Generation of a neuron-astrocyte co-culture system for neurodegenerative disease modelling

Major research project

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Abstract

Neurodegenerative diseases are a group of debilitating neurological conditions associated with progressive loss of neurons, thereby causing a wide spectrum of clinical symptoms**.** Generation of representative *in vitro* models for neurodegenerative disease is essential to get a better understanding of disease mechanisms and facilitate drug screenings. Monoculture models of neurons fail to recapitulate the important interactions between neurons and astrocytes in physiological and diseased states. The aim of this study is therefore to generate and characterize co-culture model of neurons and astrocytes to study the supportive interactions in a physiological state as well as cytotoxic effects in an inflammatory state. Commercially available cell lines as well as induced neurons and astrocytes derived from hESCs will be used for this purpose. The cells will be characterized using immunofluorescence analysis and qPCR and their functionality will be determined through calcium imaging experiments. In this study, we show that neurons support the maturational profile of astrocytes in co-culture in terms of gene expression. In an inflammatory model, activated astrocytes have a cytotoxic effect on the neurons as shown by loss of the cells in co-culture. The results from our study provide new questions and insights for further research that could lead to the optimization of neuron-astrocyte co-culture models.

Layman's summary

Neurodegenerative diseases are a group of disorders in the brain in which neurons are progressively lost. In order to better understand these diseases and find a potential cure, a lot of research is done in cell culture laboratories. In cell culture, neurons can be studied from up close. But not only neurons are important for these diseases. Also astrocytes, which are supportive cells of the brain, play a role in neurodegeneration. For better cell culture experiments, both neurons and astrocytes should be put together in one culture. In this study, neurons and astrocytes are kept together in a so-called co-culture to examine the supportive effects these two cell types have on each other. This is tested by looking at the expression of certain genes using a technique called quantitative PCR. We also do a co-culture experiment using astrocytes that are transformed into an inflammatory state, so we can mimic neurodegenerative disease. When we culture these inflammatory astrocytes together with neurons, we expect to see a loss of neurons in the culture. Neurons and astrocytes bought from companies are firstly used for these experiments, but we also wanted to try to generate neurons and astrocytes from stem cells. Stem cells are beneficial because they can self-renew unlimited amount of times and are able to become any cell type of the body. Neurons and astrocytes can be generated from these stem cells by integrating cell-specific genes into viruses and giving these viruses to the cells. The viruses will integrate the genes in the DNA of the stem cells and this will lead to differentiation into specific cell types. Several experiments are done to prove that the differentiation is successful. We measure neuron- and astrocyte-specific proteins and genes in the cells. We also want to show that the neurons and astrocytes are able to send signals. In our brain, neurons and astrocytes send signals using calcium molecules. By measuring the amount of calcium over time, we can test if the cells are active. Once the neurons and astrocytes derived from stem cells are fully mature, they can be co-cultured. The results from this study can lead new insights that are needed for optimization of future co-culture models.

1. Introduction

Neurodegenerative diseases are a group of debilitating neurological conditions associated with progressive loss of neurons, thereby causing a wide spectrum of clinical symptoms ranging from brain cognitive deficits to loss of locomotor functions [1]. Particularly in an aging population, the burden of these diseases is progressively increasing [2]. Despite tremendous research efforts, the mechanisms of most neurodegenerative diseases are still not well understood and disease-modifying treatments remain to be discovered. In order to bridge the gap between pre-clinical research and clinical trials, generation of representative *in vitro* models is essential. These models could not only provide a better understanding of disease mechanism, but also facilitate drug screenings by analyzing the potential protective role of compounds on neuronal cultures.

Traditionally, neurodegenerative disease has been most studied through monoculture models of neurons, since neurons were considered the most important cell of the human brain. However, the brain also comprises of other cells, named glial cells, which exert many supportive functions. Astrocytes are the most abundant type of glial cells and have several interactions with neurons. They facilitate neurotransmitter trafficking, play a role in nutrient and ion metabolism, release growth factors and cytokines, and protect against oxidative stress [3]. In neurodegenerative disease, however, astrocytes are often directed towards a proinflammatory phenotype [4]. These proinflammatory astrocytes subsequently contribute to the progression of neurodegeneration by the release of neurotoxic amounts of nitric oxide or glutamate and downregulation of neurotransmitter uptake. Additionally, the release of cytokines by the proinflammatory astrocytes contributes to further activation of astrocytes, creating a vicious loop of degeneration [5]. Monoculture models fail to recapitulate these interactions between neurons and astrocytes. To increase the quality of preclinical research into neurodegenerative disease, *in vitro* models should combine neurons and astrocytes in a co-culture system.

Different types of cellular resources could be used for an *in vitro* co-culture model of neurons and astrocytes. Human primary cells are directly isolated from human tissue and have been extensively used as a tool for research into disease mechanisms and preclinical drug testing. They are more physiologically relevant than cell lines obtained from rodents, which are easy to grow and maintain, but are derived from tumorous tissue and are therefore unlikely to accurately mimic the characteristics of neurons *in vivo* [6]. Drawbacks of using human primary cells are that the amount of tissue is limited, and tissue samples are difficult to obtain, especially from the human brain [1]. Furthermore, in case of astrocytes, human primary cells proliferate poorly and are prone to degeneration [7]. Therefore, other avenues should be considered for the development of *in vitro* disease models.

Embryonic stem cells (ESCs) are derived from the inner cell mass of mammalian blastocysts and may serve as a potentially unrestricted source for *in vitro* experiments because of their unlimited self-renewal capacity and ability to differentiate into cells of all three germ layers [8]. Originally, growth factor-based approaches were used to differentiate ESCs into a desired cell type. However, these protocols are often costly and time-consuming. For neurological research, this led to the development of the Lund Human Mesencephalic (LUHMES) cell line. The LUHMES immortalized cell line comprises of genetically modified neural progenitors in which terminal differentiation is halted, but can be induced through a tetracycline-controllable system [6]. The neural progenitors differentiate into post-mitotic dopaminergic neurons after exposure to a set of factors; tetracycline/doxycycline, glial cell line-derived neurotrophic factor (GDNF), and dBcAMP. LUHMES neurons are particularly suitable for research into Parkinson's disease (PD), a neurodegenerative disorder associated with progressive loss of dopaminergic neurons in the substantia nigra [9]. A drawback of using such an immortalized cell line, however, is that they are fixed to become a certain fate, and are modified in a way that makes it debatable whether they represent actual human neuronal cells.

An alternative approach would be to generate induced cell types directly from ESCs. A recently developed, more efficient method of generating induced cell types is through delivery of transcription factors. A way to introduce genes to drive differentiation into a new cell type is through lentiviral delivery. Lentiviruses have been shown to effectively integrate their DNA into the host genome, leading to translation of viral proteins and passage of viral DNA on to the daughter cells [10]. Vierbuchen *et al*. performed a screening on 19 candidate genes that could potentially reprogram somatic cells to a neuronal fate, and found that the combined expression of three transcription factors *Ascl1*, *Brn2*, and *Myt1l*, rapidly and efficiently converts mouse fibroblasts into functional induced neurons (iNeurons). While *Ascl1* on its own is sufficient to generate iNs displaying immature neuronal features, the addition of *Brn2* and *Myt1l* increases the efficiency of generating functionally mature iNs [11]. The iNs generated from this experiment, however, consist of a heterogenous population of neuronal subtypes. Later, a set of three transcription factors – *Ascl1*, *Nurr1*, and *Lmx1a* – was identified that could drive reprogramming of mouse and human fibroblasts into dopaminergic iNs specifically [12]. The same group described a protocol for the generation of induced astrocytes (iAstrocytes) from fibroblasts through forced expression of astroglial transcriptional factors *Nfia*, *Nfib*, and *Sox9* [3]. Since traditional protocols for generating astrocytes from stem cells are time-consuming, the transcription factor-based approach has subsequently been used to generate iAstrocytes from hESCs in another study [13].

The basic aim of this study is to generate a physiological co-culture model of neurons and astrocytes to study their supportive roles and interactions. Besides studying the supportive

roles in a physiological model, the neurotoxic effect of astrocytes on neurons is studied in an inflammatory co-culture model. Neurons and astrocytes are either cultured directly or via a transwell system. Supportive functions will be assessed by comparing gene expression data using quantitative PCR (qPCR) from monocultures to the co-culture results and neurotoxicity of the inflammatory model will be examined through cell counting and observations from microscopic images. LUHMES neurons and primary astrocytes will serve as cellular resources for this co-culture system, however LUHMES neurons will first be characterized in different culturing conditions. Lastly, an attempt was made to create this model entirely from ESC-derived cells using the lentivirus-based cellular reprogramming technique. Various culture conditions are tested in order to optimize the differentiation into iNeurons and iAstrocytes. The differentiated iNeurons and iAstrocytes are identified by examining the molecular and functional properties of the cells and verifying their resemblance to human primary cells. The molecular phenotype will be characterized through assessment of specific neuronal or astrocytic protein and gene expression by immunofluorescence analysis and qPCR, respectively. A functional property of neurons and astrocytes is that they exhibit spontaneous or stimulated increase in intracellular calcium levels. Changes in these levels are detected through calcium imaging experiments.

2. Materials and methods

2.1 Cell Culture

LUHMES human neural precursor cells (obtained from ATCC) were cultured as described previously [6]. Briefly, culture flasks were pre-coated with 100 µg/ml poly-l-ornithine (Sigma-Aldrich®) and 1 mg/ml fibronectin (Sigma-Aldrich®). The cells are proliferated in growth medium consisting of Advanced Dulbecco's modified Eagle's medium (DMEM)/F12, 2 mM glutamine, 1% N2 supplement (100X, Gibco™), 1% antibiotic. Growth medium was supplemented freshly with 40 ng/ml fibroblast growth factor (FGF) 2 (PeproTech®). Normal passage was done in T75 flasks when cells reached 60-70% confluency using warm 0.025% Trypsin-EDTA solution (Sigma-Aldrich®) in complete growth medium. The medium was refreshed every 2-3 days.

Primary astrocytes (obtained from Lonza) were grown on type-I collagen-coated T175 flasks in DMEM/F12 medium supplemented with 1% N2, 10% fetal bovine serum (FBS) (Life technologies), and 20 ng/ml epidermal growth factor (EGF). The cells were sub-cultured every 3 days using 0.25% Trypsin-EDTA.

H1 cells were cultured in feeder-free conditions using StemFlex™ medium (Gibco™) with daily medium changes. Cells were plated on Matrigel® (Corning®) coated six-well plates from Corning® and routinely passaged using ReLeSR™ for dissociation. For initiation of differentiation experiments, the cells were dissociated into single cells using Accutase® (Sigma-Aldrich®) when cells reached 80% confluency and kept in StemFlex™ medium supplemented with 10 µg/ml rho-kinase (ROCK) inhibitor (STEMCELL™).

2.2 Differentiation LUHMES cells

Before differentiation of LUHMES cells, the cells were normally passaged in the T75 flask. Notably, the differentiation starts from a 40 to 50% confluent cell layer. To differentiate the neural precursor cells into neurons, the medium was changed to complete differentiation medium (Advanced DMEM/F12, 1% glutamine (2mM), 1% N2, 1% antibiotic) freshly supplemented with 1 mM dBcAMP, 10 ng/ml brain-derived neurotrophic factor (BDNF), 10 ng/ml GDNF, 0.2 µM vitamin C, 1 µg/ml doxycycline and left in the incubator for two days. Thereafter, the cells were finally replated in complete differentiation medium on Matrigel-coated wells using warm 0.025% Trypsin/EDTA. The cells were plated at density 1×10^5 cells/cm^2 for immunofluorescence analysis and calcium imaging or at 1.5×10^5 cells/cm^2 for qPCR experiments. The medium was refreshed one day after the final replate and consequently changed every 2-3 days. The cells were harvested at either day 6 or day 10.

2.3 Co-culture of LUHMES cells with primary astrocytes

On day 2 of the LUHMES differentiation protocol, the LUHMES cells and primary astrocytes were both dissociated using 0.025% Trypsin/EDTA and replated directly together or separately using transwells. The cells were kept in either original LUHMES differentiation medium, co-culture N2 medium (Advanced DMEM/F12 and Neurobasal in 1:1 ratio, 1% N2, 1% GlutaMAX™, 1% AB) or co-culture B27 medium (Advanced DMEM/F12 and Neurobasal in 1:1 ratio, 1% B27, 1% GlutaMAX™, 1% AB). The media were freshly supplemented with 1 mM dBcAMP, 10 ng/ml BDNF, 10 ng/ml GDNF, 0.2 µM vitamin C, and 1 µg/ml doxycycline. For the direct-contact and transwell models, the neurons were plated at densities of 1 x 10^5 cells/cm^2 and 1.5 x 10^5 cells/cm^2, respectively, onto Matrigel® coated plates. The astrocytes densities were adjusted to obtain astrocyte to neuron ratios of 1:2, 1:3, and 1:4. The cells were kept in co-culture until day 6 of LUHMES differentiation.

2.4 Immunofluorescence

For immunofluorescence analysis, cells were plated in Matrigel-coated 24-well or 96-well Corning® glass plates. A list of the primary and secondary antibodies used in these experiments can be found in the Supplementary Tables 1 and 2. Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Thereafter, the cells are washed twice with phosphate-buffered saline (PBS) and subsequently permeabilized for 15 minutes in room temperature with either 0.01% Triton X-100 or 0.01% saponin for membrane-associated markers. After washing once with PBS, the cells are blocked for 1.5h with 3% bovine serum albumin (BSA). Primary antibodies were added to the cells in the blocking buffer and incubated overnight at 4ºC. The cells were washed three times for 5 minutes with PBS on a rocker at low speed. Secondary antibodies were subsequently added in 4% BSA and incubated in the dark for 1.5h at room temperature. For nuclear staining, the cells were incubated with 4 µg/ml Hoechst in PBS for 15 minutes in the dark at room temperature. The cells were finally washed three times with PBS. Images are obtained using the Yokogawa Cell Voyager CV7000S confocal microscope.

2.5 Gene expression

RNA from the cells was isolated using the ReliaPrep™ RNA Cell Miniprep System, which included incubation with DNase I to prevent DNA contamination. The concentrations of RNA from the samples were determined with the NanoDrop™ One spectrophotometer. The samples were reverse transcribed into complementary DNA using the Bio-Rad iScript™ cDNA synthesis kit. -qPCR was performed with Bio-Rad SYBR® Green supermix and eventually carried out using the Bio-Rad CFX96™ real-time qPCR system.

2.6 Cytokine stimulation

Primary astrocytes were incubated for 48 hours with either 5 ng/ml FGF and 5 ng/ml EGF or 30 ng/ml tumor necrosis factor (TNF) α and 10 ng/ml interleukin (IL) 1α. After harvesting the stimulated astrocytes, the cells were washed to eliminate any excess cytokines that could affect the co-culture.

2.7 Cell count

Cell density was determined using a TC20™ Automated Cell Counter (Bio-Rad). After harvesting the LUHMES neurons that were co-cultured with stimulated astrocytes, 10 µl of the cells was mixed in 1:1 ratio with Trypan blue (0.4%) and loaded into a cell counting chamber.

2.8 Generation of iNs from hESCs

On day -2, H1 cells are dissociated into single cells using Accutase®. 6 x 10^5 cells per well replated in Matrigel-coated six-well plate in StemFlex medium supplemented with 10µM ROCK inhibitor. One day later the cells are infected with lentiviral constructs containing the Ascl1, Nurr1, Lmx1a transcriptional factors and the lentiviral construct containing rtTA at 5-25 multiplicity of infection. Infection is done in StemFlex medium supplemented with 8 µg/ml polybrene and 10µM ROCK inhibitor. On day 0, the virus-containing medium is replaced with N2 medium (DMEM/F12, 1% N2 supplement, 1% GlutaMAX™ (Gibco™), 0.5% non-essential amino acid (NEAA), 1% antibiotic) containing 1 µg/ml doxycycline. One day after doxycycline initiation, a four-day antibiotic selection with 0.25 µg/ml puromycin is started. From this moment, also 200µM Vitamin C was added freshly to the N2 medium. From day 3 onwards, the medium was freshly supplemented with 20 ng/ml BDNF and 20 ng/ml GDNF. On day 7, the cells are replated on 20 µg/ml Laminin-511-coated plates at densities 1-2 x 10^5 cells/cm^2. The medium was additionally supplemented with 0.5mM dBcAMP from day 7. After 3-4 weeks, the cells were harvested for experiments.

2.9 Generation of iAs from hESCs

iAstrocytes were generated in line with the protocol described by Canals et al. [13] Briefly, when H1 cells reached 80% confluency they were dissociated into single cells and plated in the same way as described earlier for the generation of iNeurons. On day -1 the cells were infected with transcriptional factors Sox9, Nfia, Nfib and the lentiviral construct for rtTA in StemFlex™ medium supplemented with 8 µg/ml polybrene. One day after infection, the medium was refreshed with StemFlex™ containing 1 µg/ml doxycycline. The cells were followingly cultured in Expansion medium consisting of DMEM/F12, 10% FBS, 1% N2 supplement, 1% GlutaMAX™, and 1% antibiotic for two days. From day 3 to day 6 the medium was gradually transitioned from expansion medium to FGF medium consisting of Neurobasal medium with 2% B27 supplement, 1% NEAA, 1% GlutaMAX™, 1% FBS, and 1% antibiotic supplemented

freshly with 8 ng/ml FGF, 5 ng/ml CNTF, and 10 ng/ml BMP4. The cells were dissociated using Accutase® and replated in Matrigel-coated wells on day 7. From day 9 onwards, the cells were cultured in Maturation medium consisting of DMEM/F12 and Neurobasal in a 1:1 ratio with 1% N2, 1% sodium pyruvate, 1% GlutaMAX™, 1% antibiotic and freshly supplemented 5 ng/ml heparin-binding EGF-like growth factor, 10 ng/ml CNTF, and 10 ng/ml BMP4. From this timepoint, the medium was refreshed every 2-3 days and cells were kept until week 3 or week 4.

2.10 Calcium Imaging

Cells plated on Matrigel-coated 24-well or 96-well Corning® glass plates were loaded with 5 µM Fluo-4AM (abcam) and incubated at 37ºC for 30 minutes. After incubation, the cells are washed twice with the indicated medium. Live fluorescence imaging is done using the Yokogawa Cell Voyager CV7000S confocal microscope. For analysis of spontaneous calcium fluctuations, the cells were analyzed for two minutes with a 60X or 40X objective lens. Calcium peak analysis after stimulation is done by the addition of 100 µM glutamate and 100 µM ATP after 30 seconds. Images for all calcium experiments were taken every 0.5 seconds. Fluorescence intensity of the cells was quantified using ImageJ by selecting cells as regions of interest (ROIs). Fluorescence intensity for each ROI is plotted over time. Cells that exhibit changes in basal fluorescence intensity or in response to stimulation were counted manually. Fluctuations in fluorescence intensity after stimulation were standardized to the levels of fluorescence intensity before stimulation.

2.11 Statistical analysis

The data from this report are presented as means ± SD for all experiments, except calcium imaging of iAstrocytes with stimulation. Here, data is presented as means ± SEM. All statistical testing was performed using Prism. For all analyses, we used unpaired two-tailed *t* tests. For qPCR data, multiple *t*-tests were performed to compare the genes in all group combinations. The significance level was set at $P < 0.05$.

3. Results

3.1 Characterization of LUHMES neurons

LUHMES dopaminergic neurons are often used for *in vitro* models in PD research. We characterized the molecular features of these neurons using immunofluorescence analysis and qPCR experiments. Immunofluorescence analysis was performed on 6-day differentiated LUHMES cells to examine the expression of dopaminergic marker tyrosine hydroxylase (TH) – a rate-limiting enzyme for dopamine synthesis – and general neuronal markers, β3-tubulin and MAP2. We found an overall high expression of the general markers β3-tubulin and MAP2. Dopaminergic marker TH was only expressed by a fraction of the cells, which comprised of roughly 1.8% of the cells (Figure 1A, Supplementary Figure 2).

Considering that LUHMES cells will eventually be used for co-culture with astrocytes, we aimed to determine whether culturing conditions would affect the neuronal maturation of LUHMES cells in monoculture when kept in different media than described in a previous study [6]. Additionally, the cells were harvested for experiments at different timepoints to see how this would affect the gene profile of the cells. We examined the changes in expression of five transcriptional factors marking neurodevelopment and nine neuronal maturational markers. We determined the fold change in expression of these markers at two different timepoints and in three different medium conditions using qPCR. It was observed that medium conditions and timepoint of harvesting showed differential effects on the gene profile of the cells. Highest expression of a wide range of markers was found when harvesting cells cultured in co-culture B27 medium at day 10. When considering the change in gene expression between the two timepoints, expression of 7 out of 14 markers increases over time when cultured in the original LUHMES medium, while expression of 11 out of 14 markers increases when cultured in co-culture B27 medium (Figure 1C).

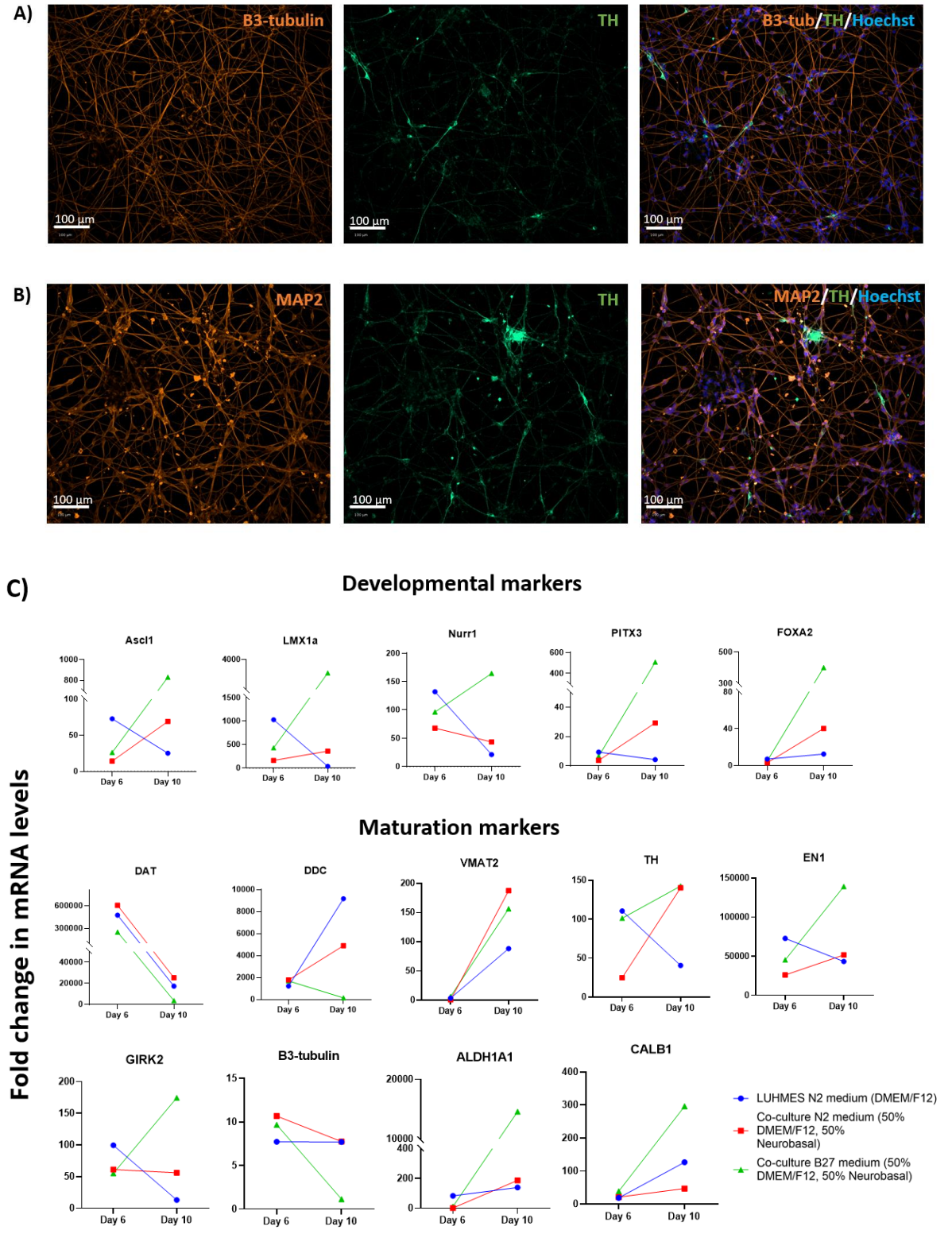


Figure 1 | Molecular characteristics of LUHMES neurons in monoculture. A) Immunofluorescence analysis of day 6 LUHMES cells using β3-tubulin and MAP2 as general neuronal markers and TH as dopaminergic marker. **B)** Fold changes in mRNA levels of several developmental and maturational neuronal markers in day 6 and day 10 LUHMES neurons in different medium conditions. Fold change is normalized to GAPDH levels and relative to undifferentiated stem cell control.

3.2 Supportive effects of neurons and astrocytes in a physiological co-culture model

Astrocytes carry out several supportive functions for neurons. Using a physiological co-culture model of neurons and astrocytes, we aimed to examine these supportive functions by comparing the expression of seven maturational markers through qPCR experiments. We did not find a significant increase in gene expression of neuronal markers TH, DAT, DDC, VMAT, PITX3 and EN1. GIRK2 expression was significantly higher in the neurons co-cultured with astrocytes in a 4:1 ratio compared to monoculture neurons (Figure 2A).

We observed a significantly higher expression of astrocytic markers S100β, GLAST, GLT-1, AQP-4, GLUD2, and vimentin in the astrocytes co-cultured with neurons in a 1:4 ratio compared to monoculture astrocytes. GFAP expression remained the same in co-culture compared to monoculture (Figure 2B). For both neurons and astrocytes, gene expression data of the co-culture in a 2 to 1 neuron to astrocyte ratio did not differ significantly compared to the monoculture.

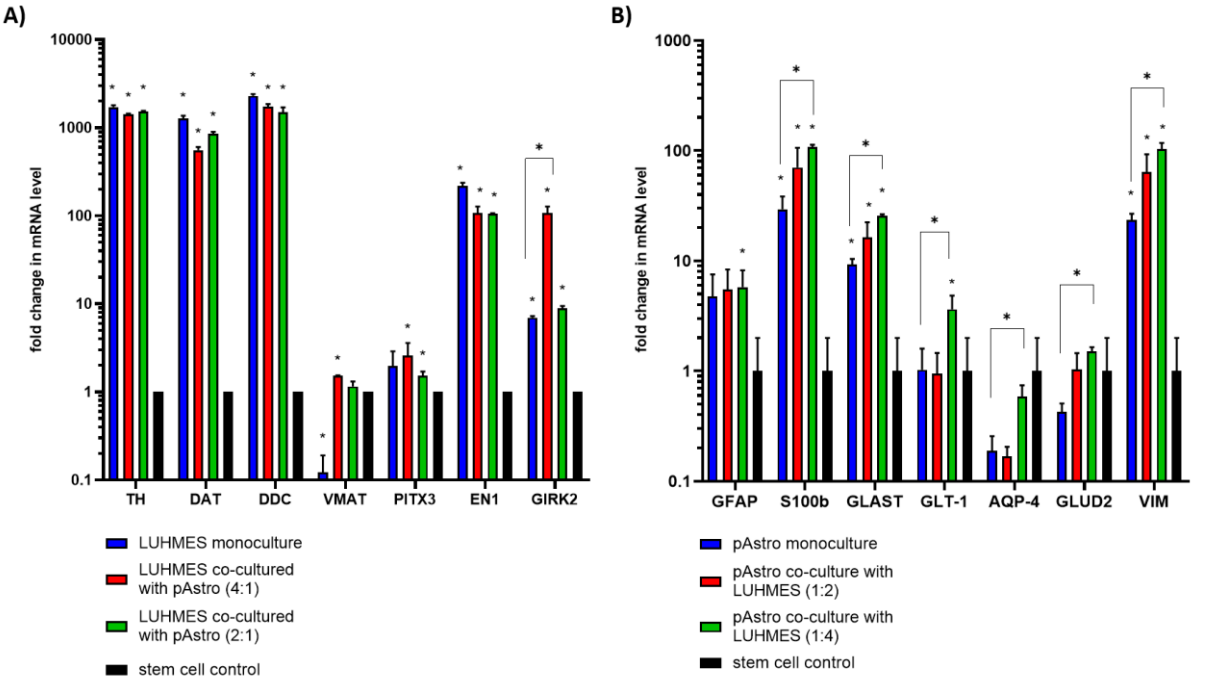


Figure 2 | Gene expression data of LUHMES neurons and primary astrocytes in a physiological co-culture model. A) Fold increase in gene expression of neuronal markers in day 6 LUHMES neurons in monoculture and co-culture in 4 to 1 and 2 to 1 neuron to astrocyte ratios. **B)** Fold increase in gene expression of astrocytic markers in primary astrocytes in monoculture and co-culture in 1 to 4 and 1 to 2 astrocyte to neuron ratios. Fold increase is normalized to GAPDH levels and relative to the control cells. * $P < 0.05$, significance symbols without brackets indicate a significant difference compared to control cells. Data is represented as means ± SD.

In order to prove that both neurons and astrocytes are present in the co-culture model and the cell types can be distinguished, we co-localized the cells using immunofluorescence analysis. For this purpose, it is important that the markers used are specific for one cell type and do not overlap. By using neuronal marker MAP2 and astrocytic marker GFAP we could find cells selective for either one of the two markers and confirm the presence of both cells in co-culture (Figure 3).

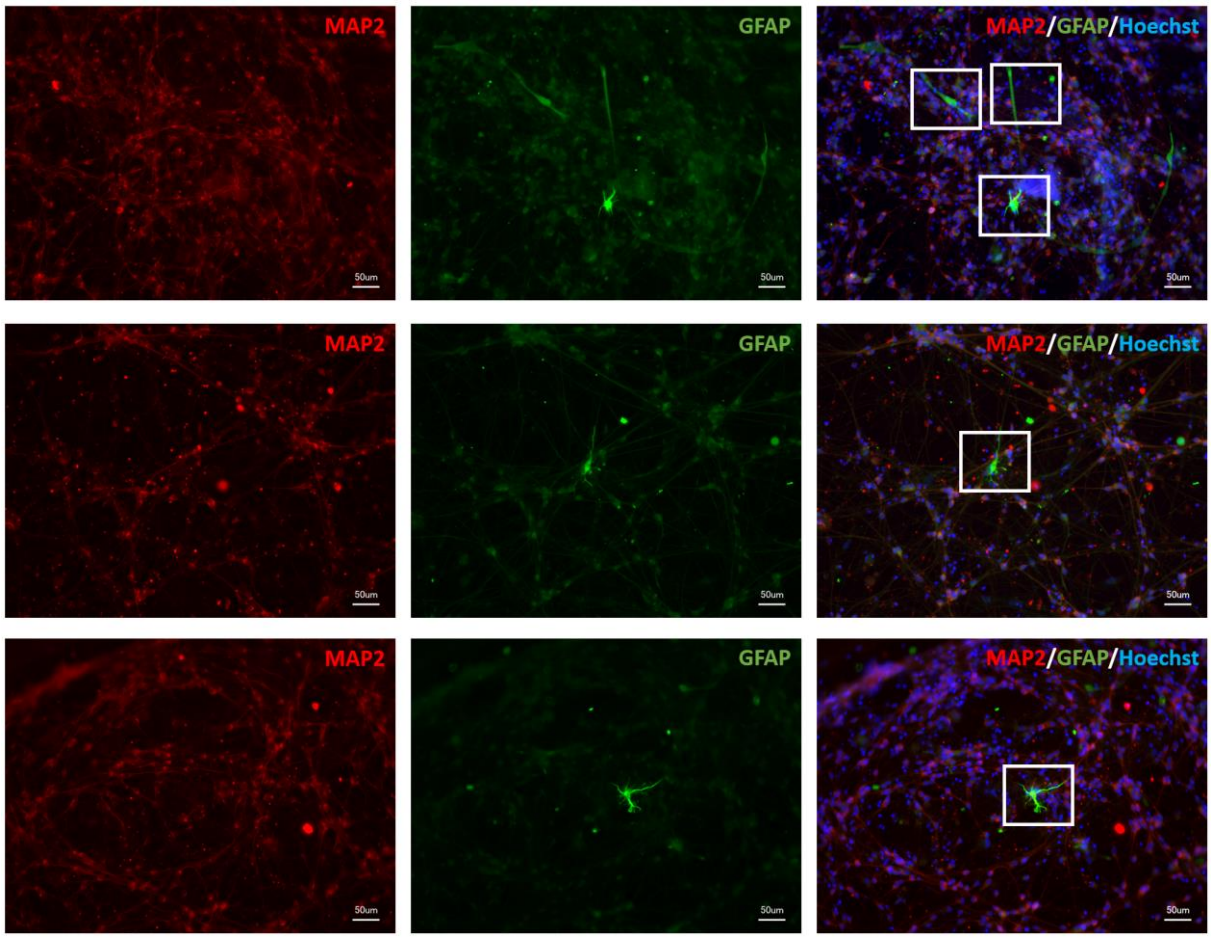


Figure 3 | Co-localization of neurons and astrocytes using immunofluorescence analysis. Immunofluorescence images of day 9 LUHMES neurons in co-culture with primary astrocytes using MAP2 and GFAP as markers. White boxes indicate the astrocytes identified by their GFAP expression.

3.3 Neurotoxic effects of reactive astrocytes in an inflammatory co-culture model

In order to create an *in vitro* model for neurodegenerative disease, we used inflammatory astrocytes in our co-culture. These reactive astrocytes are expected to have a cytotoxic effect on the neuronal culture, mimicking neurodegeneration in diseased states. Quiescent and inflammatory phenotypes were generated by stimulating the astrocytes with FGF-2 + EGF and TNF-α + IL-1α, respectively, as described in previous studies [14] [15]. Differences between the quiescent and inflammatory astrocytes can be observed in brightfield images. Most strikingly, the inflammatory astrocytes seem to be more proliferative than the quiescent astrocytes (Figure 4A).

When comparing brightfield images of the neurons in co-culture with quiescent astrocytes and reactive astrocytes, a lower density can be observed in the culture with neurons that are co-cultured with inflammatory astrocytes (Figure 4B). To quantify the difference in cell density we counted the cells after we harvested them using an automated cell counter. The cell density was significantly lower in the co-culture with inflammatory astrocytes (Figure 4C). The expression of seven out of eleven neuronal markers remains the same in co-culture with quiescent astrocytes when compared to co-culture with inflammatory astrocytes. Synaptophysin and EN1 expressions are significantly decreased in neurons co-cultured with inflammatory astrocytes. However, VAMP2 and DAT expressions are increased in the inflammatory co-culture model (Figure 4D).

Between the quiescent and inflammatory phenotypes, there might be differences in the expression of certain astrocytic markers. We performed qPCR on monocultures on FGF and TNF-α + IL-1α induced astrocytes to compare the gene profiles. The inflammatory astrocytes show a significantly higher expression of GFAP and S100β, while GLT-1 is significantly more expressed in quiescent astrocytes. When co-culturing the quiescent astrocytes with neurons, we observed an increased expression of GFAP and GLUD2, while GLAST and vimentin expression was decreased. In the co-culture of inflammatory astrocytes with neurons, we also observed a decrease in vimentin expression. The expression of AQP-4 was increased in the inflammatory co-culture model (Figure 4E).

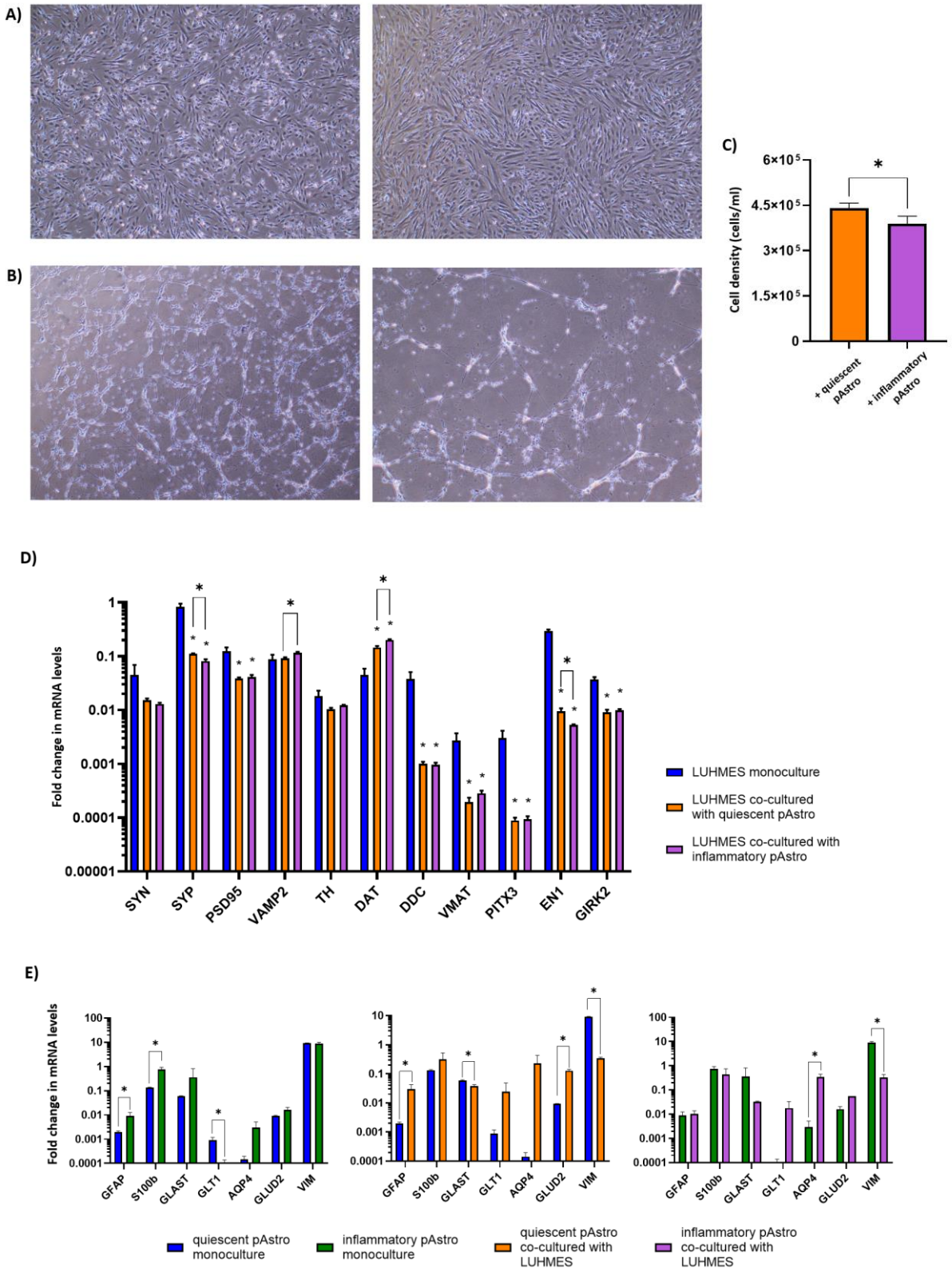
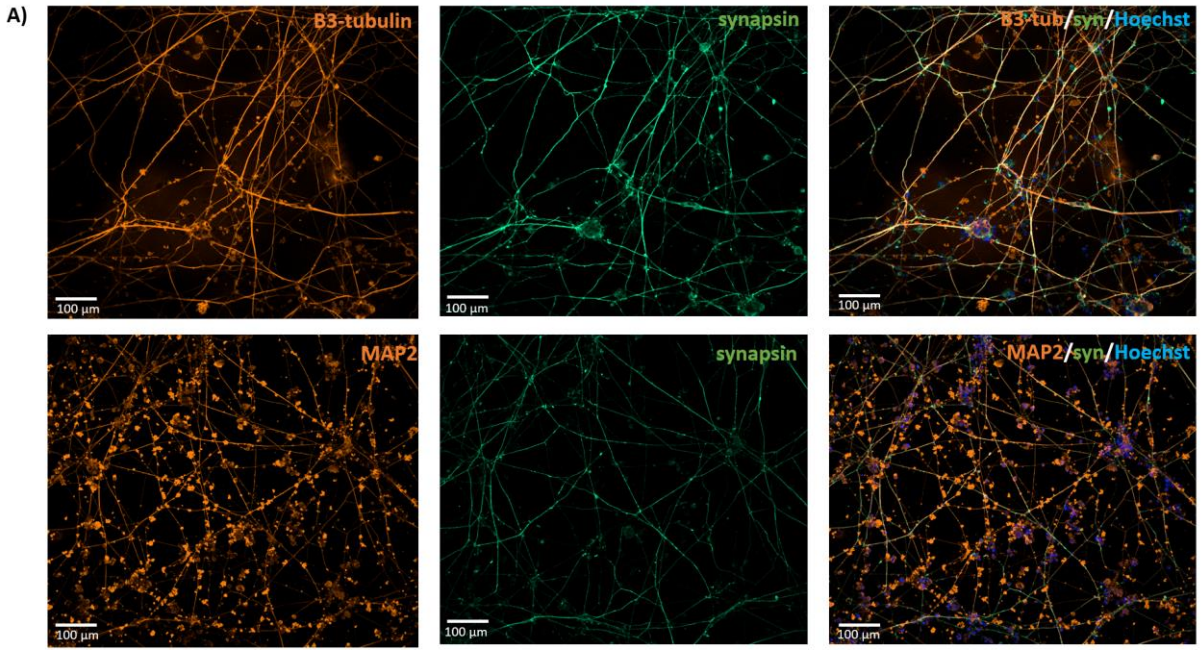


Figure 4 | Morphological and molecular analysis of a neuroinflammatory neuron-astrocyte co-culture model. A) Brightfield images of primary astrocyte monoculture. On the left are the astrocytes that underwent FGF-2 stimulation and on the right are the astrocytes that received TNF-α + IL-1α stimulation. **B)** LUHMES day 6 neurons after 4 days of co-culture with primary astrocytes. On the left are the neurons co-cultured with FGF-2 astrocytes and on the right are the neurons co-cultured with TNF-α + IL-1α astrocytes. **C)** Cell count data of LUHMES neurons

in co-culture with quiescent astrocytes compared to neurons co-cultured with inflammatory astrocytes. **D)** Gene expression analysis of LUHMES neurons co-cultured with quiescent and reactive astrocytes. **E)** Gene expression analysis of quiescent and reactive primary astrocytes in monoculture and co-cultured with LUHMES neurons. Fold change in mRNA levels is normalized to GAPDH expression levels. * $P < 0.05$; significance symbols without brackets indicate a significant difference compared to monoculture. Data is represented as means ± SD.

3.4 Induced Neurons and Astrocytes as cellular source for a co-culture model

An alternative source for generating an *in vitro* neurodegenerative disease model could be stem cell-derived neurons and astrocytes. First, we aimed to generate a pool of general neurons. For this purpose, we used *Ascl1* as only transcription factor. To confirm that the differentiation from stem cells into neurons was successful, we performed immunostaining with general neuronal markers β3-tubulin, synapsin, and MAP2. The neurons stained positive for all the general neuronal markers (Figure 5A). To be able to study PD, specifically dopaminergic neurons should be used. In order to generate a stem cell-derived model for PD research, we additionally delivered transcriptional factors *Lmx1a* and *Nurr1* to the stem cell culture. We confirmed the neuronal cell fate using immunostaining with general neuronal markers, and moreover used TH as a marker to specifically identify dopaminergic neurons, for which the neurons also stained positive (Figure 5B).



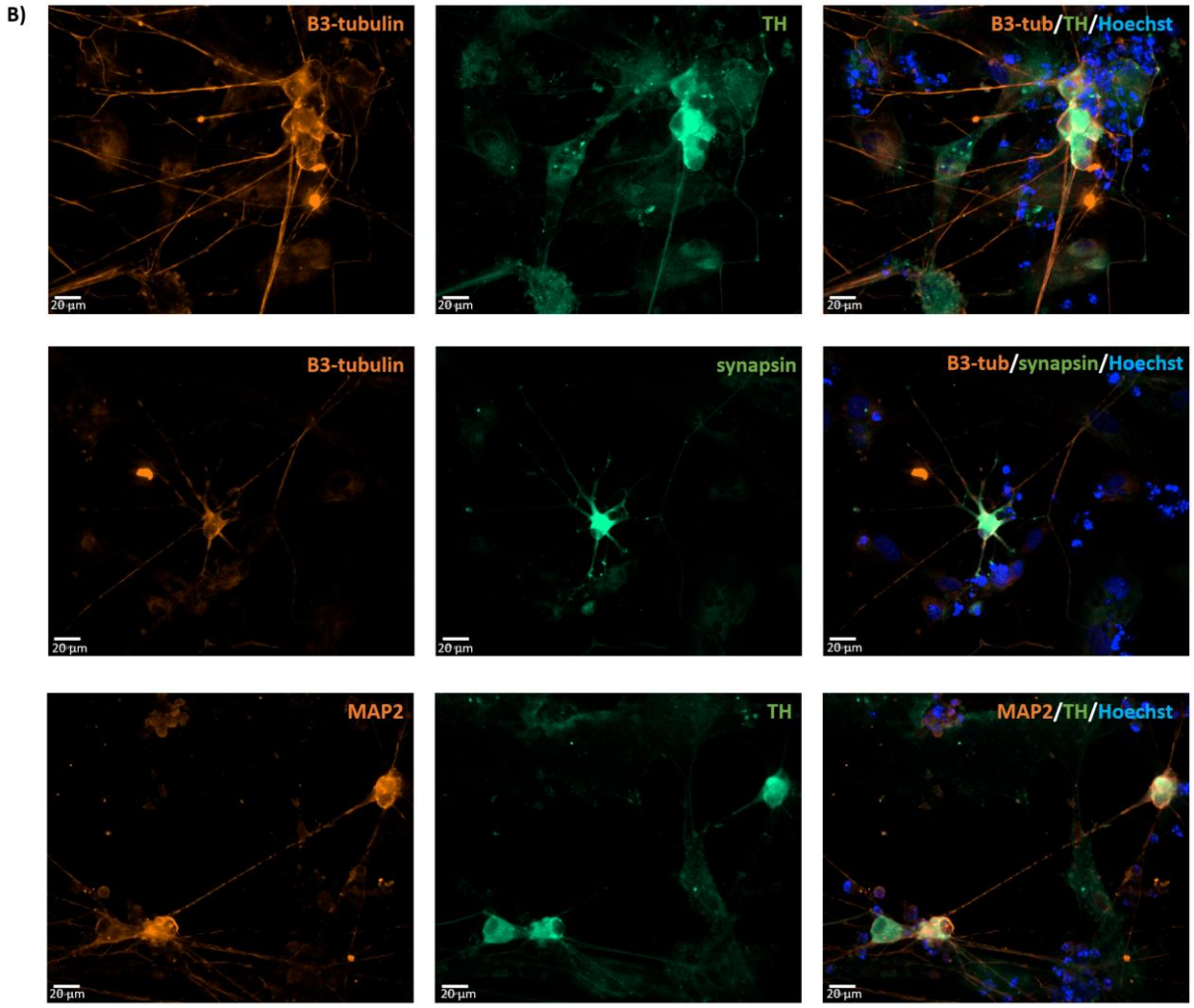


Figure 5 | Molecular characterization iNeurons. A) Immunofluorescence images of general iNeurons that received Ascl1 as only transcriptional factor. B3-tubulin, synapsin, and MAP2 were used as neuronal markers. **B)** Immunofluorescence characterization of dopaminergic iNeurons that received Ascl1, Lmx1a, and Nurr1 as transcriptional factors. Additionally to the previous markers, TH is used to identify dopaminergic neurons.

Next to molecular characterization, we proved that iNeurons are functionally active through calcium imaging experiments. A proportion of the cells show spontaneous neuronal activity as shown by the peaks in their signal intensity curves. The signal intensity curves of the active cells can be clearly distinguished from the non-active cells (Figure 6A). To test whether functional maturation is increased over longer differentiation periods, we performed calcium imaging experiments at two timepoints. We observed a significant increase in the percentage of active iNeurons in the week 4 culture compared to week 3 (Figure 6B). Within these pools of active iNeurons, the signal intensity of the peak values evoked was significantly higher in the week 4 iNeuron culture compared to week 3 (Figure 6C).

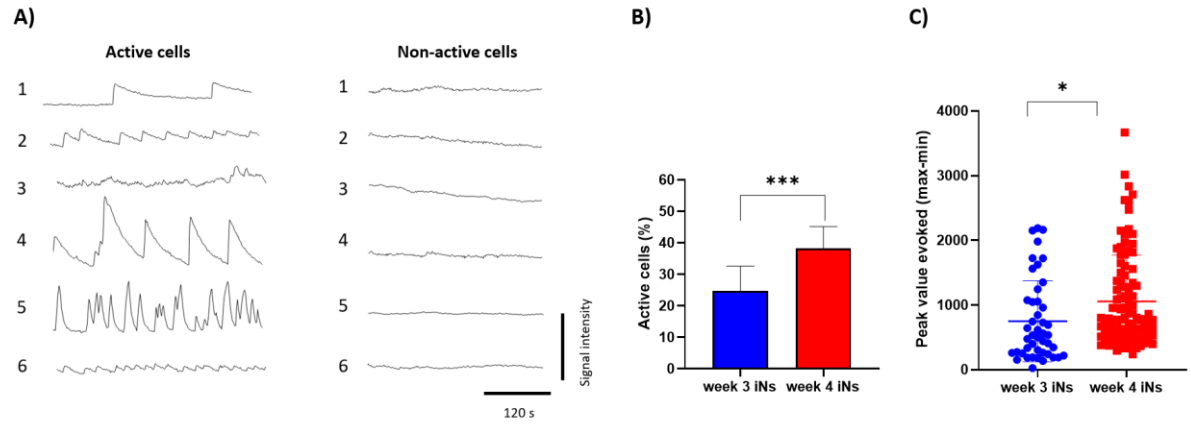
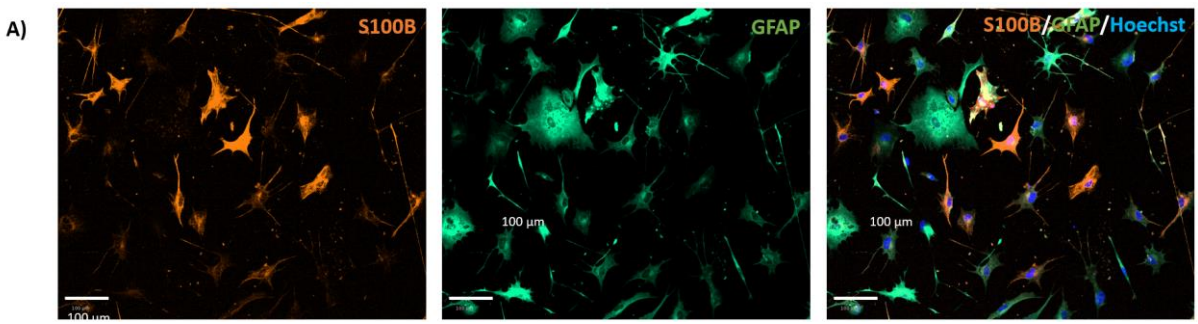


Figure 6 | Functional characterization of iNeurons using Fluo-4-AM-based calcium imaging. A) Examples of signal intensity curves derived from calcium imaging data experiments on iNeurons. Active cells show peaks in their curves over time while in the curves of non-active cells no peaks can be detected. **B)** Percentages of active iNeurons in week 3 and week 4 cultures. **C)** Signal intensity peaks from active iNeurons were quantified and compared between week 3 and week 4. *** $P < 0.001$; * $P < 0.05$. Data is represented as means ± SD.

For the identification of mature iAstrocytes, similar experiments were used as described previously for the iNeurons. Immunofluorescence analysis was performed and the presence of astrocytic markers GFAP and S100β was confirmed. These markers were, however, not uniformly expressed among all the cells and we observed different morphologies among the cells (Figure 7A, 7B). In order to generate a more homogeneous population of astrocytes, we made an attempt to direct the astrocytes to more specific cell fates by exposing the cells to different culture conditions. According to previous literature, culturing in FGF + EGF-enriched medium directs the cells towards a quiescent state, while FBS could induce an inflammatory state in the astrocytes [14] [16] [17]. We observed that the astrocytes show different morphologies when cultured in different medium conditions (Figure 8A). After 7 days of being cultured in the different media, we performed qPCR analysis to compare the gene profiles of the cells. Here we found that GFAP and S100β expression was higher in the FBS-cultured astrocytes compared to the astrocytes cultured in FGF + EGF medium, and the expression of AQP-4 was lower (Figure 8B).



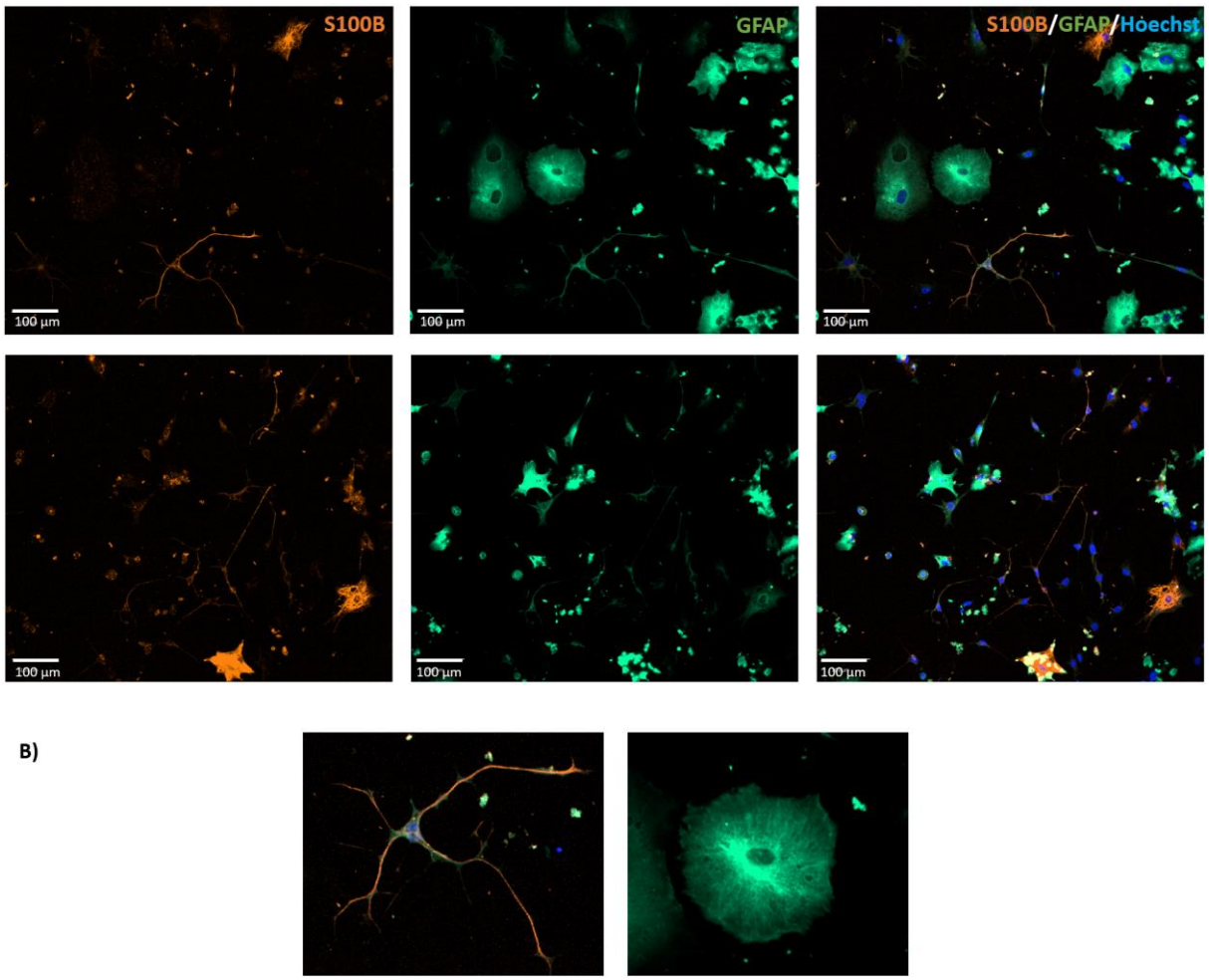


Figure 7 | Molecular characterization of iAstrocytes. A) Immunofluorescence images of day 21 astrocytes showing the expression of S100β and GFAP. **B)** Examples of different morphologies present in the iAstrocyte culture. Left shows a fibrous phenotype, right shows a protoplasmic phenotype.

Calcium is an important molecule for intracellular ATP signaling in astrocytes and calcium transients in astrocytes are known to occur in response to neuronal activity [18]. Therefore, calcium imaging can be used to examine the functional maturity of iAstrocytes. The iAstrocytes were stimulated with ATP and glutamate at a certain timepoint and expected to create a peak in signal intensity in response to these stimuli. We compared the peaks generated by the iAstrocyte culture to peaks generated by control cells, and could observe that the shape of the peaks generated by iAstrocytes in response to these stimuli differ from the control. Moreover, we found that the values of the curves were significantly different between iAstrocytes and control cells (Figure 9A). To rule out that the peak in signal intensity is caused by mechanical stress of medium dispensing onto the culture and not by a response to the stimuli, we additionally compared signal intensity curves between iAstrocyte receiving ATP or glutamate and iAstrocytes receiving medium stimulation only. With this experiment we also found that the peaks generated by stimulation with ATP or Glutamate are significantly different from stimulation with medium only (Figure 9B).

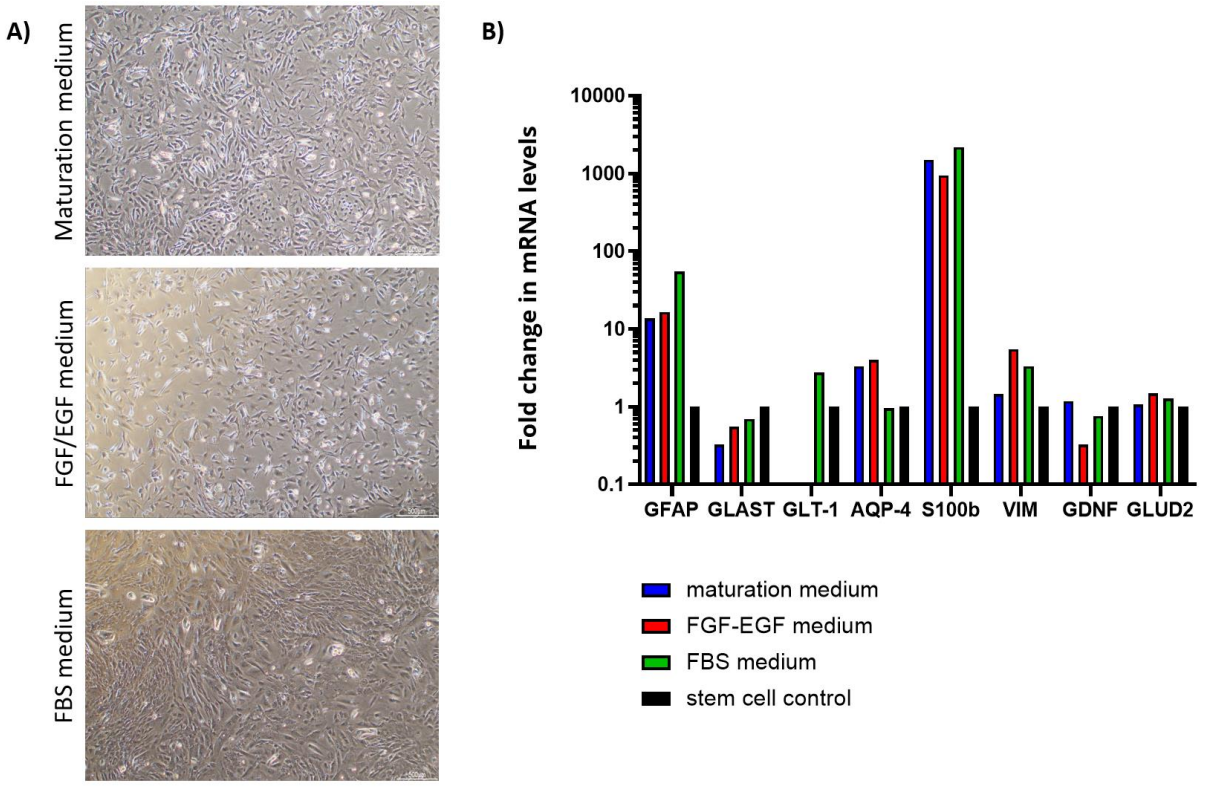


Figure 8 | Characterization of iAstrocytes cultured in different medium conditions. A) Brightfield images showing morphological differences between day 28 iAstrocytes after being cultured in different medium conditions for 7 days. **B)** Fold change in mRNA levels of several astrocytic markers after being cultured in different medium conditions for 7 days.

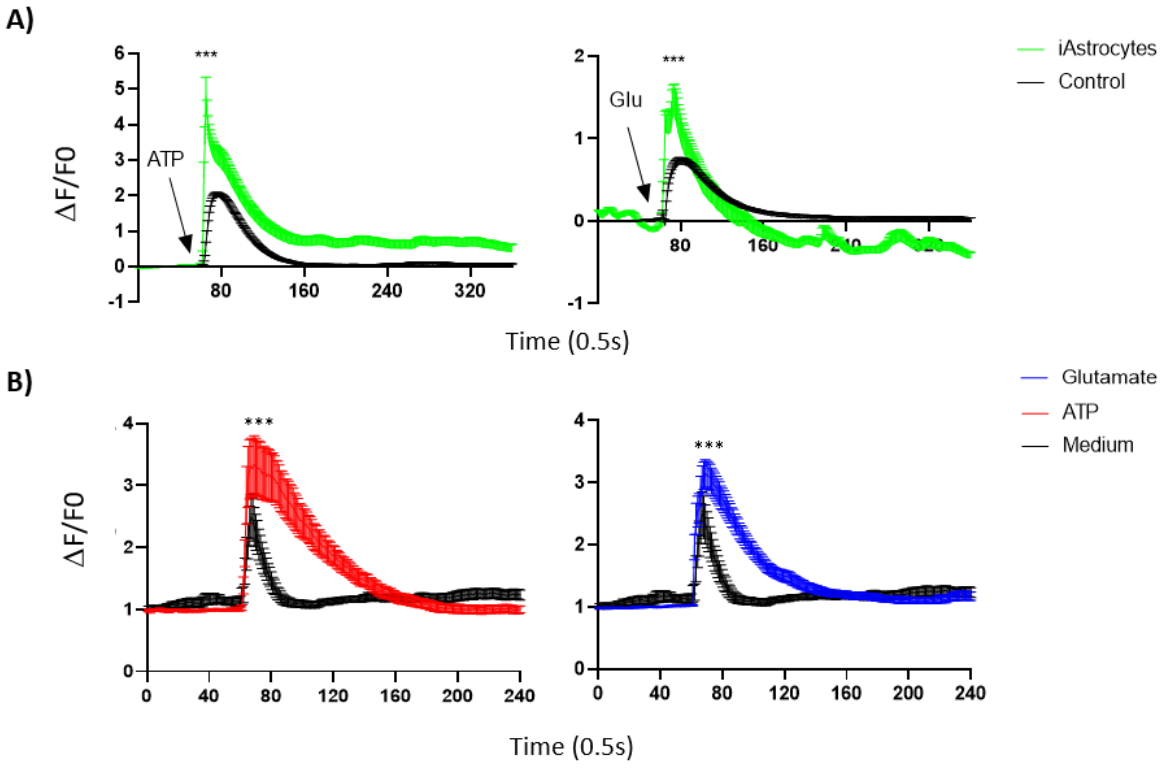


Figure 9 | Functional characterization of iAstrocytes as shown by Fluo-4-AM-based calcium imaging. A) Calcium imaging results showing fold changes in fluorescent intensity in response to ATP and glutamate in day 21 iAstrocytes and control cells (undifferentiated stem cells). **B)** Calcium imaging results showing fold changes in intensity in iAstrocytes receiving ATP or glutamate compared to stimulation with medium only. *** $P < 0.001$. Data is represented as means ± SEM. for n = 50 cells.

4. Discussion and conclusions

In this report we establish that co-culture of LUHMES neurons with primary astrocytes can effectively support the maturation profile of astrocytes in terms of gene expression of astrocytic markers. An underlying mechanism for the increased expression of these markers in co-culture might be homeostatic tuning of astrocytic genes according to neuronal needs [19]. For example, we observe a significant increase in the expression of GLAST, GLT-1, and GLUD2 in our co-culture experiment. These genes encode for important glutamate transporter proteins, and therefore play a role in a prominent supportive astrocytic function, namely glutamate uptake and recycling. Additionally, expression of AQP4, the main water channel protein expressed in the brain, is significantly higher in the co-cultured astrocytes. This protein is believed to be involved in neuroinflammatory disease and have a neuroprotective effect [20]. In contrast to the beneficial effects of co-culture that we found for astrocytic genes, we do not see a significant increase in gene expression of neuronal markers of LUHMES neurons co-cultured with astrocytes compared to LUHMES neurons in monoculture. Also previous studies have reported mixed results on the beneficial effects of astrocytes on neuronal maturation [21]. Hedegaard et al. therefore aimed to determine which astrocytic extracellular proteins play an important role in astrocyte-to-neuron interaction. Using transcriptomic analysis, a set of eight astrocytic genes encoding extracellular proteins that are linked to synaptic neuronal proteins were identified [21]. Future studies on the supportive roles of astrocytes could therefore focus on these eight astrocytic and whether they are expressed before performing a co-culture analysis.

Besides a physiological co-culture model, we aimed to mimic an inflammatory state associated with neurodegenerative disease. In the inflammatory model we established that inflammatory astrocytes, generated through stimulation with TNF-α and IL-1α, have a cytotoxic effect on the neuronal culture as shown by brightfield images and cell counting results. Differences in morphology were observed between the quiescent astrocytes and the inflammatory astrocytes. Moreover, the inflammatory astrocytes seemed to be more proliferative. We also confirmed the difference in astrocytic phenotype through qPCR experiments and found significant differences in expression of certain markers. We see that GFAP – a major constituent of astrocyte intermediate filaments – and S100β – a cytoplasmic calcium-binding protein – expression are significantly increased in the inflammatory

phenotype, which is in line with previous research [3 22]. The expression of astrocytic glutamate transporter GLT-1 is lost in the inflammatory astrocytes, a phenomenon that has also been observed within inflammatory human central nervous system lesions [23]. Therefore, these results further confirm the identity of the inflammatory astrocytic phenotype after TNF-α + IL-1α stimulation.

We could not conclude that co-culturing LUHMES neurons with TNF-α + IL-1α-stimulated astrocytes negatively impacts the expression of neuronal maturation markers. This shows that the cytotoxic effect of inflammatory astrocytes is not directly associated with a change in neuronal maturation. Therefore it is likely that neuronal maturation took place before apoptosis and was thereby left unaffected. For future gene expression studies on the effects of the inflammatory astrocytes in co-culture with neurons, apoptotic and necrotic genes could be used as markers to see whether these genes have an increased expression in the neurons co-cultured with inflammatory astrocytes. When looking at the gene expression of astrocytic markers in co-culture compared to monoculture in this experiment, we see similar effects in the quiescent and inflammatory astrocytes. What is particularly striking, is that expression levels of GLT-1 and AQP-4 are higher in co-culture. These results support the previously stated hypothesis of how neurons regulate the expression of astrocytic genes that play a neuroprotective role to maintain a homeostatic balance.

Using a lentiviral delivery approach of transcription factors, we were able to generate a pool of general iNeurons using only *Ascl1*. When additionally delivering transcriptional factors *Lmx1a* and *Nurr1*, we could successfully generate neurons expressing dopaminergic marker TH. Generation of dopaminergic neurons specifically could potentially lead to generation of a physiologically relevant *in vitro* model for PD, as this is associated with the loss of dopaminergic neurons in the substantia nigra [9]. Besides molecular characteristics, a substantial part of these iNeurons also show functional maturation as shown by calcium imaging experiments. When we compare functional properties of the iNeurons between two timepoints, we see that the functional maturation is increased over time. Culturing the iNeurons for a longer time could therefore potentially generate a more mature cell type, however a maximum could be reached at a certain timepoint where culturing the cells for longer does not have beneficial effects. To determine what this optimal timeframe is for maturation of the cells, longer experiments would have to be done including more timepoints. Moreover, additional transcription factors could be added or medium conditions could be optimized in order to generate higher percentages of functional iNeurons.

With the same approach as described above, we also successfully generated iAstrocytes by delivering transcriptional factors *Nfia*, *Nfib*, and *Sox9*. A heterogeneous pool of astrocytes

was obtained as can be concluded from the immunofluorescence analysis experiments. Within these cultures, we found astrocytes that resemble the fibrous and protoplasmic phenotype (Figure 7B). The fibrous astrocytes possess long, thin, processes, while protoplasmic astrocytes have highly branched bushy processes [24]. The iAstrocytes culture could possibly be directed towards a more specific cell fate by culturing them in different medium conditions. Our experiments show that iAstrocytes cultured in FBS-rich medium develop a different morphology and seem to be more proliferative. This increase in size and higher proliferation rate could be a sign of astrocytic hypertrophy, which is a hallmark of astrocyte reactivity [25]. No strong conclusions could be drawn from the gene expression data from our experiments, therefore more research is required to characterize the gene profiles of the iAstrocytes kept in different medium conditions.

In the process of getting towards previously stated results, multiple challenges were encountered. Optimal culturing conditions for LUHMES neurons firstly had to be determined before progressing to the co-culture experiments. Different coating materials for plating the cells were tested, and the LUHMES neurons did not seem to attach properly to PLO/laminin-111 coated well, as we observed the neurons formed clusters in this case (Supplementary Figure 1A). Also, when culturing LUHMES neurons for longer time periods, the cells start detaching from the outer walls of the wells (Supplementary Figure 1B). This made it challenging to obtain adequate immunofluorescence analysis images from LUHMES cultures, and therefore we could not analyze network complexity in LUHMES monoculture compared to co-culture with astrocytes. Moreover, there were difficulties using the induced cell-type method. The stem cells were unstable, and therefore we were not able to generate a proper co-culture model using induced cells as shown by immunostaining images (Supplementary Figure 3). However, we did prove that we were able to generate both cell types separately, therefore using induced cell types for co-culture could still be a promising approach for future studies.

The ultimate aim is that these studies into neuron-astrocyte co-culture models lay the foundation for an *in vitro* system that closely recapitulates neurodegenerative disease. Essentially, they could lead to better understanding of neuron-astrocyte interactions and possibly facilitate drug screenings. Previous research has already demonstrated that co-culture of neurons with astrocytes ameliorates the toxic effects of neurotoxic agents when added to the culture [26]. A next step would be to add possible neuroprotective compounds, such as anti-inflammatory agents, to the inflammatory co-culture to see whether these could rescue the cytotoxicity caused by the astrocytes.

5. References

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Supplementary Information

Table 1 | Primary antibodies used for immunofluorescence analysis.

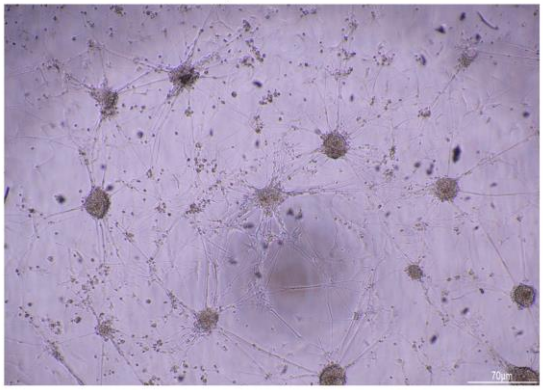
Primary antibody	**Target**	**Concentration**	**Host**	**manufacturer**
Pab	SYN	0.25 µg/ml	Rabbit	Abcam
Mab	B3-tub	1.43 µg/ml	Mouse	Thermo
Pab	GFAP	5 µg/ml	Rabbit	Dako
Pab	TH	1 µg/ml	Rabbit	Abcam
Mab	MAP2	4 µg/ml	Mouse	Sigma
Mab	S100b	2 µg/ml	Mouse	Dako

Table 2 | Secondary antibodies used for immunofluorescence analysis

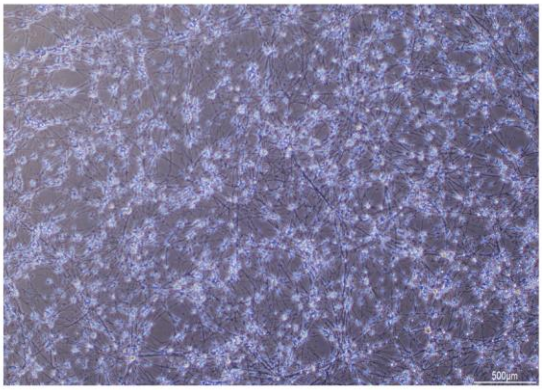
Concentration	**Antibody**	**Manufacturer**
4 µg/ml	Goat Anti Rabbit 488	Thermo
4 µg/ml	Goat Anti Mouse 555	Thermo

A)

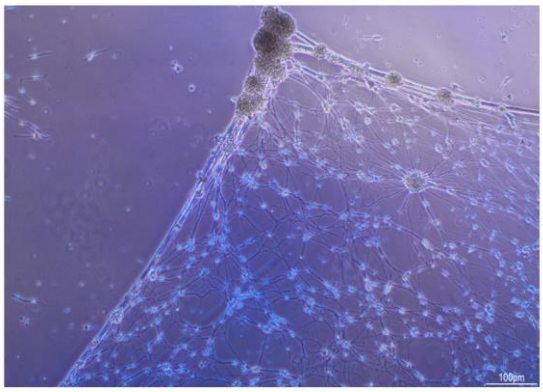
PLO/Laminin-111

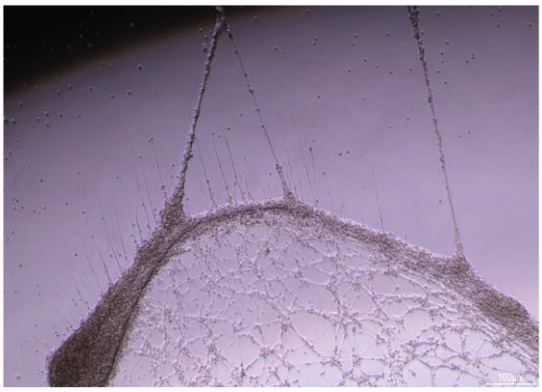


Matrigel



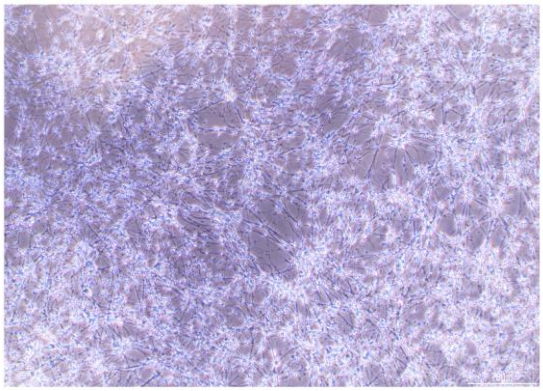
B)



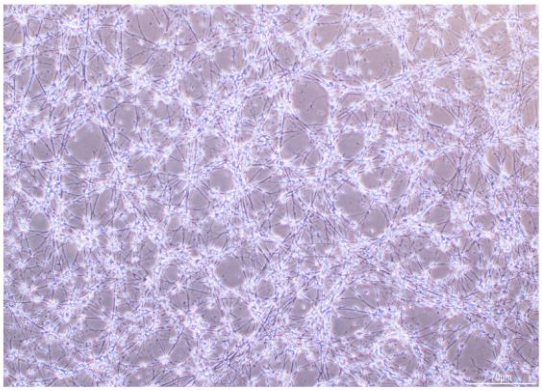


C)

Original LUHMES medium (DMEM/F12)



Co-culture N2 medium (50% DMEM/F12, 50% NB)



Co-culture B27 medium (50% DMEM/F12, 50% NB)

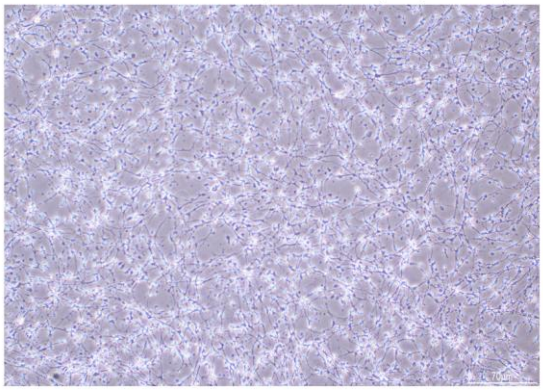


Figure 1 | Culture conditions of LUHMES neurons. **A)** Differences in behavior of LUHMES cells on different coating materials. LUHMES plated on PLO/laminin-111 coating form clusters (left), while LUHMES grow normally on Matrigel coating (right). **B)** Cells start detaching from outer wall of wells with day 10 LUHMES (left) and day 7 LUHMES (right). **C)** Morphologies of day 6 LUHMES cells in different medium conditions.

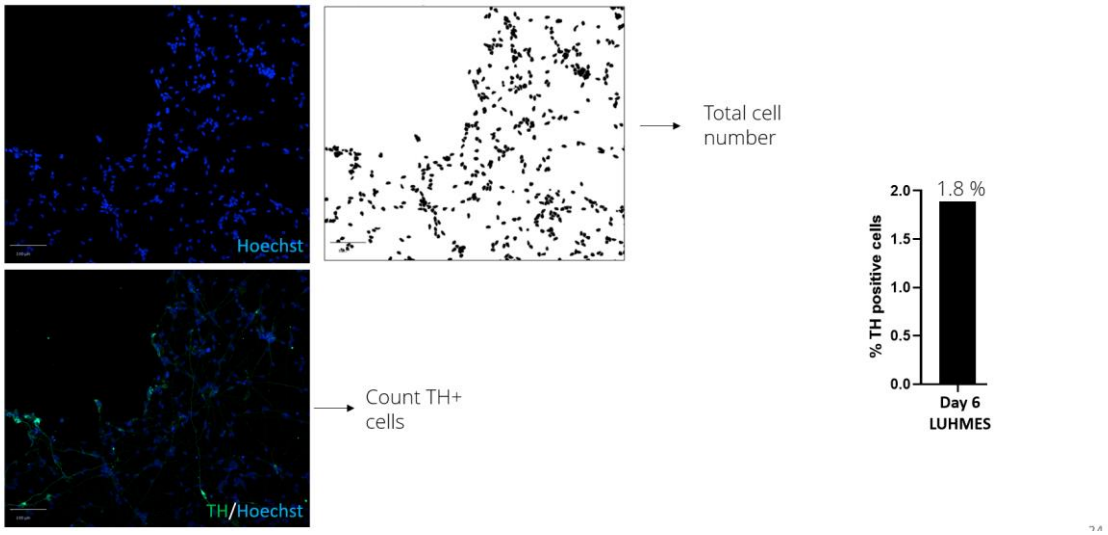


Figure 2 | Determination of TH expression in day 6 LUHMES through immunofluorescence analysis. Using nuclear-staining images, cells were counted with ImageJ software. TH expressing cells were manually counted and the eventual percentage of TH positive cells was 1.8%.

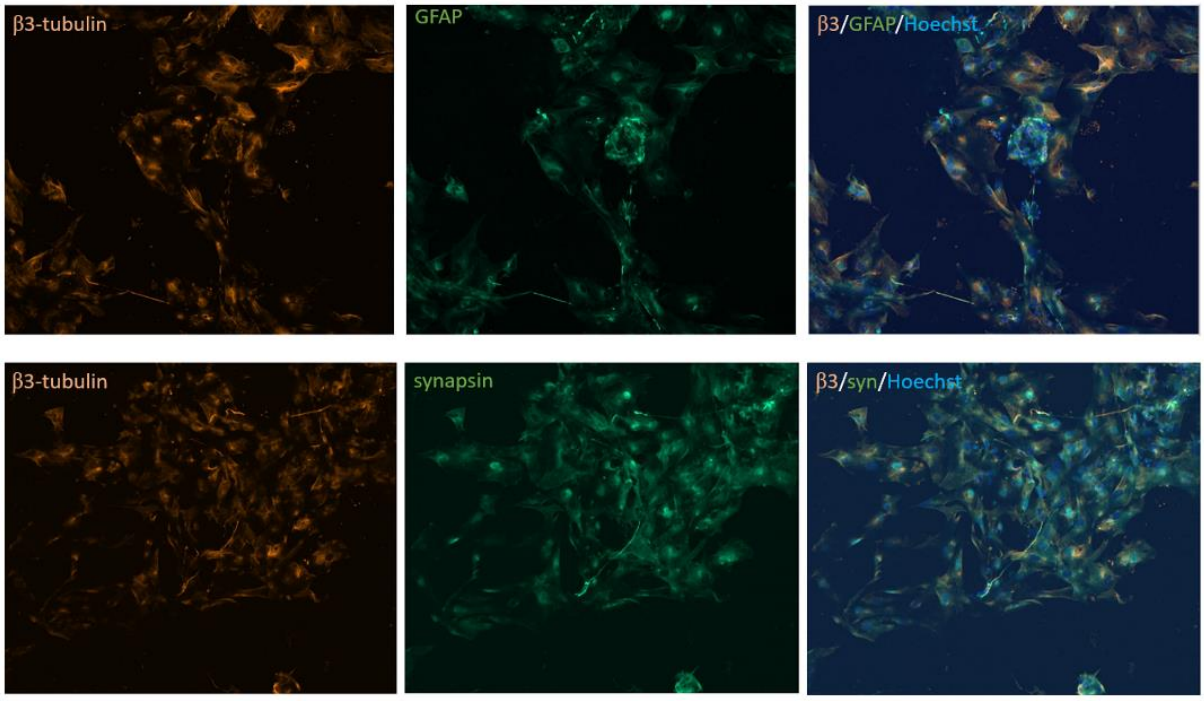


Figure 3 | Immunofluorescence imaging of day 14 co-culture with iNeurons and iAstrocytes. Neuronal markers β3-tubulin and synapsin and astrocytic marker GFAP were used to characterize iNeurons and iAstrocytes in co-culture. No distinct cell types could be identified from this experiment.

List of abbreviations

ESCs	Embryonic stem cells
Lund Human Mesencephalic	LUHMES
Glial cell line-derived neurotrophic factor	GDNF
Parkinson's disease	PD
Induced neurons	iNeurons
Induced astrocytes	iAstrocytes
Achaete-scute homolog 1	Ascl1
LIM Homeobox Transcription Factor 1 Alpha	LMX1a
Nuclear receptor related 1	Nurr1
SRY-Box Transcription Factor 9	Sox9
Nuclear factor I a/b	Nfia/b
Quantitative PCR	qPCR
Dulbecco's Modified Eagle's Medium	DMEM
Fibroblast growth factor	FGF
Fetal bovine serum	FBS
Epidermal growth factor	EGF
Rho-kinase	ROCK
Brain-derived neurotrophic factor	BDNF
Bovine serum albumin	BSA
Phosphate-buffered saline	PBS
Tumor necrosis factor	TNF
Interleukin	IL
Non-essential amino acid	NEAA
Region of interest	ROI
Tyrosine hydroxylase	TH
Microtubule-associated protein 2	MAP2
Dopamine active transporter	DAT
Drosophila dopa decarboxylase	DDC
Vesicular monoamine transporter	VMAT
Pituitary homeobox 3	PITX3
Engrailed Homeobox 1	EN1

S100 calcium binding protein β	S100β
Glutamate/aspartate transporter	GLAST
Astrocytic glutamate transporter 1	GLT-1
Aquaporin 4	AQP-4
Glutamate dehydrogenase 2	GLUD2
Glial fibrillary acidic protein	GFAP
Vesicle-associated membrane protein 2	VAMP2