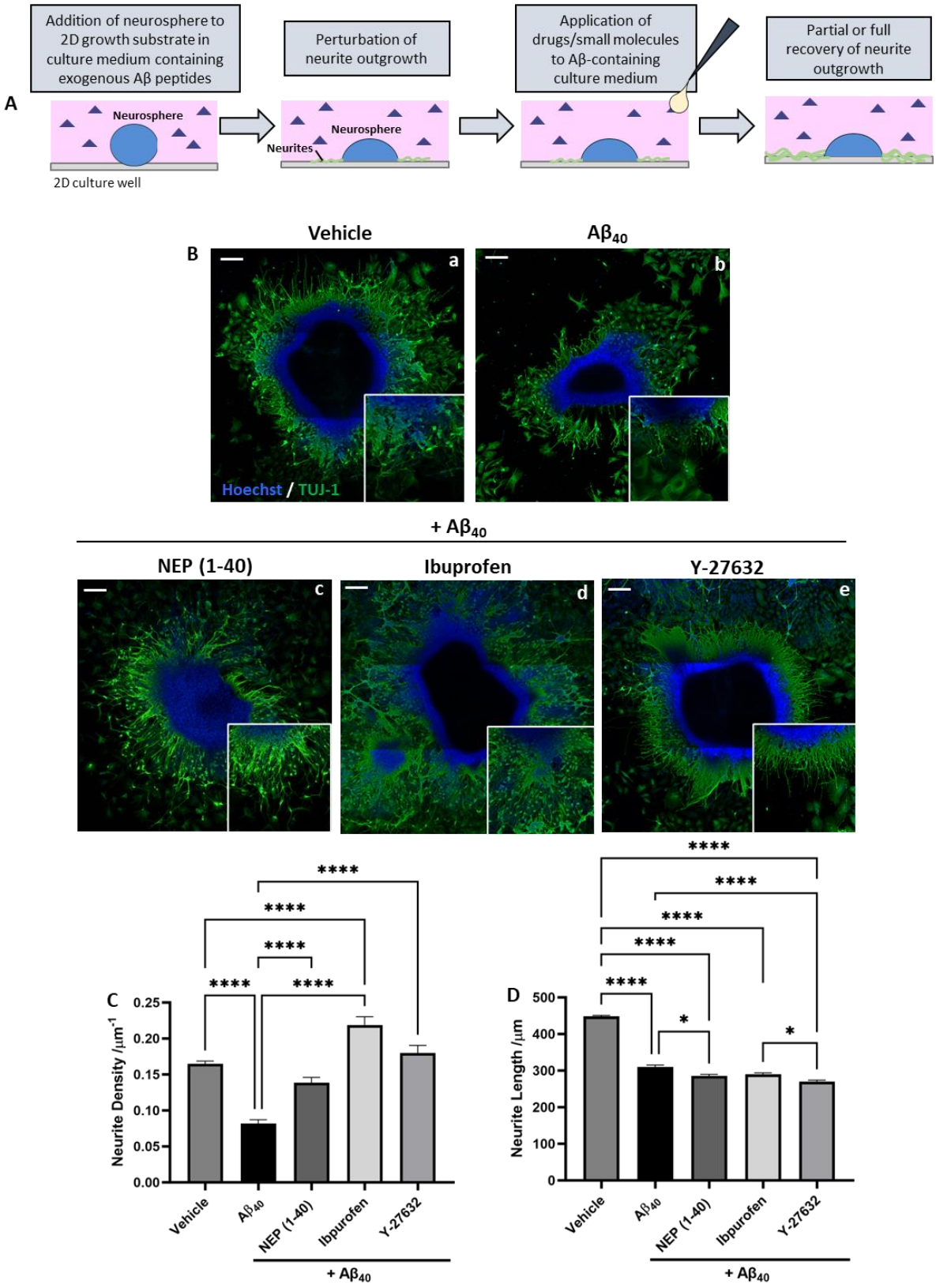


Figure 3: Modulation of NgR signalling restores neurite outgrowth in the presence of inhibitory Aβ40

Inhibitors of aspects of NgR and its downstream signalling cascade were added to 2D cultures of embryonal carcinoma (EC) cell-derived neurospheres for 10 days during which

time neurites extended (A). Immunofluorescence analysis (B) for the pan neuronal marker TUJ-1 (green) highlights neurite outgrowth, whilst nuclei are stained by Hoechst (blue). Neurite density (C) was significantly reduced by $A\beta_{40}$ treatment compared with the vehicle matched control, 1 μM NEP (1-40) and 10 μM Y-27632 both restored neurite density to a point that did not significantly differ from the vehicle control, whereas 100 μM ibuprofen treatment significantly enhanced neurite density (data represent mean ± SEM, n=9, one-way ANOVA with Tukey's multiple comparisons, $p<0.0001$). Neurite length (D) was significantly reduced by all treatment conditions compared with the vehicle matched control (data represent mean ± SEM, n=9, one-way ANOVA with Tukey's multiple comparisons, $p<0.0001$). Scale bar: 100 μm.

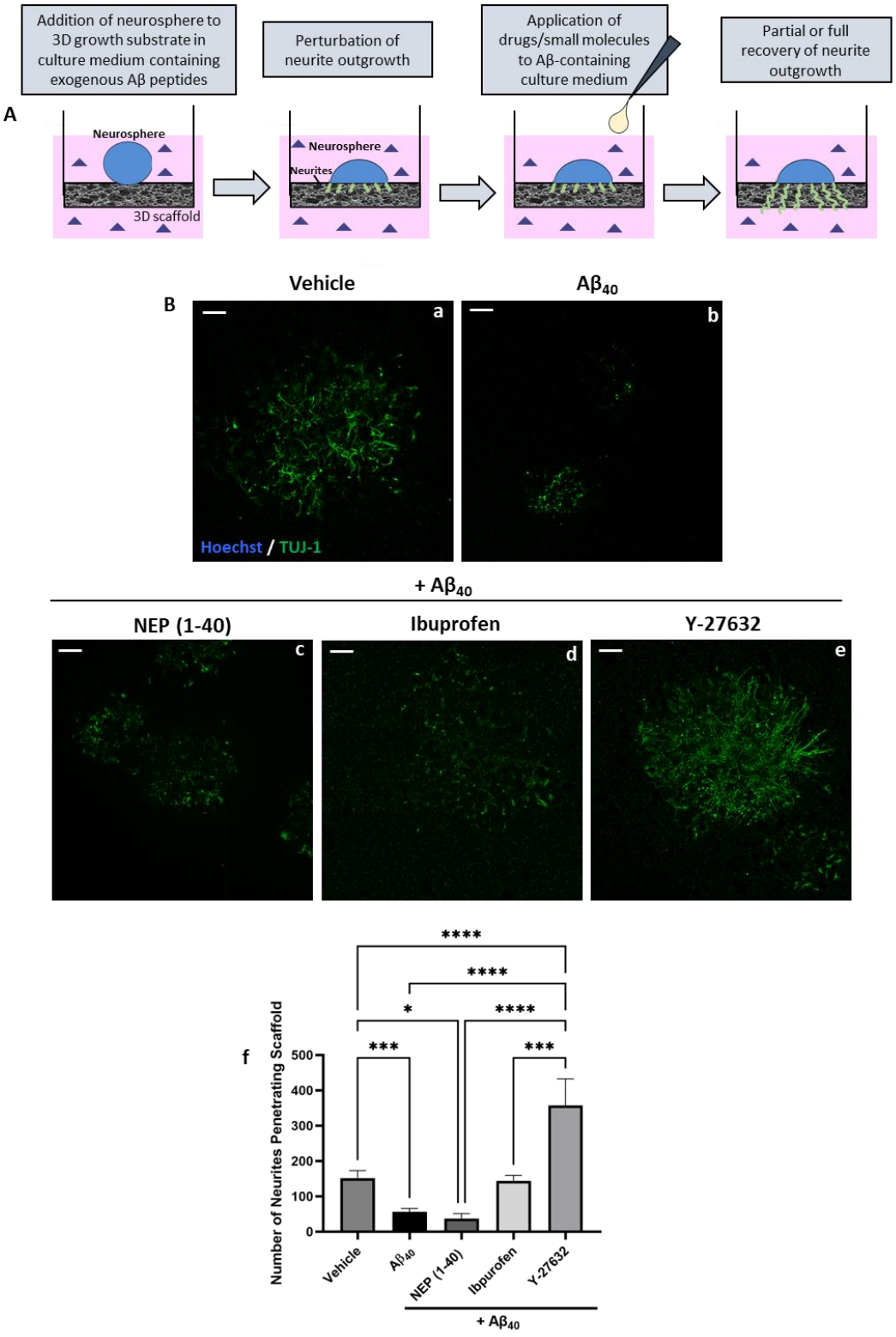


Figure 4: Modulation of Rho A/ROCK signalling attenuates Aβ40-mediated neurite inhibition in 3D culture

Embryonal carcinoma cell (EC) derived neurospheres were seeded onto 3D culture substrates in the presence of culture medium supplemented with 1 μM $A\beta_{40}$ alone or in combination with test compounds: 1 μM NEP (1-40), 100 μM ibuprofen or 10 μM Y-27632 (A). Neurite penetration was visualised as neuritic TUJ-1 positive (green) extensions from the underside of the scaffold (B). Quantification of neurite penetration (C) (data represent mean ± SEM, n=9, one-way ANOVA with Tukey's multiple comparisons) revealed a significant reduction in the presence of $A\beta_{40}$ alone ($p=0.0010$) and in combination with NEP (1-40) ($p=0.0360$), whereas ibuprofen restored neurite penetration to control levels and Y-27632 significantly enhanced neurite penetration ($p<0.0001$). * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$. Scale bar: 100 μm.

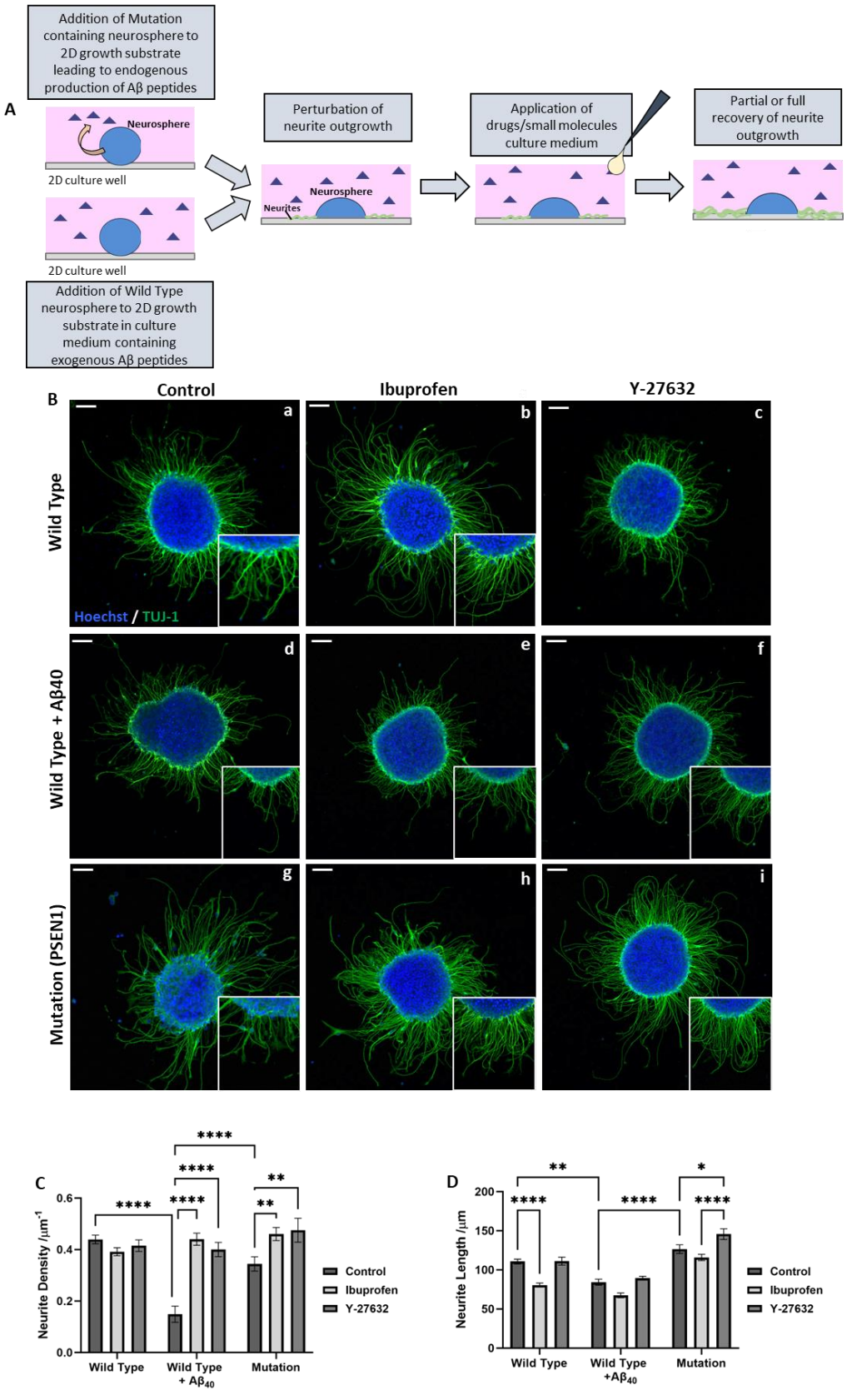
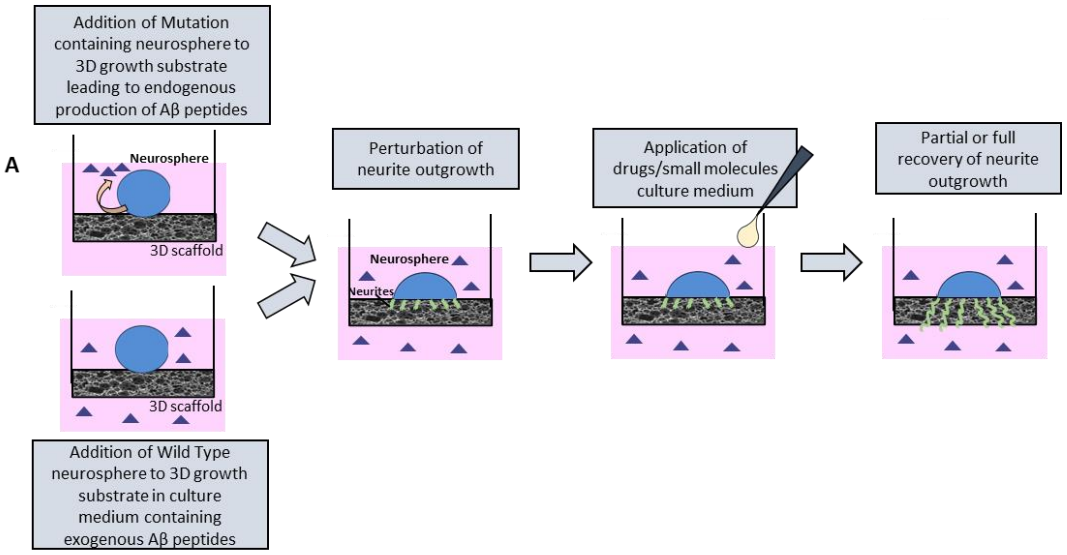
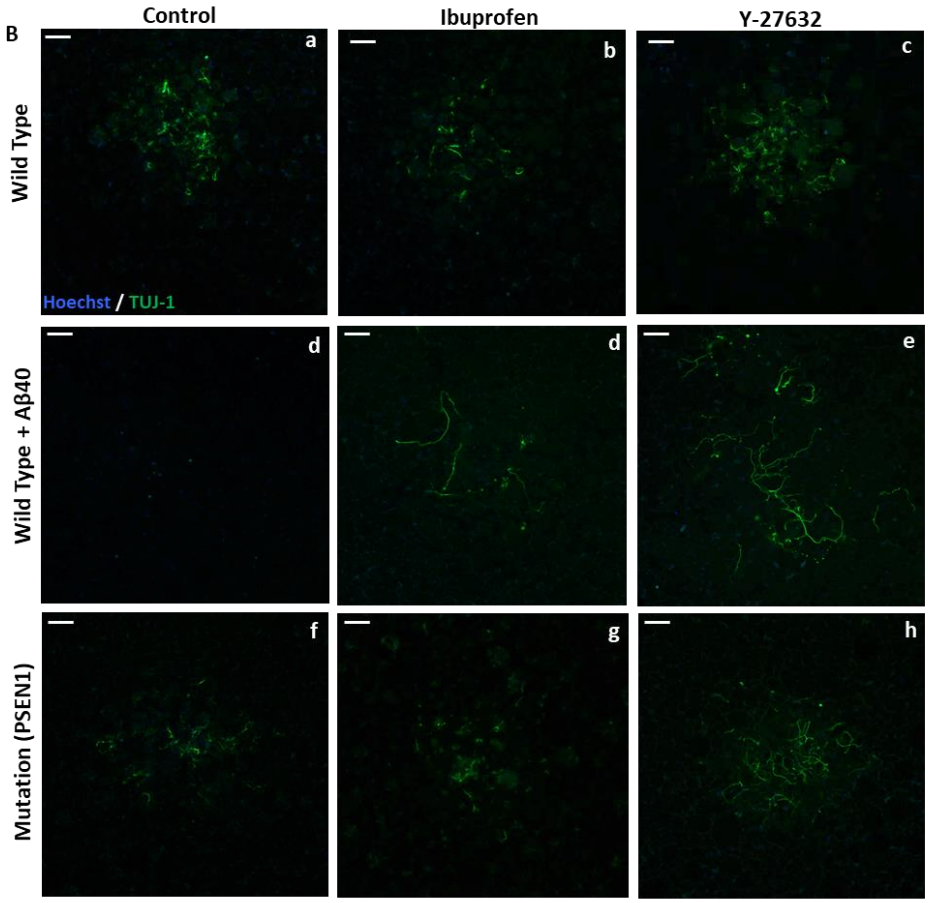


Figure 5: Neurite outgrowth from neurospheres that possess AD-associated mutation is reduced and rescuable through Rho A/ROCK modulation in 2D culture

Neurospheres were formed from induced pluripotent stem cell (iPSC) derived neuroprogenitor cells from either wild type (WT) or cells transfected with an AD-associated

mutation in presinillin-1 (Mutation) phenotypes. Neurospheres were seeded onto 2D culture substrates and treated with a range of medium supplementations: 1 µM $A\beta_{40}$, 1 µM NEP (1-40), 100 µM ibuprofen or 10 µM Y-27632 for 10 days prior to neurite outgrowth analysis (A). TUJ-1 positive (green) neurites radiate from a central neurospheres mass, where nuclei containing perikarya reside (blue) (B). Neurite density (C) (data represent mean ± SEM, n=9, two-way ANOVA with Tukey's multiple comparisons) was significantly reduced in WT cells supplemented with $A\beta_{40}$ ($p<0.0001$) and both ibuprofen and Y-27632 treatments enhanced neurite density in WT + $A\beta_{40}$ and Mutation containing cells. Neurite length (D) (data represent mean ± SEM, n=9, two-way ANOVA with Tukey's multiple comparisons) was significantly reduced in the in WT + $A\beta_{40}$ ($p=0.0055$) condition and negatively affected by ibuprofen in all cases. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Scale bar: 100 µm.


A
Addition of Mutation containing neurosphere to 3D growth substrate leading to endogenous production of Aβ peptides
Neurosphere
3D scaffold
Addition of Wild Type neurosphere to 3D growth substrate in culture medium containing exogenous Aβ peptides
3D scaffold
Perturbation of neurite outgrowth
Neurosphere
Neurites
Application of drugs/small molecules culture medium
Partial or full recovery of neurite outgrowth


B
Control
Ibuprofen
Y-27632
Wild Type
Wild Type + Aβ40
Mutation (PSEN1)
a
b
c
d
d
e
f
g
h
Hoechst / TUJ-1

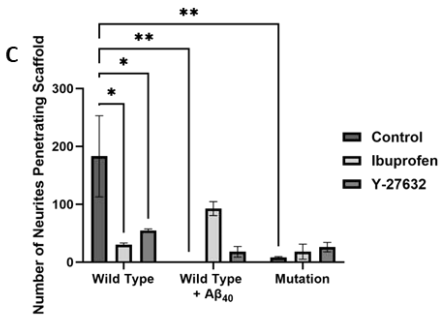

C
Number of Neurites Penetrating Scaffold
300
200
100
0
**
**
*
*
Wild Type
Wild Type + Aβ40
Mutation
Control
Ibuprofen
Y-27632

Figure 6: Neurite outgrowth in 3D is reduced from neurospheres that possess AD-associated mutation

Neurospheres comprised of induced pluripotent stem cell (iPSC) derived neuroprogenitor cells were seeded onto 3D growth substrates with a combination of media additives: 1 µM $A\beta_{40}$, 1 µM NEP (1-40), 100 µM ibuprofen or 10 µM Y-27632 for 10 days prior to neurite outgrowth analysis (A). Neurite penetration (TUJ-1, green) was visualised from the underside of the scaffold (B). Quantification of neurite penetration (C)) (data represent mean ± SEM, n=9, two-way ANOVA with Tukey's multiple comparisons) revealed a significant reduction in both WT + $A\beta_{40}$ ($p=0.018$) and Mutation ($p=0.0030$) containing samples. * = $p < 0.05$, ** = $p < 0.01$. Scale bar: 100 µm.

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Conflicts of interest: SP is affiliated with the company Reprocell Europe. KG declares that there is no conflict of interest to disclose.

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