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Developing and Investigating New Methods for Alzheimer's Disease Modeling Using iPSC Derived Co-cultures of Cortical Neurons and Astrocytes

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Abstract

Alzheimer's disease (AD) is the most common cause of dememtia. Models that mimic the human brain and AD pathology, for drug discovery and developing treatments for AD, are needed. One such model is possibly co-cultures of human induced pluripotent stem cells (HiPSC) derived neurons, astrocytes and microglia. In this project, a protocol for differentiating astrocytes from HiSPCs, was implemented. Immunocytochemistry stainings showed that astrocyte markers were expressed more the longer into differentiation and the more mature the astrocytes got. Functional analysis done through live cell calcium imaging on mature astrocytes, showed that 90% of cells responded after stimulation with ATP. It was shown that mature astrocytes could survive in different neuronal medias, where BrainPhys media gave rise to more expression of astrocyte markers. Co-cultures of mature neurons and astrocytes were created to investigate survival and functionality of neurons when cultured together with astrocytes, and alone. Both cells were found to survive together, where neurons and neurite maturation and proliferation seemed to be improved in the co-cultures. Furthermore, synaptic protein expression was found to be higher in neuron and astrocyte co-cultures compared to neuron mono-cultures and total neurite lengths per cell were slightly improved in co-cultures cultured in neural maintenance media. In a future perspective, the possibility to differentiate astrocytes from HiPSCs may give rise to models, for example co-cultures of neurons, astrocytes and microglia, that mimics the human AD pathology better than models that have been used so far.

Contents

1
Introduction

1.1 Background

Alzheimer's disease (AD) is a progressive neurological disorder and accounts for between 60 to 70% of dementia cases, which makes it the most common cause of dementia [1]. Symptoms usually start with mild memory loss in the early stages of disease. In later stages, AD patients might develop a lack of response to the environment and lose conversation ability [2]. Furthermore, shrinkage or atrophy of the brain along with brain cell death are the pathological characteristics in the latest stages of the disease [1]. In the United States AD is the 6th leading cause of death. The estimated survival after diagnosis is 4 to 8 years, however upwards a life-span of 20 years is not impossible [2].

One of the main characteristics of AD in the brain is the formation of amyloid plaques. These plaques are a result from the cleavage of amyloid precursor protein (APP) forming toxic β-amyloid42 (Aβ42). Aβ42 will aggregate and accumulate between neurons, in a process that leads to disruption of cell functions [3]. A reverse correlation between Aβ42 levels in the brain versus the cerebrospinal fluid (CSF) has been found [4, 5]. This correlation points to decreased Aβ42 in CSF of AD patients and may be due to the plaques trapping Aβ42 [4]. Another protein relevant to AD is Neurogranin, which is a synaptic protein. This protein has been shown to decrease in brains with AD compared to controls, along with another synaptic protein, synaptotagmins. This seems to be more prominent in early AD compared to late AD [6]. However, synaptic proteins in CSF showed the opposite as made clear from two papers published in 2014 [7, 8]. An increase of neurogranin in CSF was noted in three independent clinical cohorts, on top of this, the increase was also noted in early stages of AD [7, 8]. These results suggests that synaptic proteins in CSF are potential biological markers (biomarker) that could be used to screen for AD early on. The mechanism for this synaptic protein release, along with its association with Aβ accumulation is not known.

There is currently no cure for AD and the death related cases of Alzheimer's is not slowing down [2, 9]. Although recently, an antibody treatment, aiming at removing Aβ from the brain, was approved in the US and seems to reduce cognitive and functional decline in early AD. Efforts are now being taken worldwide to help find better ways to treat, delay and prevent AD [2], but also to detect it as early as possible for the treatment to be effective.

In the past decades most AD clinical trials have been unsuccessful with 99.6% failure rate in the period 2002 to 2012[10], likely due to poor understanding of the disease[11]. There have been a lack in models that could effectively recreate pathological, biochemical and behavioural characteristics. Earlier models to investigate AD included CNS organotypic cultres developed from rat hippocampus and cortex. Embronynic spinal cords and ganglia from rats were also used to study differentiation and electrohpysiological properites of cells. Problems with these models are that that the cells are hard to maintain in 2D cultures, along with this, tissues

varies from sample to sample and lowers the repeatability of experiments[11]. There are also other experimental models based on for example mice, non-human primates and zebrafish. All models have strengths and weaknesses, but one thing all share is the ability to only mimic limited aspects of the human AD pathology [12] and the translational value from pre clinical efficacy to clinical activity is poor [13]. This is potentially due to features in brain structures that are species specific, such as cerebral cortex which is believed to be important to cognitive functions specific to humans [13]. SH-SY5Y is a human derived cell line that is frequently used in research of neurodegenerative diseases [14]. An advantage compared to animal based models is that SH-SY5Y expresses human protein [14]. The cell line is originally derived from a metastatic bone tumor biopsy. With this follows that SH-SY5Y has cancerous properties that can influence differentiation fate viability and metabolic properties [15].

In 2007 Shinya Yamanaka published an article in which they successfully reprogrammed differentiated human somatic cells into pluripotent stem cells [16], which 5 years later won him the Nobel prize [17]. Induced pluripotent stem cells (iPSCs) are derived from somatic skin and blood cells [18]. The somatic cells are made pluripotent by forced induction of genes and factors for the cells to keep embryonic stem (ES) cell properties [19]. The possibility to generate stem cells from somatic cells have made novel *in vitro* AD models possible. Since the discovery iPSCs have been differentiated into neurons, astrocytes and microglia, all three representing cell types present in the human brain. This can be a useful resource in research of neurodegenrative diseases, AD included, since models that mimics the human brain can potentially be created. More accurate models can be a tool to identify new therapeutic targets to treat AD [13]. As already mentioned, deposits of Aβ plaques is the pathological hallmark for AD. The focus in research has been to investigate neurons, due to them containing these pathological markers for AD [20].

One model that could possibly mimic the human brain better than single cell- and animal models is to create co-cultures of iPSC-derived neurons, astrocytes and microglia. Astrocytes have been shown to accumulate large amounts of Aβ42 in co-cultures with neurons. Instead of digesting the Aβ42 the astrocytes store it intracellularly [21]. Along with this, in 2007, the astrocytic changes from neurodegeneration were noted in post-mortem AD patients, where senile plaques and blood vessels containing Aβ, were surrouned by reactive hypertrophich astrocytes [20]. The fact that astrocytes are involved in AD is not a new discovery but that there have not been more advancements in AD research on astrocytes shows there is a need for more adequate methods [20]. Astrocytes is a star shaped type of glial cell located in the nervous system, with the main role role to support other CNS cells by providing physical and trophic support, for synaptic maturation and signaling to work as intended[22]. Taking on a regulatory role, astrocytes are functional in both neuro- and synaptogenesis, as well as controlling the permeability of the blood-brain barrier and maintaining extracellular homeostasis [23]. Co-cultures of that character have been done before [24]. Neuronal and microglia cultures are already established in the Zetterberg lab, but protocols for astrocyte differentiation and co-culture have not been implemented.

1.2 Aim

The aim is divided to four points

1. Implement a protocol for differentiating mature human astrocytes.
2. Characterize astrocyte maturation.

3. Optimize neuron-astrocyte co-culture.
4. Assess the effect on neuronal maturation in the absence/presence of astrocytes.

In the long term this can lead to potentially better models to investigate AD. Further understanding of the impact of Aβ exposure to synaptic biomarker release can be gained. This is important for the development and biological understanding of novel biomarkers.

1.3 Delimitation

There are 4 main types of cells in the human brain, and this project, with the time that is available, will only cover the culturing and differentiation of neurons and astrocytes from iPSCs.
A further limitation is the choice of iPSCs, while one of the cell lines is already proven to work for neuron differentiation, the other line has not been as much tested for this, and there is not time to find more optimized cell lines. When it comes to astrocytes, a protocol does not exist for astrocyte differentiation at the laboratory, which is why a protocol for these cells will be developed.

1.4 Social and Ethical Aspects

When it comes to social and ethical concerns in biotechnology there are certain fields that have been widely controversial and criticized, for example antibiotic handling, stem cell research and genetic modification (GMO), more commonly perhaps in the agricultural field where GMO crops are more prevalent to the market. While genetic modification of somatic cells can be used to treat medical conditions, genetic germ cell modification can in contrast enable modified genes to be carried from one individual to another individual. This is therefore prohibited in 75 countries [25]. In this project however no genetically modified cells will be used.

The world has however progressed significantly and has started to accept biotech research more and more, because of all the great things that come from research; medicines to varying diseases and knowledge into bodily mechanisms to just name a few.

Most ethically controversial could be embryonic stem cell research, since in order to obtain these cells, destruction of the embryos is necessary. It is widely controversial when life begins and has been linked to abortion debates [25].

In our project we will handle and culture stem cells, namely induced pluripotent stem cells. As already mentioned iPSC are reprogrammed somatic cells and are useful for disease modeling. Compared with embryonic stem cells, iPSC escapes some of the ethical issues attached with this since neither embryos nor oocytes are used. A skin biopsy is however needed to harvest the somatic cells, but compared with oocyte donation, there are less risks [26]. The patients from which the somatic cells are harvested for iPSC have given consent and their privacy is highly respected. For cells that are purchased, most often than not, researchers will not know from whom their cells are originating. The iPSCs will however keep the genetic buildup or DNA from the donors, and great care needs to be taken to not infringe on the patients genetic information.

Another pressing issue in biotech research is antibiotic resistance buildup in germs. This is a phenomenon where bacteria, for example, develop a resistance to the drugs that are meant to eliminate them, and instead continue to grow [27]. This issue is often due to overuse of

antibiotics, and contrary to popular belief, if a bacteria even develops resistance to only one antibiotic could have serious implications. Antibiotic resistance could potentially lead to us not being able to treat infections and control public health treats [27]. However in this project no added antibiotics in the mediums for iPSC or astrocyte culturing is used, instead proper aseptic techniques and rigorous sterility should ideally ensure that no unwanted bacteria grows in the cell cultures. For the induction to neural cells a medium containing penicillin and streptomycin is used. This is because of the long protocols (up to 90 days) and a potential germ contamination would be devastating. With this said, and considering that the experiment is conducted in a proper lab environment. All the biowaste, and especially the biowaste containing potential antibiotics will be discarded properly and not thrown in the sink where it could potentially end up in the environment. The risk of antibiotic resistance spreading outside of the lab is therefore small.

2
Methods and Materials

This chapter, goes through the culturing and differentiation process of neurons and astrocytes, along with seting up the co-cultures. Furthermore are the experimental setup also presented. All reagents, chemicals used and media compositions along with specified protocols mentioned in this chapter can be seen in appendix A.

2.1 Neuron and Astrocyte Differentiation

In this section the procedure of differentiation neurons and astrocytes from iPSCs is explained. Figure 2.1 and 2.2, neuron respectively astrocyte, shows which media was used for which time period. For neuron differentiation, figure 2.1, Neural Induction Media (NIM) was used from day 0 to 10, Neural Maintenance Media (NMM) supplemented with FGF_2 from day 10 to 19, NMM from day 19 to 32. At day 32 the cells were seeded onto ibidi slides and in wells to mature in NMM until day 75. For astrocytes, figure 2.2, NIM was used from day 0 to 13, Glial Expansion Media (GEM) from day 13 to 32, Astrocyte Induction Media (AIM) from day 32 to 47, Astrocyte Maturation Media(AMM) from day 47 to 75 and Astrocyte Media (AM) from day 75 to 110. More details of the differentiation procedures follows in section 2.1.1-2.1.4.

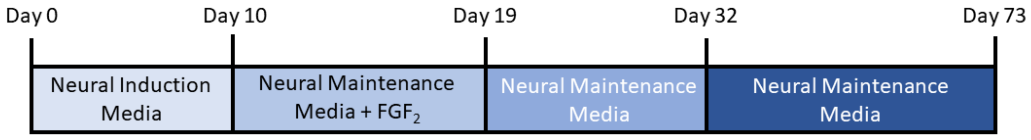

Figure 2.1: Overview of differentiation of neurons from iPSCs, including media used at what time frame.

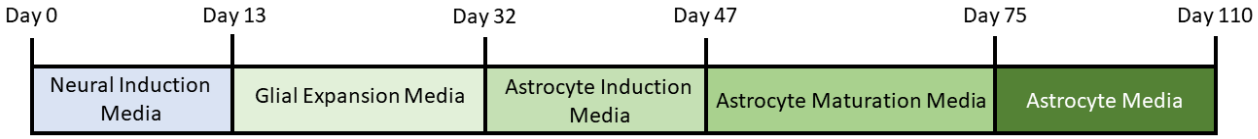

Figure 2.2: Overview of differentiation of astrocytes from iPSCs, including media used at what time frame.

2.1.1 Culturing of Human iPSCs

Differentiation of both neurons and astrocytes starts from two human iPSC lines, Ctrl1 and 004iC2, both coming from two healthy individuals. The cell lines were taken up from nitrogen storage tanks and thawed according to protocol A.3. In brief, the two cell lines were thawed and plated on two different 6-well primaria plates coated with matrigel according to protocol A.5.1, in 2ml mTeSR+ medium each. The following medium changes were done the day after

thawing/passage and then every second day with 2ml mTeSR+. The cells were passaged with EDTA according to A.6 when 80% - 100% confluency were reached. All plates were coated with matrigel according to A.5.1. The cells were cultured in mTeSR+ for around 14 days prior to start of differentiation, passaged a total of 4 times, every 3-5 days.

2.1.2 Differentiation of Neural Stem Cells from Human iPSCs

The protocol for differentiation of iPSCs into neural stem cells (NSC) followed the protocol developed by Shi et al [28] with slight modifications. When the iPSCs reached 70% - 90% confluency, the cells were detached with EDTA and merged 2:1, which means that the total worth of cells for two wells were seeded in one single well. Induction to NSC begins with change of media to Neural Induction Media (NIM) containing SB431541 and Noggin for dual inhibition of SMAD signaling. Worth noting is that two different variations of NIM were used, one containing B-27 with vitamin A (+V.A) and one and without vitamin A (-V.A). This was to see if it is possible to differentiate astrocytes from NSCs from the same starting point as for the protocol for neurons. In total four 6-well plates, two for each cell line with +V.A and -V.A, were seeded with the variations of NIM. NIM was changed every day for 8 to 12 days, while monitoring the appearance of cells with neuroepithelial morhpology. The cells should become small, uniform and "shine" with a brownish/yellow color. If cells start to detach, they should be passaged before day 10 to 12.

After 8 days, induction ended for all Ctrl1 wells due to the cells started to detach from the plate. Induction ended after 10 days for the 004iC2 +V.A wells and 13 days for 004iC2 -V.A. (The reason for the extra days were that the cells neuroepithelial morphology was not clear enough.) On the end of inductions, the wells were passaged 1:2, with dispase according to A.7. In brief the Ctrl1 and 004iC2 cells cultured in absence of vitamin A, where all passaged without deviations and plated in 2ml glial expansion media (GEM) on laminin 2020 coated 6-wells. These were to be differentiated into astrocytes. In figure 2.3, an overview of pictures taken from the day of thawing of iPSCs until start of NPC induction can be seen.

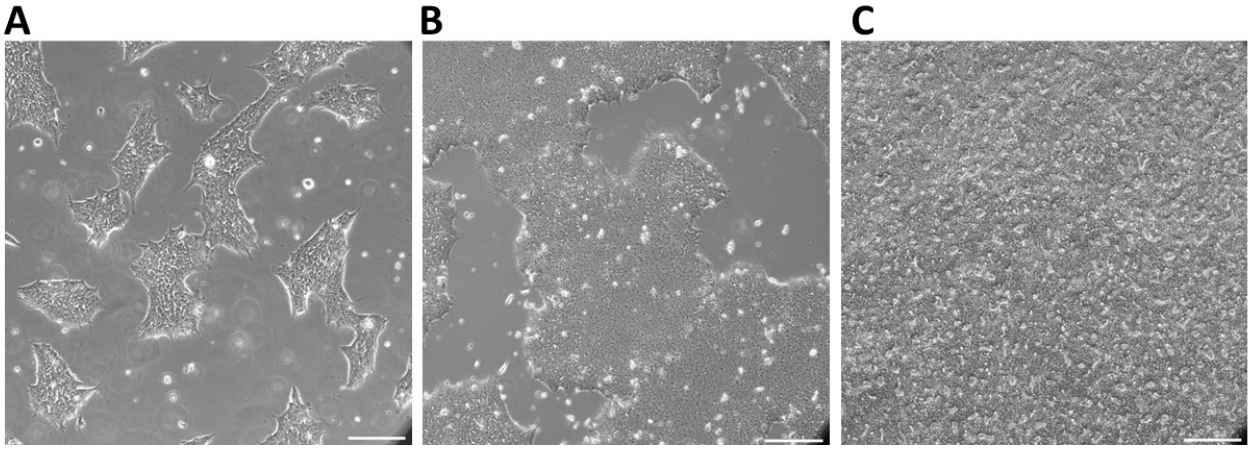

Figure 2.3: Overview from iPSC culturing and NPC differentiation. **(A)** 004iC2 cell line, day after thawing. **(B)** 004iC2 cell line, 70% confluency. **(C)** Ctrl1 cell line, one day post merge, 100% confluency.
Scale bar: 200µm

The Ctrl1 and 004iC2 cells cultured in the presence of vitamin A, were also passaged 1:2, but further divided, into neuronal and astrocyte differentiation. Protocol A.6 was therefore adjusted by splitting the Ctrl1 +V.A cell suspension in two tubes after the last wash step and

spinning it quickly in the centrifuge before resuspending again and seeded in 2ml neural maintenance media (NMM) supplemented with 20 ng/ml FGF_2 or GEM, respectively on laminin 2020 coated 6-well plates. For the 004iC2 +V.A well, protocol A.6 was refined even more. After the second wash the cell suspension was split in two 15 ml falcon tubes and the rest of the washes were carried out as normal, lastly being seeded in 2ml NMM supplemented with 20 ng/ml FGF_2 or GEM on two different laminin 2020 coated 6-wells. Below is a table containing the plate and well setup leading into neural progenitor cell (NPC) and glial progenitor cell (GPC) inductions for neuron and astrocyte differentiation respectively.

Table 2.1: Plate setup for neuron and astrocyte differentiation

Plate Setup				
Plate	**Cell Line**	**(+/-) Vitamin A**	**Differentiate Into**	**Total Wells**
1	Ctrl1	+ Vitamin A	Neurons	1
2	Ctrl1	+ Vitamin A	Astrocytes	1
3	Ctrl1	- Vitamin A	Astrocytes	2
4	004iC2	+ Vitamin A	Neurons	1
5	004iC2	+ Vitamin A	Astrocytes	1
6	004iC2	- Vitamin A	Astrocytes	2

2.1.3 Differentiation of Mature Neurons from Neuronal Progenitor Cells

Cells from plate 1 and 4 were cultured in NMM supplemented with 20 ng/ml FGF_2 for a total of nine days after induction. Medium was then changed to NMM solely for the rest of differentiation. Medium changes were done every second day and the day after passage. Dispase passages of 1:2 were performed when cells were 80% - 100% confluent (every 2-4 day) and plated on laminin 2020, according to protocols A.7 and A.5.2. Around day 22-25 after the start of induction, the cell cultures were fairly clean and could be passaged with accutase according to protocol A.8. The Ctrl1 cells were passaged 1:1 to 1:1.5 with accutase and plated in NMM on laminin 521 coated 6-well plates. 004iC2 cells were, however, manually picked according to protocol A.9 due to problems with non-neuronal cells and plated as Ctrl1. Ctrl1 cells were continually cultured in NMM for around 10 days, with medium changes every second day and after passages. Accutase passages were conducted when cells reached 80% - 100% confluency.

004iC2 cells were cultured in NMM up until day 28 after induction when the cells started to look contaminated so a dispase and accutase passage were performed right after the other in an effort to clean them up. In brief, dispase passage was performed according to protocol A.7 and instead of plating after the final wash, the cells were respuspended in 500µl accutase and an accutase passage were performed according to protocol A.8.

On day 31 after start of induction, the 004iC2 neurons were discarded due to them being still very contaminated. Ctrl1 cells were however passaged with accutase a final time, before being seeded out on laminin 521 and poly-L-ornithine coated 24-well plates and 8-well ibidi-slides. In brief, the cells were passaged with accutase according to protocol A.8. Then counted and plated to 50000 cells/cm^2 in 500µl and 250µl NMM on 36 24-wells and 23 ibidi-8-wells (for staining), respectively. In figure 2.4 an overview of the cells can be seen around these days.

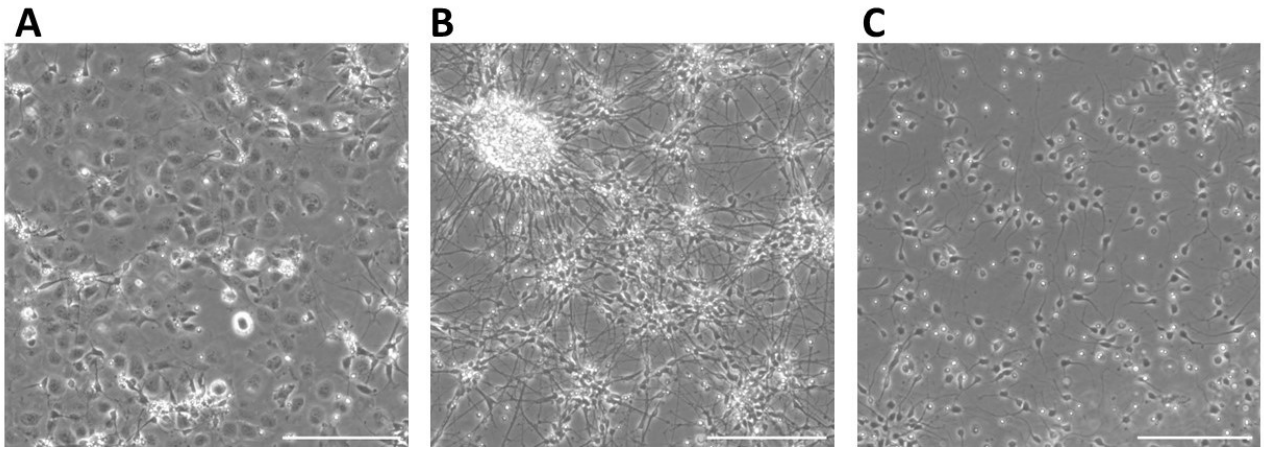

Figure 2.4: Images taken around the days when final seedout for the neurons occurred. **(A)** 004iC2 neurons day 31 after induction and discarding day. **(B)** Ctrl1 neurons day 32 after induction and day of final seedout **(C)** Ctrl1 neurons day 33 after induction and one day after final seedout.
Scale bar: 200µm

The seeded neurons were cultured in NMM for 6 weeks, with medium changes every second day. Around the tenth day of culturing the NMM were spiked with 10µl/ml laminin 521 of total media volume to ensure that the cells did not detach. Day 73 after start of induction, Ctrl1 neurons were co-cultured with the 004iC2 -V.A astrocytes. Ctrl1 neurons, differentiated for 74 days can be seen in figure 2.5.

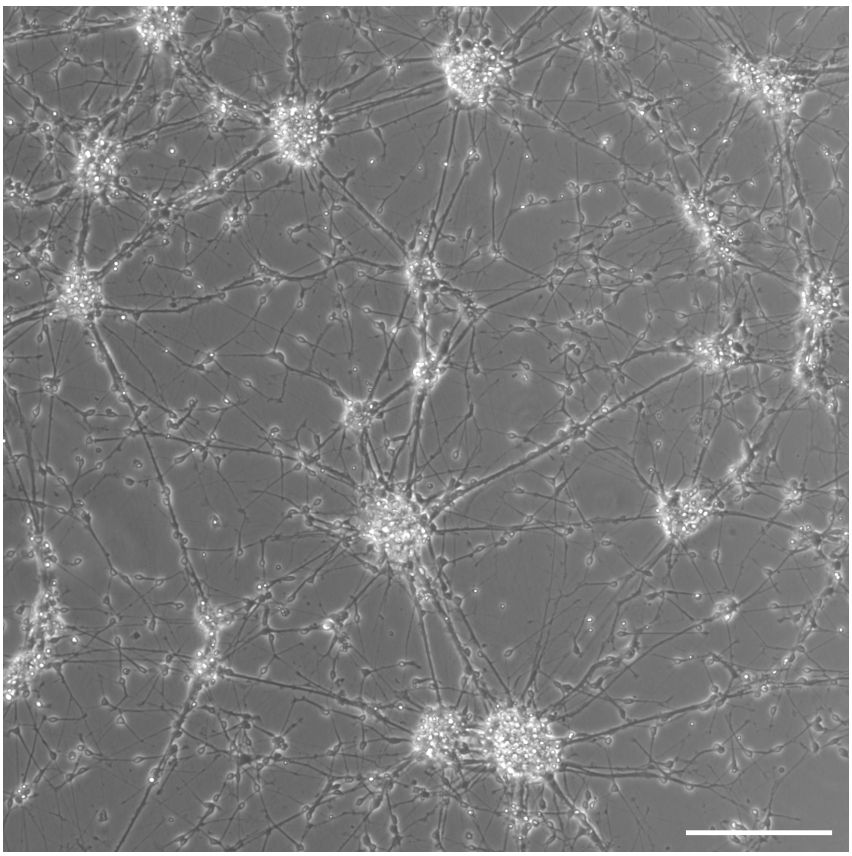

Figure 2.5: Ctrl1 neurons day 74 after induction.
Scale bar: 200µm

2.1.4 Differentiation of Glial Progenitor Cells into Mature Astrocytes

Following the differentiation of NSCs from chapter 2.1.2, cells from plate 2,3,4,5 and 6 were all differentiated towards astrocytes based on a protocol developed by Perriot et al [14] with some modifications. The cells were cultured in GEM for 19 days, and manually picked according to A.9, to then be plated in astrocyte induction medium (AIM), in which the cells were cultured for 14 days. Medium changes for both GEM and AIM were done every second day and/or the day after passage. Dispase passages according to protocol A.7 were performed during GEM culturing and accutase passages, according to protocol A.8, during AIM culturing. The passages were done when cells were 80% - 100% confluent (every 2-4 day) and plated on laminin 2020. Cells from the Ctrl1 cell line, with and without vitamin A, were frozen at day 19 in differentiation according to protocol A.4, to be thawed and used to differentiate towards astrocyte again, as a second experiment.

Following culturing in AIM the cells were cultured in astrocyte maturation media (AMM), containing ciliary neurotrophic factor (CNTF), for 4 weeks, starting at day 47 after start of induction. Medium changes for the first 18 days were done every second day and/or the day after passages. Cells were passaged with accutase according to protocol A.8, and plated to a cell density of 40000cells/cm^2 only when the cells were 90% - 100% confluent. Since at this point it is important not to passage cells too often, the cells can be allowed to overgrow to some extent. However if non astrocyte growth occurs a passage can be done at lower confluency to "eliminate" these other cells. After 18 days of AMM culturing medium changes were done only twice a week, and celldensity after accutase passages were kept at 20000 cells/cm^2. Worth noting is that plate coating for the 4 weeks of AMM culturing were laminin 2020 according to A.5.2 for the first 9 days, then the rest of the culturing coating were switched to matrigel according to protocol A.5.1. After end of the maturation period of 4 weeks, on day 75 the 004iC2 -V.A astrocytes were co cultured with the Ctrl1 +V.A neurons or prepared for characterization. The rest of the 004iC2 -V.A astrocytes were cultured in astrocyte medium (AM) for 35, until day 110. An overview of pictures taken during astrocyte differentiation of the 004iC2 cell line can be seen in figure 2.6. The results from the astrocyte differentiation and characterization are presented in chapter 3.

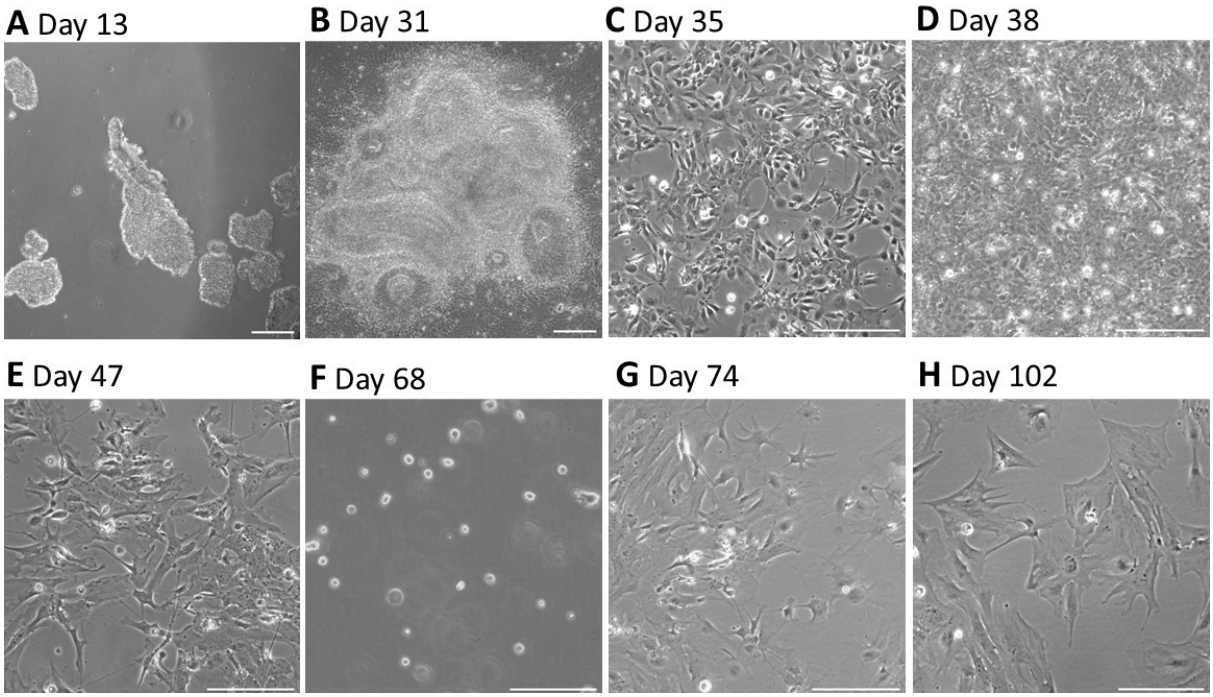

Figure 2.6: Images from astrocyte differentiation of the 004iC2 -V.A cells
(**A**) Day 13, first dispase passage in GEM (**B**) Day 31, day after manual picking of rosettes in GEM(**C**) Day 35, day after second accutase pasaage in AIM(**D**) Day 38, 100% confluency(**E**) Day 47, end of AIM, start of AMM (**F**) Day 68, right after an accutase passage in AMM (**G**) Day 74, day before co-culturing with Ctrl1 neurons (**H**) Day 102, mature astrocytes
Scale bar: 200µm

2.2 Co-Cultures of Mature Neurons and Astrocytes

Co-cultures of Ctrl1 +V.A neurons differentiated for 73 days and 004iC2 -V.A astrocytes differentiated for 75 days, were done by passaging the mature astrocytes with accutase according to protocol A.8. The astrocytes were then seeded on the neuron 24-well plates with a cell density of 25,000 cells/cm^2. 10 wells per plate, with an even combination of neurons only and neurons + astrocytes containing two different medias, NMM or Brainphys media (BPM), were achieved. An overview of the experimental setup with respective plate and amount of wells for each media and test condition can be seen in table 2.2 below.

Table 2.2: Experimental setup of co-cultures with neurons and astrocytes and neuron mono-cultures.

Plate Number	Cell Lines	Cell types	Media	Total Wells
1	Ctrl1 +Vit. A & 004iC2 -Vit. A	Neurons + Astrocytes	NMM	5
1	Ctrl1 +Vit. A & 004iC2 -Vit. A	Neurons + Astrocytes	BPM	5
2	Ctrl1 +Vit. A	Neurons	NMM	5
2	Ctrl1 +Vit. A	Neurons	BPM	5

NMM and BPM were changed every second day, where all NMM was replaced every time, but only of BPM was changed at a time. This is due to BPM producing active neurons which produce valuable proteins, which is to some extent still in media if only half is changed. The wells containing neurons were continuously spiked with 10µl/ml laminin 521 of total media volume every tenth day.

The 24-well plates with neurons and astrocytes were cultured for 8 days before media, RNA and protein were collected according to protocol A.11.1, In brief, 500 μl media from 5 wells, RNA from two wells, and protein from three wells per condition was collected.

2.3 Western Blot

Western blot is an important cell and molecular analytical technique used for identifying and separating certain proteins and peptides in cell mixtures. Western blot can be separated into three main events. Where first the proteins will be separated by size through gel electrophoresis, to then be transferred and immobilized on solid support usually a nitrocellulose or polyvinylidene difluoride membrane, before lastly being visualized by letting a primary and secondary antibody bind to it [29, 30].

Astrocytes (A) differentiated for 75 days were seeded in 24-wells and cultured in AM, NMM and BPM for 8 days after which protein samples were collected from the wells according to protocol A.11.3. Protein samples were also collected from the seeded co-cultures (N + A) of astrocytes (day 75) and neurons (day 73) along with the neuron (day 73) control plates (N) after 8 days of culturing in NMM and BPM. The protein samples were lysated in RIPA buffer according to A.12 and protein were measured using PIERCE BCA protein assay according to protocol A.13. The concentrations are presented in table B.1 in appendix B, where two replicates per samples can be noted.

Western blot were then performed on the two highest protein concentrations of the three samples (from table B.1) according to protocol A.14. In brief, RIPA buffer were added to the specified volume sample presented in table 2.3 to a total volume 18µl, in order to get 1.5µg of sample weight. 6µl load buffer and 1.25µl DTT were also added to the samples before being loaded on the gel, total volume 25µl. SeeBlue^{TM} Plus2 Pre-stained Protein Standard were added for reference. Worth noting here is that sample 7 and 19 theoretically need a volume greater than 18µl to achieve 1.5µg protein weight. This is not possible so the volume added was capped at 18µl, which lead to lower concentrations.

Table 2.3: Concentration and volumes of samples and reagents for western blotting.

Sample	Plate & Well	Mean Conc. (µg/ml)	Weight Protein in 18µl (µg)	Volume sample for 1.5µg (µl)
1	N+A NMM P1	165.46	2.97828	9.1
3	N+A NMM P3	197.108	3.531186	7.6
4	N+A BPM P1	208.742	3.757356	7.2
6	N+A BPM P3	97.51	1.75518	15.4
7	N BPM P1	69.586	1.252548	21.6 (18)
8	N BPM P2	89.366	1.608588	16.8
10	N NMM P1	113.8	2.0484	13.2
11	N NMM P2	91.228	1.642104	16.4
14	A AM P2	261.566	4.708188	5.7
15	A AM P3	186.636	3.359448	8.0
16	A NMM P1	194.547	3.501846	7.7
18	A NMM P3	343.477	6.182586	4.4
19	A BPM P1	81.221	1.461978	18.5 (18)
20	A BPM P2	113.334	2.040012	13.2

The gels were ran at 200V, starting at 120V until samples entered the gels. The Gels were then blotted onto 0.2 uM nitrocellulose membranes using trans blot turbo (Biorad) and stained with primary and secondary antibodies, which can be seen in table 2.4 along with band sizes and dilution. For information about supplier and identifier, see A.1.

Table 2.4: Primary and secondary antibodies, along with respective size and concentration, used for western blotting.

	Name	Band (kDA)	Dilution	Source
Primary Antibody				
	PSD95(rb)	~ 85	1:1000	Abcam[31]
	SNAP25(rb)	~ 25	1:20000	Sigma Aldrich[32]
	GAPDH	~ 36	1:20000	Novus Biologicals[33]
	EAAT1(rb)	~ 60	1:1000	Abcam[34]
Secondary Antibody				
	Anti-rabbit, HRP-linked		1:8000	Cell Signaling Technology[35]
	Anti-mouse, HRP-linked		1:8000	Cell Signaling Technology[36]

The membranes were then developed in a Chemidoc instrument (Biorad). After pictures were taken the membranes were stripped and re-stained with GAPDH, according to protocol A.14.3, used for reference to normalize data. Densiometry measurements of the optical density per band were then performed in ImageJ and normalized against the GAPDH optical density. The results from the Western blots are presented in section 3.2.3 for astrocytes and 3.3.2 for co-cultures.

2.4 Immunocytochemistry

Immunocytochemistry or (ICC) is a method applied to cells in order to visualize and detect proteins and antigens with the use of antibodies. A fluorescent or colored signal is created when the antibodies are directly or indirectly linked to a reporter protein. ICC usually follows

four steps. Seeding on cells on glass slides or bottom plates followed by half an hour to 24 hours incubation times. Then, the cells are immunostained, before the two last steps which includes antigene visualization using a confocal microscopy and image analysis [37].

ICC was executed according to protocol A.10. In brief, astrocytes in different stages of their differentiation, were seeded on matrigel coated ibidi 8-slides at 25,000 cells/cm^2 (note: lamin2020 instead of matrigel for day 49 astrocytes and 10,000 cells/cm^2 for 109 day astrocytes) and cultured for 1-3 days in AMM or AM before being fixed and stained. For the Co-cultures, astrocytes differentiated for 75 days were seeded on the mature neurons differentiated for 73 days, in ibidi-8-wells at 25,000 cells/cm^2 and cultured in BPM or NMM for 8 days before being fixed and stained. All neurons used in co-cultures were seeded on ibidi-8-slides at day 32 at 50,000 cells/cm^2, note, some wells were not seeded with astrocytes for comparison and controls.

Pictures were taken of all stained ibidi-8-slides with a Nikon Eclipse Ti confocal microscopy and then analyzed in ImageJ. In brief, astrocyte pictures were visually analyzed and for the co-culture pictures, cell nuclei were counted, antigen intensity were measured, neurite length and area were measured. A threshold set using mean error per picture were used when counting cells to better find all nuclei, a fixed threshold of 1300 to 65535 for the intensity measurments and a manual threshold for the neurite length in order to get a good representation of stainings. Note, one replicate experiment were done with 5 pictures taken at random places per immunostained slide. Macros used for the analysis of the co-cultures in ImageJ can be seen in appendix C.
An overview of the general experimental setup for ICC can be seen in table 2.5 were the cell lines along with the days of differentiation after start of induction with NIM and how long culturing in respective media before fixation occurred.

Table 2.5: Experimental ICC setup for astrocytes (A), co-cultures of neurons and astrocytes (N + A) with the specified cell- lines and types, days differentiated when fixed and how many days from seedout in ibidi-8-slides with specified media prior to fixation.

Cell line	**Cell type**	**Days Differentiated When fixed**	**Days Cultured (Days) (Media)**	**Primary Antibodies**
Ctrl1	Astrocyte	47	1 Day in AMM	EAAT1, GFAP, Pax6, Nestin, Tuj1
004iC2	Astrocyte	49	1 Day in AMM	EAAT1, GFAP, Pax6, Nestin, Tuj1
004iC2	Astrocyte	59	3 Days in AMM	EAAT1, GFAP, Pax6, Nestin, S100, Tuj1
Ctrl1 & 004iC2 (Co-Cultures)	Neuron & Astrocyte	79 & 81	8 Days in NMM or BPM	S100, Tau
004iC2	Astrocyte	77	2 Days in AM	EAAT1, GFAP, Pax6, Nestin, S100, Tuj1
004iC2	Astrocyte	109	1 Day in AM	EAAT1, GFAP, Pax6, Nestin, S100, Tuj1

Table 2.6 shows which primary and secondary antibodies that were used in ICC, what species each antibody targets and dilution used. In parenthesis the host species is specified, m is mouse, rb rabbit and ch chicken. For information about supplier and identifier, see A.1. Four neuronal markers and three astrocyte markers are used, marked N for neuronal and A for astrocyte. The neuronal markers are β-III tubulin (tuj1), nestin, pax6 and tau. Tuj1 and tau is localized in the cytoskeleton [38, 39], nestin in intermediate filaments [40] and pax6 in the nucleus [41]. The astrocyte markers are EAAT1, GFAP and S100. EAAT1 is localized in the cellular membrane [34], GFAP in the cytoplasm [42] and S100 in cytoplasm and nucleus [43].

Table 2.6: Primary and secondary antibodies, type of marker, concentration source.

	Name	Neuron(N)/Astrocyte(A) Marker	Dilution
Primary Antibody			
	β-III tubulin(Tuj1)(m)	N	1:1000
	EAAT1(rb)	A	1:250
	GFAP(ch)	A	1:1000
	Nestin(m)	N	1:50
	Pax6(rb)	N	1:500
	S100(rb)	A	1:400
	Tau(ch)	N	1:2000
Secondary Antibody			
	goat anti-rabbit Alexa488		1:400
	goat anti-mouse Alexa647		1:400
	goat anti-chicken Alexa647		1:400

2.5 Calcium Imaging

Calcium imaging is a method that uses labeled calcium indicators, which are molecules that upon binding to calcium ions exhibit an increase in fluorescence [44]. Release of intracellular calcium ions can be induced by extracellular factors such as glutamate and ATP. This has been shown to be true for astrocytes too [45], which is advantageous since one of many roles for astrocytes is homeostasis of calcium ions [46].

Calcium imaging were performed, on 004iC2 -V.A astrocytes differentiated for 87 and 109 days, according to A.15. In brief, astrocytes differentiated for 83 days were seeded on glass at 25,000 cells/cm^2 and cultured in AM for 4 days, whereas astrocytes differentiated for 108 days were seeded out in ibidi-dishes at 9,000 cells/cm^2 and cultured in AM for 1 day. Cells were washed with HBSS and incubated with 2 µM Fluo-4 for 15 min. Cells were then washed and imaged live, where 3 µM ATP were added after 30s, using an Axiovision Observer.Z1 inverted microscope taking pictures every 683ms. Images were then analyzed in ImageJ using a "time series analyser" plugin. Images and data are presented in section 3.6.

3
Results

In this chapter, results from the differentiation and characterization of astrocytes are presented. First with an overview of the differentiation process in section 3.1. Followed by results from the characterization, functional analysis and culturing in different neuronal media in section 3.2. To lastly end with the results from immunocytochemistry and synaptic expression analysis of the co-cultures of neurons and astrocytes, in section 3.3.

3.1 Astrocyte Differentiation

In this section, results from the astrocyte differentiation experiments of the two cell lines are presented. Both in table 3.1, where an overview of the outcome of culturing can be seen and further down images from cell culturing in the form of phase contrast microscopy.

Table 3.1: Outcome for astrocyte differentiation experimental results for Ctrl1 and 004iC2 cell lines, were Day are representing days after start of induction (with NIM). Dispase (D), accutase (A) and manually picking (MP) are presented as written. The day on which experiments were deemed successful or unsuccessful/discarded are noted with GOOD or BAD, respectively.

Cell line (+/-) Vit. A	**Start NIM**	**End NIM Start GEM**	**Passages During GEM**	**End GEM Start AIM**	**End AIM Start AMM**	**End AMM Start AM**
Ctrl1 +Vit. A	Day 0	Day 8	D D A A	Day 19	Day 33	Day 57, BAD
Ctrl1 -Vit. A (1)	Day 0	Day 8	D D A A	Day 19	Day 22, BAD	———
Ctrl1 -Vit. A (2)	Day 0	Day 8	D A A A	Day 19	Day 33	Day 57, BAD
Ctrl1 +Vit. A thawed	Day 0	Day 8	D D A A	Day 20	Day 34	Day 71, BAD
Ctrl1 -Vit. A thawed	Day 0	Day 8	D A A A	Day 20	Day 34	Day 47, BAD
004iC2 +Vit. A	Day 0	Day 10	D D D MP A	Day 27	Day 41	Day 59, BAD
004iC2 -Vit. A	Day 0	Day 13	D D D MP A	Day 32	Day 47	Day 75, GOOD

Table 3.1 shows results from the astrocyte differentiation experiments. In general, what manly differs is the days in which the cells were cultured in NIM and GEM, and how many dispase passages compared with accutase passages were done during GEM culturing. Both 004iC2 cell lines were manually picked before plated in AIM, whilst the other cells lines were passaged normally with accutase. Here, only the 004iC2 -Vit. A treated cell differentiated successfully towards astrocytes, the rest of the repeats differentiated towards neurons, or got contaminated with other cells. Images of the around the days before discarding can be seen in figure B.1 in appendix B. Ctrl1 +/- thawed were cells from the same Ctrl1 +/- Vit. A cell lines, which were frozen on day 19 after induction. These cells were taken up and plated on matrigel for the rest of the differentiation, and differentiated towards neurons in the end which lead to discarding of the cells.

3.2 Characterization of Astrocytes

In this section. Results from characterization of astrocytes are presented, in the form of images from ICC, phase contrast microscopy (PCM) and Western blot. The ICC images are taken

from Ctrl1 and 004iC2 cell line differentiated +Vit. A 49 days after induction and 004iC2 cell line differentiated -Vit. A, 59, 77 and 109 days after induction. EAAT1 and GFAP poisitve astrocytes are estimated at day 77 and 109 after induction. The Western blot is of the 004iC2 cell line 83 days into differentiation without vitamin A, of which the last 8 days the wells had different media, AM, NMM and BPM to see if it were possible to grow astrocytes in cell media which, normally are used for neurons, and are presented as Western blot images and data from ImageJ analysis. Lastly, results of functional analysis from live cell calcium imaging is presented.

3.2.1 Immunocytochemistry Staining During Differentiation

In the following section the results from PCM images taken during culturing of astrocyte differentiation will be presented. Followed by immunocytochemistry results, including images from confocal microscopy, estimation of EAAT1 and GFAP positive cells.
Cells differentiated with vitamin A during induction with NIM, these being 004iC2 +Vit. A and Ctrl1 +Vit. A, started to look like they were differentiating towards neurons instead of astrocytes a seen in figure 3.1.

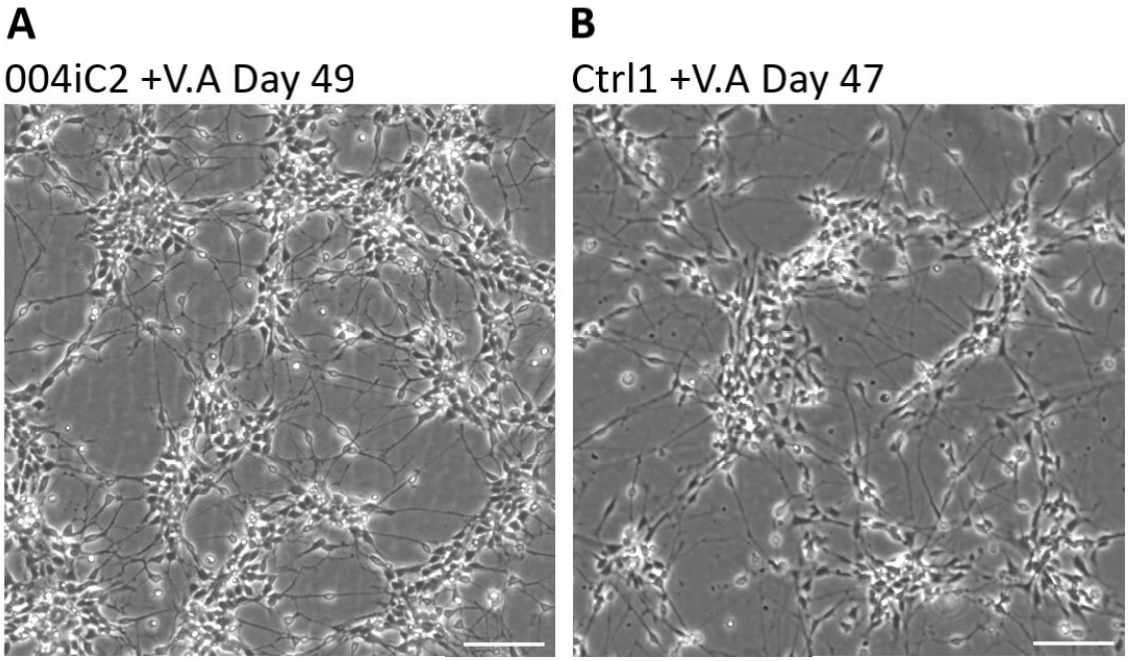

Figure 3.1: **A** 004iC2 and **B** Ctrl1 cell lines, +Vit. A, fixed 49 and 47 days after induction, respectively.
Scale bar: 100µm

In figure 3.1 it is quite clear that the cells are neuronal like, with prominent neurites between cell bodies. An ICC staining as seen in figure 3.2, was therefore conducted on Ctrl1 and 004iC2 cell lines differentiated with vitamin A fixated 47 and 49 days after induction, with the primary antibodies EAAT1, GFAP, Pax6 and nestin, the first two are astrocyte markers and the two latter are neuronal markers.

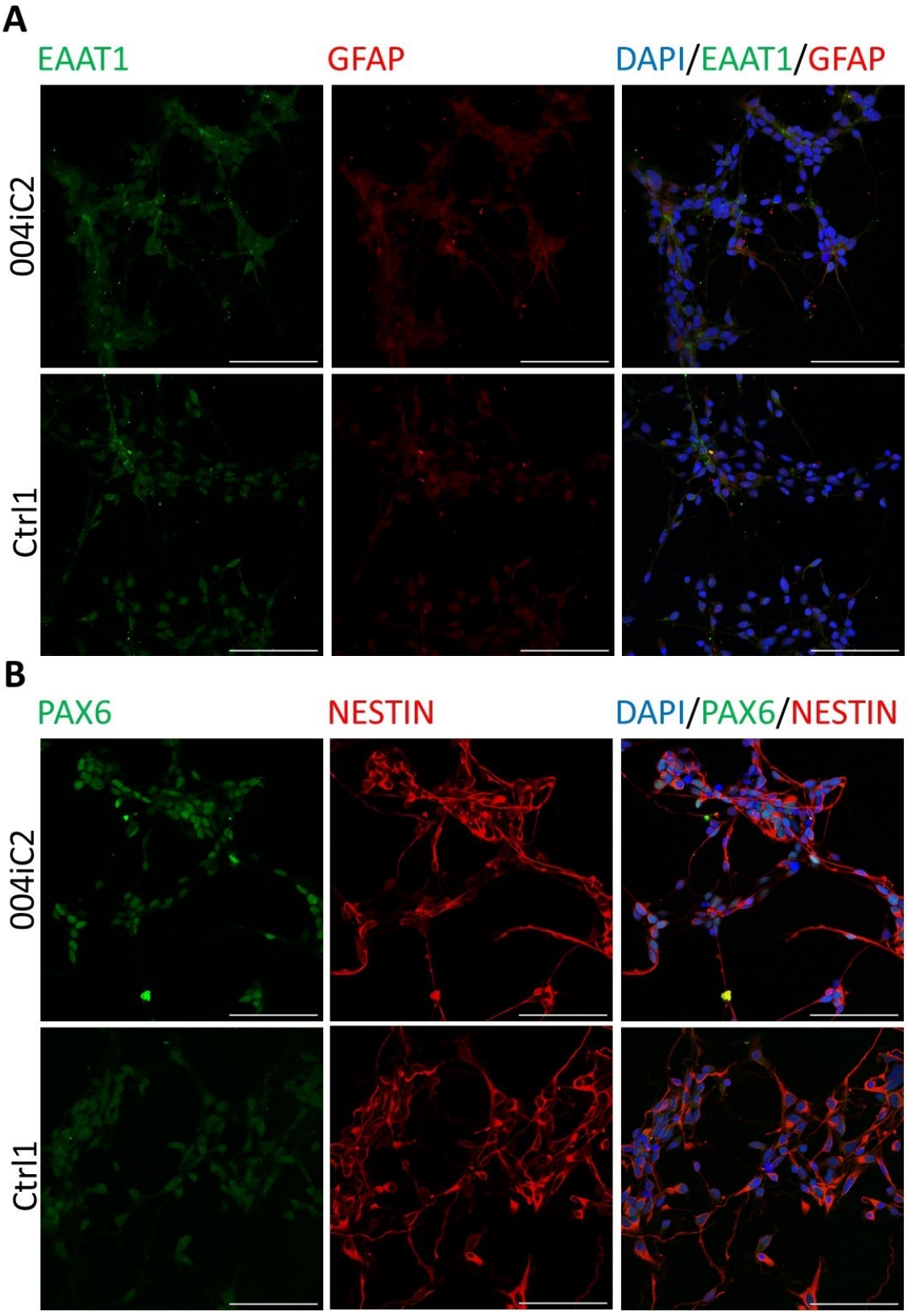

Figure 3.2: 004iC2 and Ctrl1 cell lines, +Vit. A, fixed 49 and 47 days after induction, respectively. Both cells are stained with nuclei marker DAPI (blue) **A** astrocyte markers, EAAT1 (green), GFAP (red) and **B** neuroprogenitor markers PAX6 (green), nestin (red).
Scale bar: 100µm

Figure 3.2 shows the ICC from both 004iC2 and Ctrl1 cell lines differentiated with vitamin A and fixed 49 and 47 days after induction, respectively. Both cell lines were stained as can be seen in figure 3.2. This confirmed that the cell lines indeed differentiated towards neurons, since

neuroprogenitor markers were prominent. The absence of astrocyte markers and presence of neuroprogentitor markers led to the decision to eventually discard these cells (Ctrl1 and 004iC2 with vitamin A) around day 57 and day 59 as seen in figure B.1 in appendix B, and continue the differentiation with 004iC2 without vitamin A. (Note: Ctrl1 - Vit. A were cultured for 10 more days, before also being discarded due to similar neuron proliferation instead of astrocytes, as the two -Vit. A variants. This can also be seen in figure B.1 in appendix B.)
Cells from the 004iC2 cell line differentiated without vitamin A, looked as they differentiated towards astrocytes through PCM investigation as seen in figure 3.3.

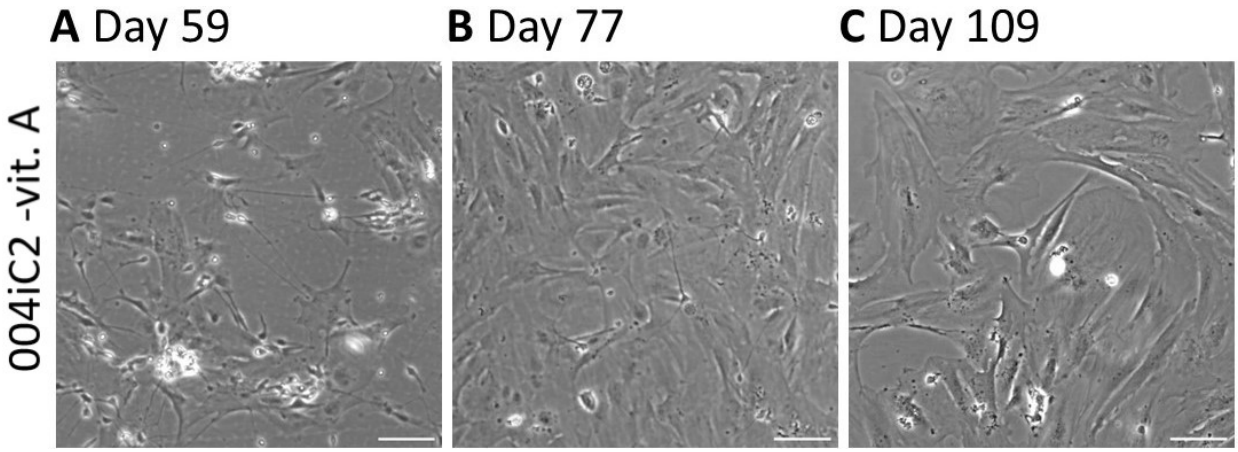

Figure 3.3: 004iC2 cell line, -Vit. A, fixed at **A** 59, **B** 77 and **C** 109 days after induction
Scale bar: 100µm

The 004iC2 cells without vitamin A in figure 3.3 had very different morphology compared to the cells with vitamin A. ICC was therefore done, on the same time points as the PCM images in figure 3.3, during the differentiation. Figure 3.4 shows the ICC staining of the 004iC2 cell line differentiated towards astrocytes without vitamin A on said time points, stained with the astrocyte markers EAAT1, GFAP and S100 along with an estimation of the percentage EAAT1 and GFAP positve cells from day 77 and 109 in differentiation.

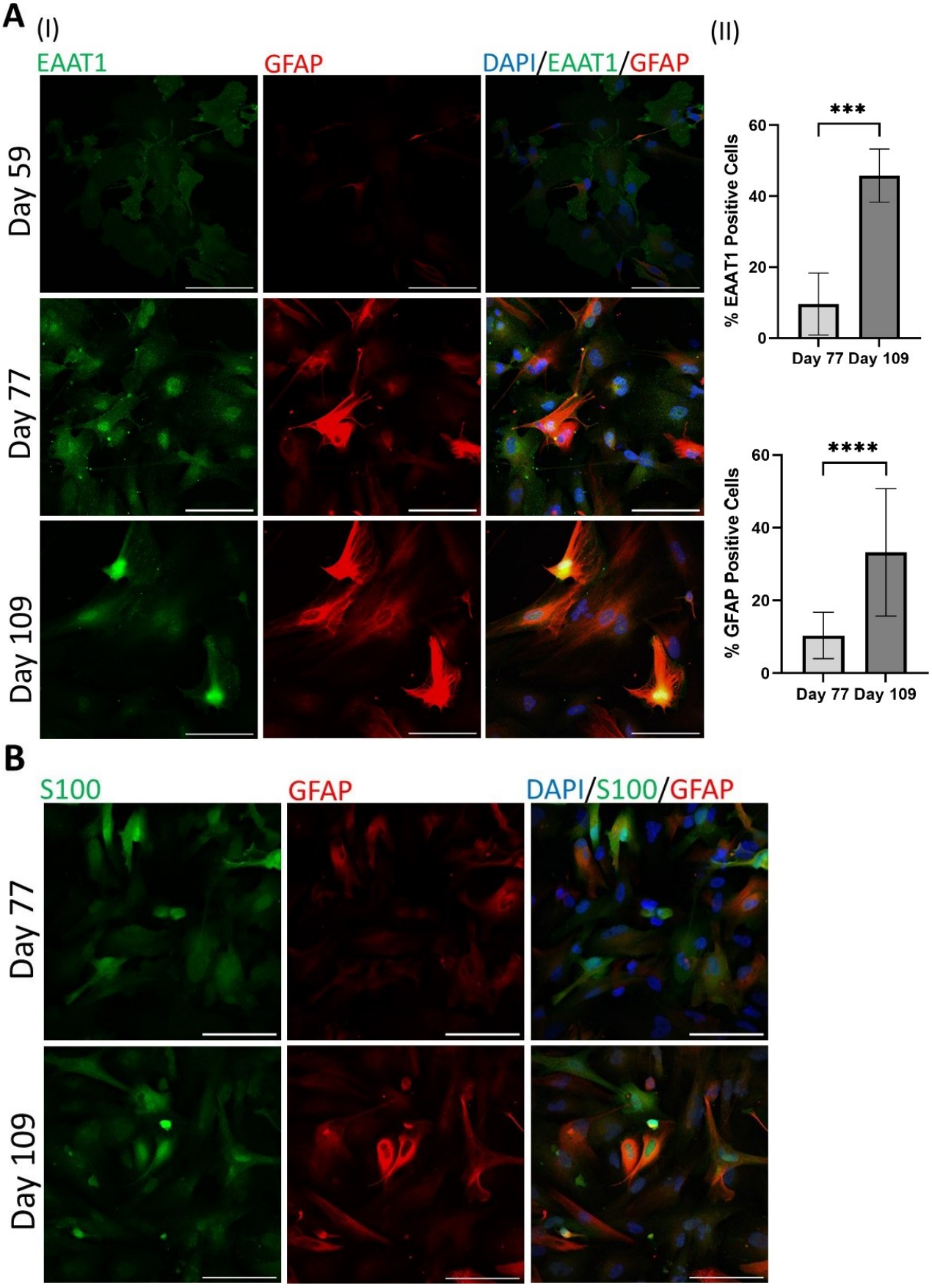

Figure 3.4: 004iC2 cells without vitamin A fixed at 59, 77 and 109 days after induction and stained **A**(I) with the astrocyte markers EAAT1 (green), GFAP (red) and DAPI (blue) for nuclei staining, and in **B** astrocyte markers S100 (green), GFAP (red) and DAPI (blue) were used.
Scale bars: 100µm

(Figure text continued from previous page) **A**(II) shows graphs with and estimation of the percentage EAAT1 and GFAP positive cells on day 77 and 109. Bars represent mean +/- S.D, from 15 and 30 images from three experiments for EAAT1 and GFAP, respectively, for day 77. (Note, day 109 had only one experiment done, with 5 and 10 images, respectively.) All images were analyzed by two persons, independently. Mean values were analyzed with Mann-Whitney test ***p≤ .001 and ****p ≤ .0001.

In figure 3.4 **A**(I), a gradual increase of all markers can be noted the further in the differentiation the cells were. The estimation of the percentage EAAT1 and GFAP positive cells from day 77 and 109 in figure 3.4 **A**(II)showed an significant increase of both markers on day 109 compared to day 77, with an increase of roughly 30 and 20 percentage points, respectively. Figure 3.5 also shows the 004iC2 cell line differentiated without vitamin A at 59, 77 and 109 days after induction, stained with the neuron markers pax6, nestin and tuj1, and the astrocyte marker S100.

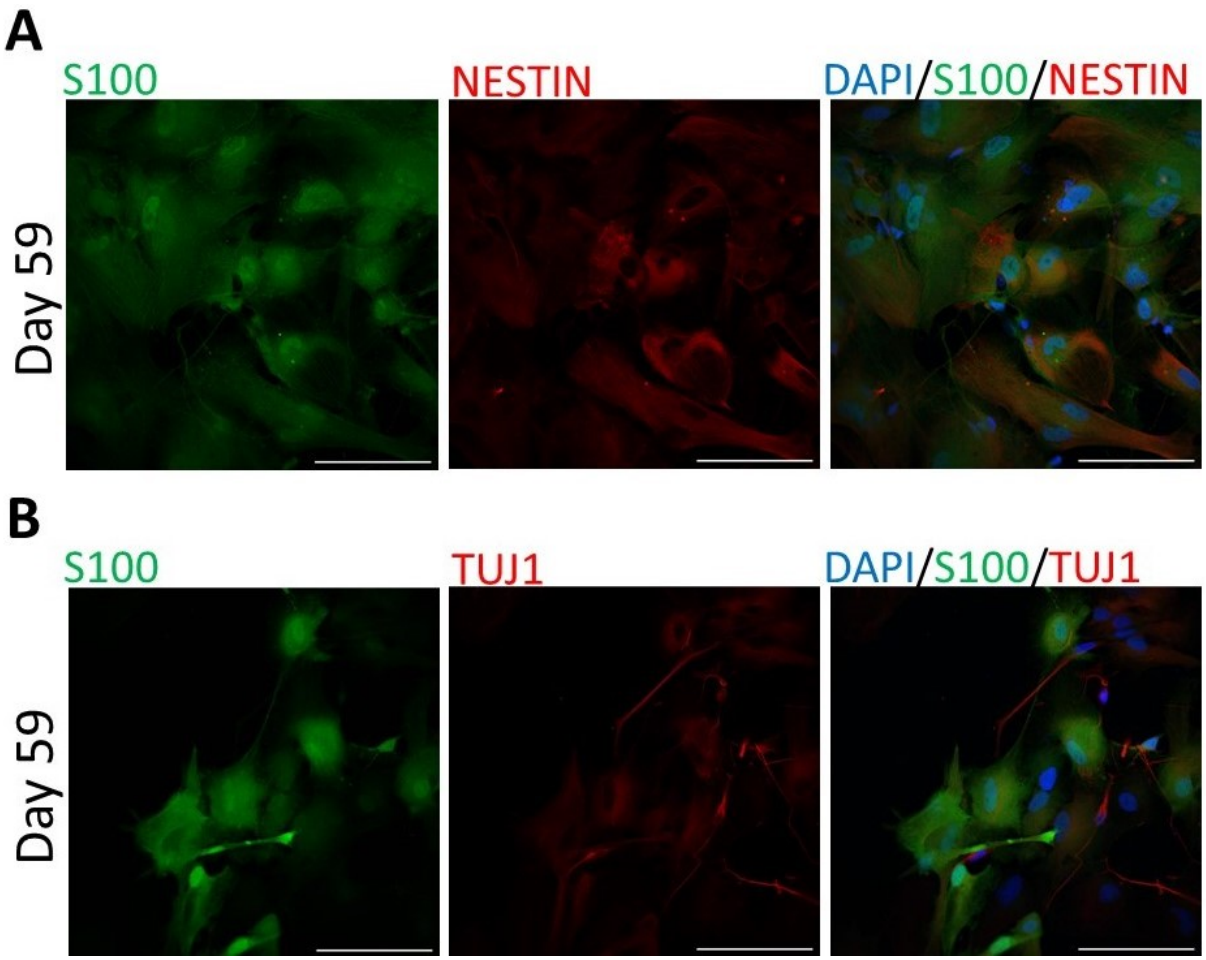

Figure 3.5

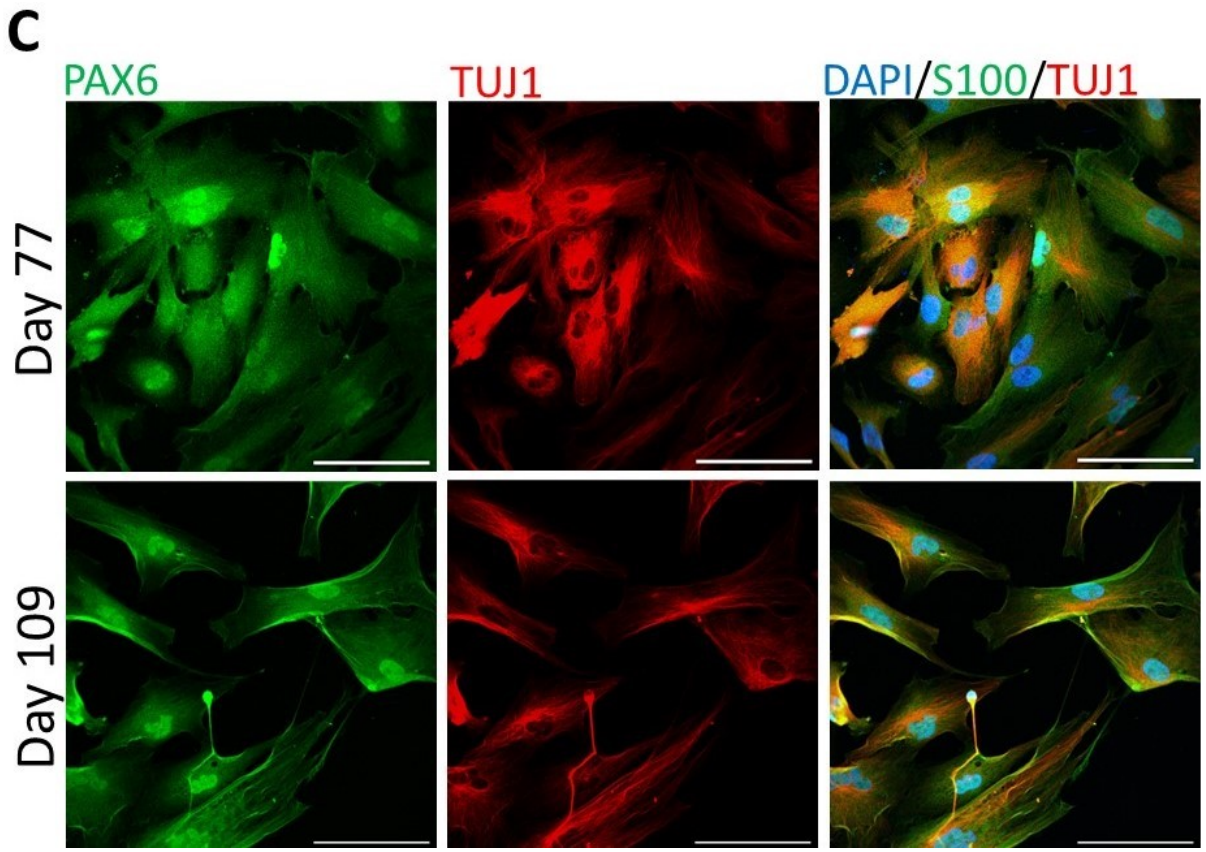

Figure 3.5: 004iC2 cell line differentiated without vitamin A fixed at 59, 77 and 109 days after induction and stained with the neuron markers Pax6 (green), nestin (red) and tuj1 (red), the astrocyte marker S100 (green) and DAPI (blue) as nuclei staining. **A** and **B** shows 004iC2 at day 59 after induction stained with S100, Nestin, Tuj1. **C** shows 004iC2 at day 59 after induction stained with Pax6, Tuj1 and Dapi.
Scale bar: 100µm

It can be noted in figure 3.5 that neuron markers are present. However, Pax6 and nestin look differently compared to staining of 004iC2 and Ctrl1 +Vit. A on day 49 as seen in figure 3.2 **B**, where Pax6 was concentrated to the nuclei and nestin showed clear neurites. However at both day 77 and 109 pax6 and nestin more smeared, and pax6 is also located to the cell soma.

3.2.2 Functional Analysis

Here follows the results from the functional analysis, in the form of live cell calcium imaging, conducted on the astrocytes, through investigation if cells react to ATP stimulation by getting a increased flow of calcium. This is done since astrocytes are involved in calcium homeostasis. Results are in the form of images and data from the live imaging. Images were analyzed in ImageJ by making an region of interest (ROI) in every cell body in the images and using a "time series analyser" plugin where the average intensity of fluorescence after addition of ATP were measured at every time point. Cells with a clear increase followed by a decrease of fluorescence, after ATP addition, were counted as a cell that reacted to ATP stimulation by releasing a calcium ion wave. In figure 3.6 images taken during the calcium imaging with the corresponding data can be seen, aswell as an example graph of a valid calcium wave. Worth noting is that astrocytes for day 87 only has one replicate while astrocytes for day 109 has 4 replicates.

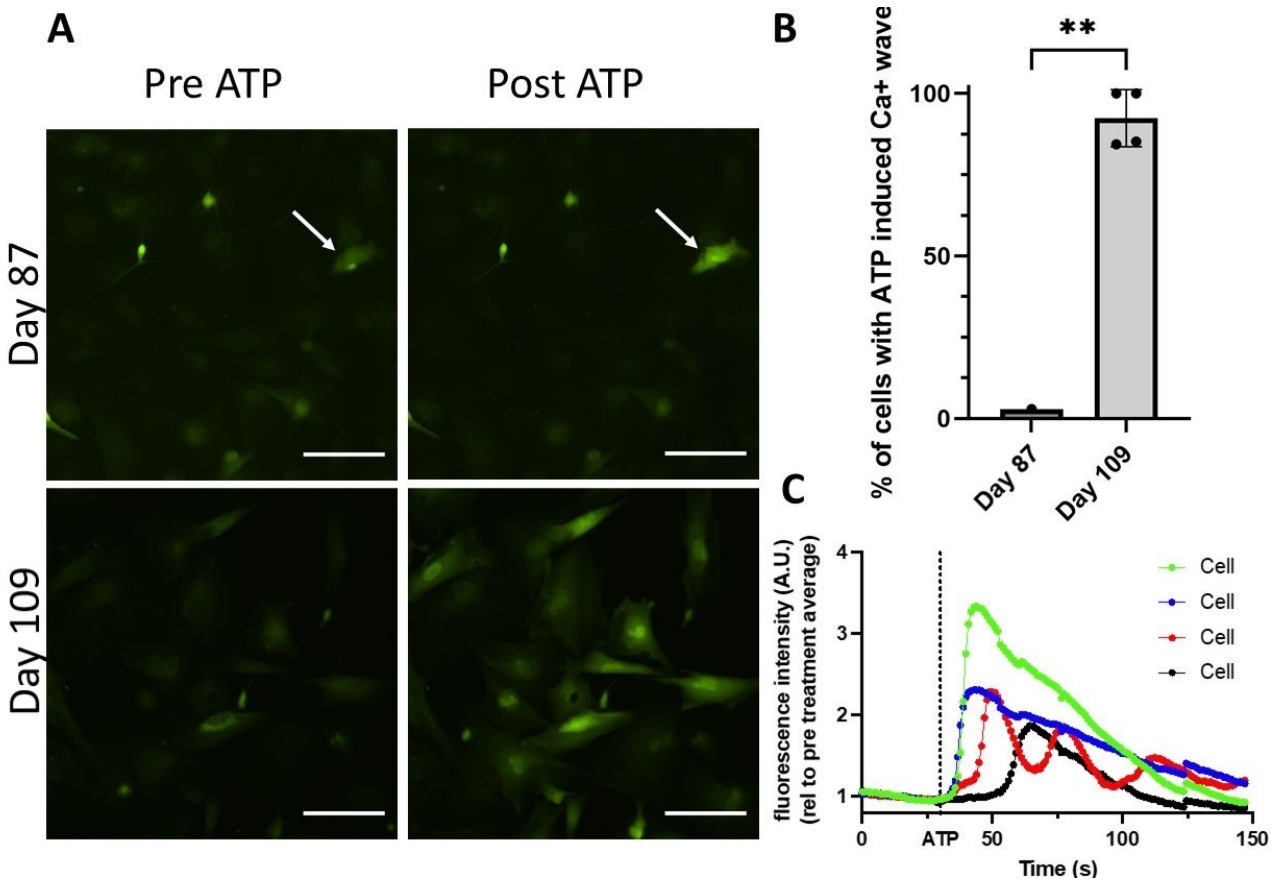

Figure 3.6: **A** astrocytes differentiated for 87 and 109 days pre and post ATP stimulation. Scale bar: 50µm **B** percentage of cells that responded with ATP induced Ca+ wave (top). Bars represent mean with +/- S.D for 4 replicates of one experiment for day 109. (one replicate for day 87). Mean values were analyzed with an Unpaired t-TEST $**p \leq .001$ **C** example traces of valid calcium wave with fluo-4 fluorescence intensity over time.

Figure 3.6 **A** shows one cell on day 87 after induction with NIM lighting up with fluo-4 fluorescence from ATP stimulation, compared with multiple cells at day 109. There is a significant difference of the percentage of cells with ATP induced calcium waves for day 87 and 109, with 3% and 90%, respectively,. As seen in figure 3.6 **B** and could suggest that the cells are functional astrocytes.

3.2.3 Culturing in Different Media

In this section, results from the Western blots done on the protein collected from the 004iC2 -Vit. A astrocytes in different media, are presented. One replicate experiment were performed. Results are in the form as PCM images and Western blot images and data. All membranes were developed in Chemidoc and are presented in figure 3.7, along with the PCM images from the day protein was collected.

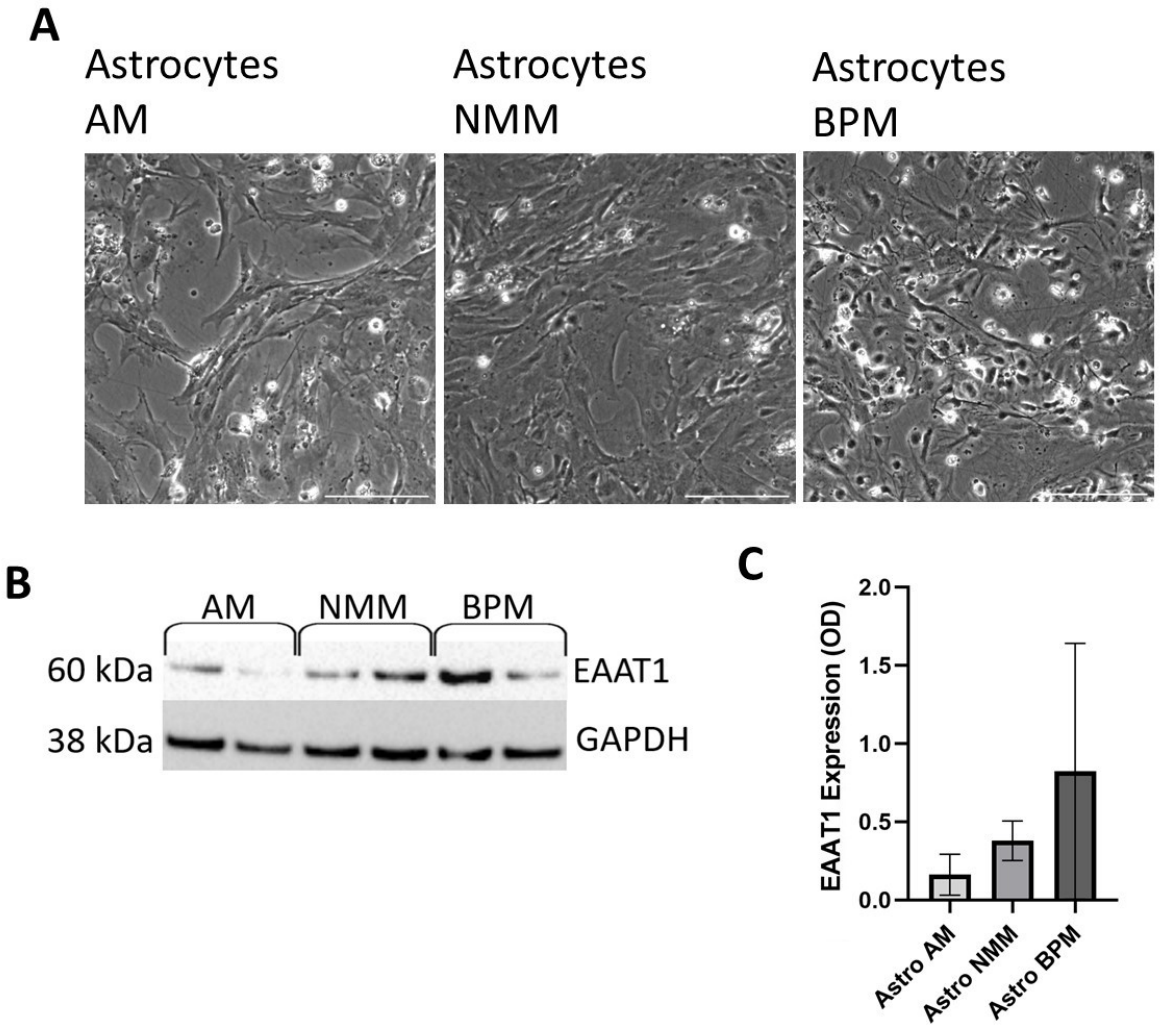

Figure 3.7: PCM images from the day protein was collected, membranes from one experiment along with densiometry data from ImageJ, for astrocytes differentiated for 83 days in AM, NMM and BPM. **A** PCM images of astrocytes in AM, NMM and BPM. Scale bar: 200µm **B** Membranes stained with EAAT1, and with GAPDH used for reference, which shows at around 60kDa and 38kDa, respectively. **C** optical density for EAAT1, normalized against GAPDH, were bars represent mean +/- S.D from 2 replicates of one biological repeat.

From the PCM images of astrocytes differentiated in different media in figure 3.7**A**, it can be seen that the astrocytes cultured in BPM has changed morphology compared to figure 3.3**B**(day 77), while astrocytes cultured in AM and NMM have not changed morphology. For the membrane stained with EAAT1 in figure 3.7**B**, it can be noted that astrocytes cultured in NMM and BPM display higher amounts of EAAT1. This is further showed in figure 3.7**C**, were densiometry measurements were performed in ImageJ. Here the optical density of EAAT1 for astroytes cultured in BPM has higher mean values of around 0.82 followed by astrocytes in NMM at 0.38 and lastly astrocytes in AM at 0.16. An increase of 412% and 138% for BPM and NMM, respectively compared to AM.

3.3 Co-Cultures of Neurons and Astrocytes

In this section results from characterization of co-cultures are presented with images from ICC, PCM and Western blot. ICC images for co-cultures are Ctrl1 neurons (79 days after induction)

and 004iC2 astrocytes (81 days after induction), of which the last 8 days the cells were in different media, NMM respectively BPM. Mono-cultures with neurons were also included for comparison. Intensity of astrocyte markers and neurite lengths were measured in ImageJ. Western blot were performed on the different cultures and different media, and optical density for SNAP25 and PSD95 were measured with ImageJ.

3.3.1 Immunocytochemistry Stainings of Co-Cultures

In this section, images from cell culturing of neuron mono-cultures and, neuron and astrocyte co-cultures, in the form of PCM images are presented in figure 3.8 **A**. Along with results from ICC staining of one replicate ibidi-8-slide with 5 images taken at different positions, in figure 3.8 **B**(I) and 3.8**C**(I) . Mean intensity and integrated density for S100 per cell aswell as total and per cell neurite lengths, as seen in in figure 3.8 **B**(II) and 3.8**C**(II), were analyzed in ImageJ for the stained ICC images. The mean intensity of S100 is calculated for the whole picture area, meanwhile the integrated density for S100 is calculated from a fixed threshold against the respective area of this threshold. Tau is analyzed from skeletonized versions of the staining and gives an approximation of the neurite lengths.

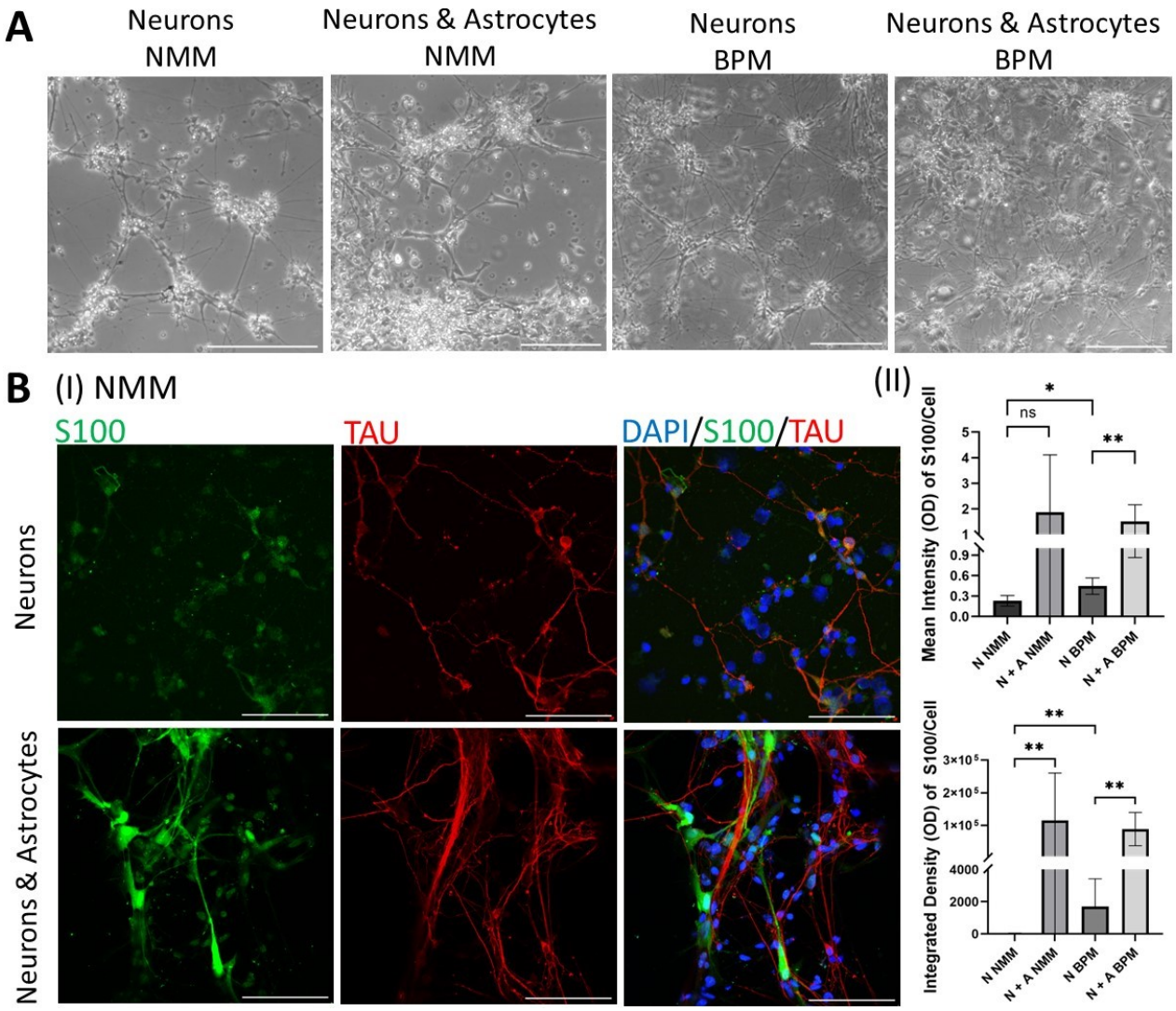

Figure 3.8

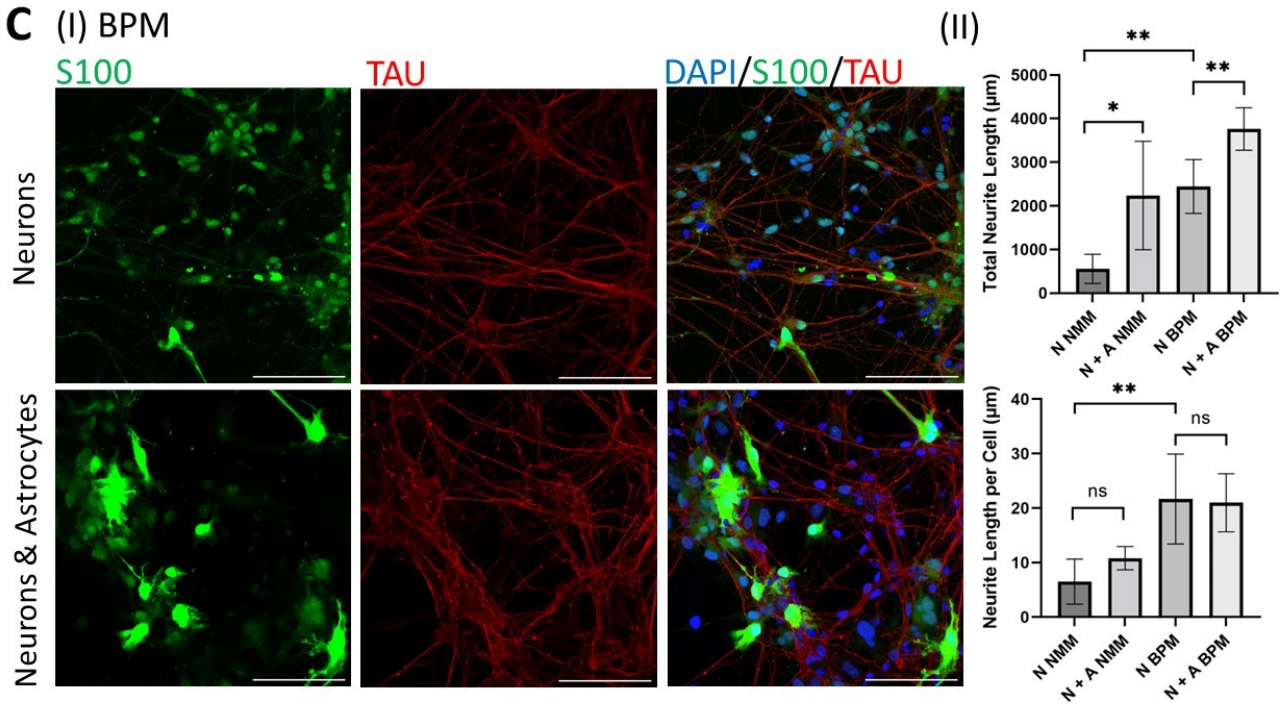

Figure 3.8: Ctrl1 Neurons differentiated for 79 days, alone and co-cultured with 004iC2 astrocyes differentiated for 81 days, in NMM and BPM for 8 days when fixed. Neuron mono-cultures (N) and neuron and astrocyte co-cultures(N + A), **A** with PCM images in NMM and BPM. Scale bars: 200µm **B**(I) and **C**(I) with ICC images, stained with DAPI (blue), S100 (green), Tau (red). Scale bars: 100µm. For **B**(I) NMM, and **C**(I) BPM. Neurons cultured alone and with astrocytes with **B**(II) mean intensity and integrated density of S100 per cell both in units of optical density (OD). Bars represent mean +/- S.D for 5 images from one experiment. Mean values were analyzed with Mann-Whitney test $*p \leq .05$ and $**p \leq .001$. **C**(II) total and per cell neurite lengths in units of µm, with logarithmic scale. Bars represent mean +/- S.D for 5 images from one experiment. Mean values analyzed with Mann-Whitney test $**p \leq .001$.
Scale bars: 100µm

Figure 3.8 **A** shows the PCM images for neurons, alone and co-cultured with astrocytes in NMM and BPM. From the images, the amount of live astrocyte cells and neurons, in co-cultures seem to be higher compared to neurons cultured without, for both NMM and BPM. Neurites for neurons and astrocyte co-cultures look more dense than neurons alone, in NMM. This might suggest that astrocytes help with neuron development and maturation. ICC images in figure 3.8 **B**(I) and figure 3.8 **C**(I) also suggest this. Figure 3.8 **A**(II) shows that co-cultures of neurons and astrocytes in both NMM and BPM had more astrocytes compared to neurons alone, as the mean intensity and integrated density of S100 was significantly higher in co cultures compared to neurons alone. Except for mean intensity of neurons and astrocytes in NMM compared with neurons, where no statistical significance was shown, due to high SD. With this in mind, the comparisons suggest that astrocytes survive being cultured together with neurons in NMM and BPM and does not die off. Furthermore the mean intensity and integrated density for S100 is barley higher in the co-cultures in NMM compared with BPM, with an increase of 30% and 23.5%, respectively, and contrary to neuronal controls where the opposite is shown. Here a significant increase of 200% and 7700% for mean intensity and integrated density of S100, respectively, can be seen. In figure 3.8 **C**(II) data from calculations in ImageJ on intensity for total and per cell skeletonized length for tau can be seen. This shows that the total neurite lengths are significantly higher for co-cultures of neurons and astrocytes in BPM compared with the neuronal control, for NMM the data follows suit, were a significant difference between test conditions is also shown. When, however, looking at the normalized data per cell its worth

noting that the difference for BPM is not as significant as it seemed in the previous graph. Here neurite lengths are around the same form PM, in favour of neurons alone. For NMM, the difference smaller but still in the favour of the co-cultures. For both graphs, however, a significant difference in total and per cell neurite length can be seen when comparing NMM and BPM for neurons alone. It is worth noting that the neurites per cell results does not differentiate between the cell count for neurons and astrocytes separately. With all data taken into account, a suggestion that astrocytes might help with neurite development in co-cultures with neurons, can be made.

3.3.2 Synaptic Protein Expression

In this section, results from the Western blots done on the protein collected from the co-cultures of neurons and astrocytes in different media, are presented. For comparison, were neurons cultured alone in the same media for the same amount of days. Two replicate experiments were performed. All membranes, were developed in Chemidoc and densiometry were analyzed in ImageJ, and are presented in figure 3.9.

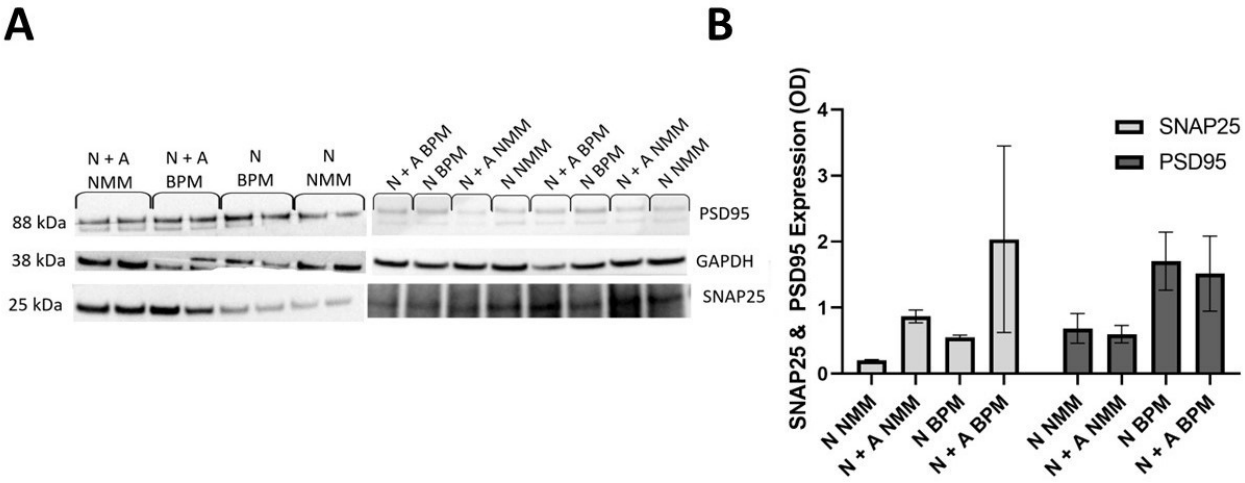

Figure 3.9: Membranes from two separate experiments with densiometry data from ImageJ, for neurons alone (N) and co-cultured with astrocytes (N + A) in NMM and BPM. **A** Membranes stained with PSD95, SNAP25 with GAPDH used for reference, which shows at around 88kDa, 25kDa and 38kDa, respectively. **B** Optical density for SNAP25 and PSD95, normalized against GAPDH. were bars represent mean +/- S.D from 4 replicates of one biological repeats (2 replicates for SNAP25).

Before going into what data from figure 3.9 **(B)** repersents, it is worth noting that SNAP25 behaved oddly during the second experiment with a smeared appearance, and densiometry analysis was not possible. Which lead to only one biological repeat with two replicates for data analysis, while PSD95 had four replicates. The values of optical density for SNAP25 were 330% and 270% higher for the protein collected from neuron and astrocyte co-cultures, in NMM and BPM, respectively, compared with neurons alone. This could suggest a positive benefit for synaptic function when culturing neurons with astrocytes. For PSD95 a decrease of 12.6% and 11.2% for co-cultures in NMM and BPM were instead seen. In general there was an average increase of around 150% (134%-175%) of SNAP25 and PSD95 for BPM when comparing to NMM. This might suggest that BPM gives rise to more synapses compared to NMM since higher expression of synapses are shown.

4
Discussion

4.1 Differentiation and Characterization of Astrocytes

Differentiation of astrocytes gave mixed results. One biological repeat succeeded in differentiating towards astrocytes. This was 004iC2 -Vit. A. The rest of the repeats all failed in some way during different steps of differentiation.

Early on, +Vit. A variants of both the Ctrl1 and 004iC2 lines started to look as if the cells were contaminated with NPCs in the plates. An early ICC were therefore conducted which confirmed the hypothesis, and the cells were then discarded. Differentiation continued with the -Vit. A variants instead. Considering that only 1 experimental repeat, out of 7 conducted, succeeded gives a fairly low percentage of successful repeats. However with this in mind there were only two cell lines, and three of the five repeats were variants of Ctrl, with the two latter being frozen and thawed. All Ctrl1 cells however had the same starting points with days in respective media up until introduction of AIM at day 19 where some were frozen, all were grown for a total of 11 days in GEM, where EGF is introduced. This can be compared with the successful repeat 004iC2 -V.A that were cultured in GEM for 19 days, more than a week extra over all Ctrl1 repeats. All repeats were cultured in AIM for 14-15 days and perhaps if the Ctrl1 cells were cultured in either GEM or AIM with glial and astrocyte inducing factors present for a bit longer, the outcome would have been different. What also differed was that both 004iC2 repeats were manually picked, which was due to the cultures not becoming clean enough with only the use of dispase. This should not really matter but perhaps the Ctrl1 lines appeared clean when going over to accutase and some contaminated cells were transferred as well, or accutase passages were started too early. Around day 55 during differentiation, an updated protocol for astrocyte differentiation were found were matrigel coating of plates were advised to culture in instead of laminin2020. This lead to a switch. The Ctrl1 -Vit. A cells up until this point had however already differentiated towards neurons, which lead to this being discarded as well. Matrigel consists of multiple growth factors compared to Laminin2020, that makes astrocytes more favoured in cultures, since neurons do not proliferate as much in matrigel compared to other media. If this coating were introduced earlier the outcome would maybe have been different.

This is why two repeats of the frozen Ctrl1 +/- Vit. A cells were thawed. To see if there was a difference in outcome if culturing in matrigel coated plates from day 22 when starting with AIM. However, as seen in the results these two repeats also differentiated towards neurons, and were ultimately discarded. This leaves, for a future study of examining different days in cultivation of the varying media. For the 004iC2 -Vit. A astrocytes matrigel seemed to work, some neuronal-like cells showed up in the plates. But after passages these cells would disappear and become less frequent as the astrocytes matured. Sidenote, the reasoning for freezing the two Ctrl1 lines at day 19 came from the fact that the 004iC2 lines had issues in getting clean for accutase passages and the the manual picking was a sort of last resort in hopes of the cultures

cleaning up, which they did. In hindsight it would have made sense to freeze the 004iC2 cell line as well since the, -Vit. A repeat at least, performed better. Perhaps for a future induction this can be done.

As the 004iC2 -Vit. A cell line progressed in differentiation it could be seen from the ICC images that the astrocyte markers became stronger further into differentiation. This implies that the cells had differentiated towards astrocytes. Estimation of EAAT1 and GFAP positive astrocytes showed an increase from day 77 to 109. For the second experiment done on day 109, the cell density/cm^2 where halved compared to the first experiment on day 77, and might suggest that lesser density helps with maturation and proper development, since there is more space for the astrocytes to spread out. Western blot showed that the astrocytes expressed EEAT1 in all 3 media, most in BPM and least in AM, and suggests that both NMM and BPM are valid media for growing mature astrocytes in. This is helpful if co-cultures with neurons are to be made where neurons can only be cultured in NMM or BPM. Functionality of the astrocytes were tested with calcium imaging since previous studies suggested that ATP from astrocytes gives rise to calcium waves [46]. Cells were imaged live at 2 time points, of which the latter had approximately 90% response after stimulation with ATP compared to 3%. It shall be noted there was only comparable data, with the second time point, from one replicate from the first time point. This was mostly due to protocol improvement during the first experiment, which was used during experiment two. The data from the second experiment suggest heavily that the astrocytes are functioning well. The cell density/cm^2 for the second experiment were roughly 3 times smaller compared with the first and might again suggest that lesser density helps with maturation and development.

Passages for the 004iC2 astrocytes were not as frequent when AMM were introduced, and cell were kept around 40,000 cells/cm^2. After maturation cell density were kept at 20,000 cells/cm^2 and only passaged when it was absolutely necessary. The decreasing density is because astrocytes become bigger when mature and cells should be allowed to proliferate for a good yield. It was, however, noticed that astrocytes from day 75 seemed to not proliferate as much. When passages were done, the yield would correspond to what was seeded at the previous passage. This made it difficult to expand the cells at this stage and experimental planning (with amount of cells) should ideally be done before the astrocytes are mature and starting with AMM. An even lower cell density of 10,000 cells/cm^2 were experimented withh at a later stage with inconclusive results however.

4.2 Co-Cultures of Neurons and Astrocytes

From the results of the co-cultures with neurons and astrocytes, it is not only clear that intensity of S100 was expressed to a greater extent for both media compared to neuron mono-cultures. Considering that astrocytes were added to the neurons, and S100 is an astrocyte markers, there should be, according to the hypothesis at least. These results, however, more importantly show, that astrocytes survive being cultured with neurons in NMM and BPM. That is for the eight days in which this experiment were conducted. It is, however, reasonable that both cell types survives since astrocyte-synaptic interactions is crucial for a functional nervous system, according to Chung *et al*[47]. A subject for future research would be to have astrocytes and neuron co-cultures for a longer period and investigate the impact on both neurons and astrocytes. From the PCM images taken at the same days of staining, the co-cultures seemed to look more dense and rich with cells, suggesting that astrocyte might help with neuronal proliferation. However a quantifiable way of determining this instead of only visual analysis

need to be done to achieve further evidence of this.

Longer neurites were found in the co-cultures. However, when normalized to the amount of cells present, the difference became smaller and not statistically significant. BPM had close to no change, in favour of neurons only, however in NMM, there seemed to be a small benefit of co-culturing neurons and astrocytes for improved neurite lengths. Cells grown in BPM, for both cultures showed an increased amount of neurite length per cell compared with NMM. However for all the results of neurite lengths per cell, it is worth noting that the cell count from which the data is normalized against, contains both astrocyte and neurons combined, and is not separated. Perhaps the difference is bigger still. This is something worth investigating further, with image analysis. This leads to the analysis of the Western blot, where synaptic protein expression of SNAP25 and PSD95 also were greater for both cultures in BPM compared to NMM, which suggests that BPM enhances synaptic function better than NMM. This coincides with Satir *et al*'s[48] findings that BPM increases synaptic function and increases expression of cortical neuron markers. Furthermore synaptic the specific expression of SNAP25 for co-cultures in both NMM and BPM, were slightly increased compared with neurons only, and might further propose that astrocytes helps with synaptic function with better membrane fusion for neurite synaptic vesicles and neuron plasma membranes. Expression of PSD95 showed a small change in favour of neuron monocultures when compared to the co-cultures. However the biggest difference can be seen for the BPM media for both cultures compared with NMM, where it is further suggested that BPM affects synaptic maturation and function.

5
Conclusions

Firstly, the implemented protocol for differentiation of astrocytes from hIPSCs, yielded mature human astrocytes. Although this is true for only one cell line, it however gives a good steppingstone in developing and optimizing the protocol further. Astrocytes showed ever changing morphology during differentiation, with significantly increasing intensity and spread of astrocyte markers and change of expression of neuroprogentior markers. The mature astrocytes showed to be functional, were 90% of cells sent out waves of calcium when stimulated with ATP. Furthermore the astrocytes were shown to survive in neuronal medias by successfully proliferating, both NMM and BPM, during the culturing period of 8 days. The medias also gave rise to higher expression of astrocyte marker EAAT1 for both NMM and BPM compared to AM.

Lastly, co-cultures were done with mature neurons differentiated for 73 days and mature astrocytes differentiated for 75 days. Astrocytes were shown to survive together with neurons for the culturing period of 8 days which cultures of cells. There were significantly more expression of astrocyte markers in the co-cultures. In the images the co-cultures looked denser, both with cells and neurites in mind, and the total length of neurites for the co-cultures compared with neurons only, were significantly longer. Overall, cells cultured in BPM gave rise to significantly longer neurites per cell for neurons, compared to NMM. It is therefore suggested that astrocytes might help with neuron proliferation, where mainly, BPM might be helping with better synaptic maturation, which agrees to previous studies. More studies on the effect astrocytes have on neurons however need to be made to receive qualitative data for comparison. Furthermore, expression of the synaptic protein SNAP25 were found to be higher in co-cultures compared with neuron mono-cultures, and we propose that astrocytes might help with membrane fusion for neurite synaptic vesicles and neuron plasma membranes. The synaptic protein PSD95 along with SNAP25 were expressed more for both cultures in BPM compared to NMM, which further supports the advantage of BPM for synaptic development compared to NMM.

6
Future Studies

From this project, there are several things that would be of interest for future studies. Method development takes time and a 5 month master thesis gives room for little error. A new induction of 004iC2 cell line would be the next step in optimizing the astrocyte protocol. It would be interesting to investigate if different time frames when adding the various growth factors to the media, could possibly achieve a higher yield of astrocytes. Even though the cells that successfully differentiated to astrocytes in the project were plated on laminin for a while, it would probably be better to use matrigel throughout the whole process.

Neurons and astrocytes were in co-culture for 8 days in our project. It would be interesting to investigate what the effect would be on survival and functionality if the co-cultutes were kept for a longer period of time. Also, adding microglia to the co-culture would be a topic for future studies.

There are some methods that would be interesting to test. For example multi electrode array (MEA), which is a method to measure action potential in cells. Neurons alone and in co-cultures with astrocytes could be seeded on MEA-plates to investigate both the affect media have on the cells as well as to further examine if astrocytes have an impact on neuronal signals. This way there would be quantifiable measured data for analysis.

Furthermore, when the protein samples were collected from the co-cultures, RNA and media from the wells were also collected as mentioned in section 2.2. Two methods could therefore be applied in form of qPCR, which could be used to quantify the expression of RNA in the mono- and co-cultures and mesoscale to measure accumulation of Aβ in the media.

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A
Appendix - Materials and Methods

A.1 Reagents and Resources

Table A.1: Table of reagents and resources used.

Reagent or Resource	Source	Identifier
Primary antibodies		
β-III tubulin (Tuj1)(m)	Abcam[38]	ab18207
EAAT1(rb)	Abcam[34]	ab416
GAPDH HRP conjugated	Novus Biologicals[33]	NB300-328H
GFAP(ch)	Abcam[42]	ab4674
Nestin(m)	R&D Systems[49]	MAB1259
Pax6(rb)	Nordic Biosite [50]	PRB-278P
PSD-95(rb)	Abcam[31]	ab18258
S100(rb)	Dako[51]	Z0311
SNAP25(rb)	Sigma Aldrich[32]	S9684
Tau(ch)	Biorbyt[52]	orb175815
DAPI	Sigma Aldrich[53]	D3571
Secondary antibodies		
anti-rabbit, HRP-linked	Cell Signaling Technology[35]	7074
anti-mouse, HRP-linked	Cell Signaling Technology[36]	7076
goat anti-rabbit Alexa488	Thermo Fischer Scientific[54]	A11070
goat anti-mouse Alexa647	Thermo Fischer Scientific[55]	A21236
goat anti-chicken Alexa647	Thermo Fischer Scientific[56]	A21449
Chemicals, peptides, and proteins		
DMEM/F-12 (1:1)(1X) + Glutamax	Gibco, Thermo Fisher Scientific	CAT#31331-028
Matrigel hESC-Qualified Matrix	Corning	CAT#354277
mTeSR^{TM} PLUS Medium	Stemcell Technologies	CAT#100-0276
Laminin 2020	Sigma Aldrich	L2020
Biolaminin 521	BioLamina	Ln521
Poly-L-Ornithine 0.01%	Sigma Aldrich	CAT#P4957
FGF_2	PeproTech	CAT#100-18B
Noggin/FC-Chimera	R&D Systems	CAT#3344NG
SB431542	Stemcell Technologies	CAT#72232
Insulin (9.5-11-5 mg/ml)	Sigma Aldrich	CAT#I9278
Beta-Mercaptoethanol (50mM)	Thermo Fisher Scientific	CAT#31350-010
MEM-NEAA (100X)	Thermo Fisher Scientific	CAT#11140-023
Sodium-Pyruvate (100mM)	Sigma Aldrich	CAT#S8636
Penicillin-Streptomycin (100X)	Hyclone	CAT#SV30010

Continued

Reagent or Resource	Source	Identifier
DPBS (1X) +$MgCl_2$/+$CaCl_2$ (+/+)	Gibco, Thermo Fisher Scientific	CAT#14040-083
Dispase	Thermo Fischer Scientific	CAT#17105-041
Accutase	Thermo Fischer Scientific	CAT#00-4555-56
EDTA	Thermo Fischer Scientific	CAT#AM9912
DMEM (1X)	Gibco, Thermo Fisher Scientific	CAT#1190-044
Neurobasal medium (1X)	Gibco, Thermo Fisher Scientific	REF#12348-017
N2 supplement (100X)	Gibco, Thermo Fisher Scientific	REF#17502-042
B-27 supplement (50X)	Gibco, Thermo Fisher Scientific	REF#17504-044
B-27 supplement without Vitamin A (50X)	Gibco, Thermo Fisher Scientific	REF#12587-010
Pierce™ BCA Protein Assay Kit	Thermo Fischer Scientific	REF#23225
Restore™ PLUS Western Blot Stripping Buffer	Thermo Fischer Scientific	CAT#46430
TBS-Tween™	Medicago	Art NO#9-7510-10
NuPAGE™ MES SDS Running Buffer (20X)	Thermo Fischer Scientific	CAT#NP0002
NuPAGE™ LDS Sample Buffer (4X)	Thermo Fischer Scientific	CAT#NP0007
SuperSignal™ West Dura Extended Duration Substrate	Thermo Fischer Scientific	CAT#34076
SeeBlue™ Plus2 Pre-stained Protein Standard	Thermo Fischer Scientific	CAT#LC5925
Histofix	Histolab	01000
Triton™ X-100	Sigma Aldrich	CAS#9036-19-5
Nonfat dried milk powder	PanReac AppliChem	A0830,1000
Trans-Blot Turbo Mini 0.2 μm Nitrocellulose Transfer Packs	Bio-Rad	CAT#1704158
ibidi Mounting Medium	ibidi	CAT#50001
Fluo-4, AM, cell permeant	Thermo Fischer Scientific	CAT#F14201
DL-Dithiothreitol solution (DTT)	Sigma Aldrich	CAT#43816
Goat serum	Sigma Aldrich	CAT#G9023
BrainPhys™ Neuronal Medium	Stemcell Technologies	CAT#05790
NeuroCult™ SM1 Neuronal Supplement	Stemcell Technologies	CAT#05711
N2 Supplement-A	Stemcell Technologies	CAT#07152
BDNF	PeproTech	CAT#450-02
GDNF	PeproTech	CAT#450-10
Dibutyryl cAMP	Sigma Aldrich	CAT#D0627
L-ascorbic acid	Sigma Aldrich	CAT#A0278
rh EGF	Stemcell Technologies	CAT#78006.1
rh LIF	Stemcell Technologies	CAT#78055
rh CNTF	Stemcell Technologies	CAT#78010
Cell lines		
hiPSC line 004iC2 from healthy individual	In House Zetterberg Lab	Ethical Approval Appendix:D
hiPSC line Ctrl1 from healthy individual	T. Sposito *et al.**	[57]
Other		
Mr. Frosty	Thermo Fischer Scientific	CAT#5100-0050

*Recieved from a partner in London.

A.2 Materials

Table A.2: Ingredients and amounts for Neural Induction Medium (NIM)

Neural Induction Medium	
Ingredient	**Amount**
DMEM/F-12 (1:1)(1X) + Glutamax	250ml
Insulin (9.5-11-5 mg/ml)	125µl
Beta-Mercaptoethanol (50mM)	500µl
MEM-NEAA (100X)	2.5ml
Sodium-Pyruvate (100mM)	2.5ml
Penicillin-Streptomycin (100X)	1.25ml
N2 supplement (100X)	2.5ml
B-27 supplement w or w/o vitamin A	5ml
Glutamax (100x)	2.5ml
Neurobasal medium (1x)	250ml
Noggin	2.5ml
SB431542	5ml
FGF_2	2.5ml

Table A.3: Ingredients and amounts for Neural Maintenance Medium (NMM)

Neural Maintenance Medium	
Ingredient	**Amount**
DMEM/F-12 (1:1)(1X) + Glutamax	250ml
Insulin (9.5-11-5 mg/ml)	125µl
Beta-Mercaptoethanol (50mM)	500µl
MEM-NEAA (100X)	2.5ml
Sodium-Pyruvate (100mM)	2.5ml
Penicillin-Streptomycin (100X)	1.25ml
N2 supplement (100X)	2.5ml
B-27 supplement (50X)	5ml
Glutamax (100x)	2.5ml
Neurobasal medium (1x)	250ml

Table A.5: Ingredients and amounts for Glial Expansion Medium (GEM)

Glial Expansion Medium	
Ingredient	**Amount**
DMEM/F-12 (1:1)(1X) + Glutamax	50ml
N2 supplement (100X)	500µl
B-27 supplement without Vitamin A (50X)	1ml
FGF_2 10ng/ml	5µl
rh EGF 10ng/ml	5µl

Table A.4: Ingredients and amounts for BrainPhys Medium (BPM)

BrainPhys Medium	
Ingredient	**Amount**
BrainPhys™ Neuronal Medium	50ml
NeuroCult™ SM1 Neuronal Supplement	1ml
N2 Supplement-A	500µl
BDNF	10µl
GDNF	10µl
100mg/ml dibutyryl cAMP	250µl
50µl/ml L-ascorbic acid	35µl
Penicillin-Streptomycin (100X)	125µl

Table A.6: Ingredients and amounts for Astrocyte Induction Medium (AIM)

Astrocyte Induction Medium	
Ingredient	**Amount**
DMEM/F-12 (1:1)(1X) + Glutamax	50ml
N2 supplement (100X)	500µl
B-27 supplement without Vitamin A (50X)	1ml
rh EGF 10ng/ml	5µl
rh LIF 10ng/ml	5µl

Table A.7: Ingredients and amounts for Astrocyte Maturation Medium (AMM)

Astrocyte Maturation Medium	
Ingredient	**Amount**
DMEM/F-12 (1:1)(1X) + Glutamax	50ml
B-27 supplement without Vitamin A (50X)	1ml
rh CNTF 20ng/ml	10µl

Table A.8: Ingredients and amounts for Astrocyte Medium (AM)

Astrocyte Medium	
Ingredient	**Amount**
DMEM/F-12 (1:1)(1X) + Glutamax	50ml
B-27 supplement without Vitamin A (50X)	1ml

Table A.9: Ingredients for Radioimmunoprecipitation assay(RIPA) buffer

RIPA buffer
Ingredient
20 mM Tris-HCl, pH 7.5
150 mM NaCl
1 mM EDTA
1% Triton X-100
0.5% sodium deoxycholate
0.1% SDS

A.3 Thawing of neuroepithelial cells during differentiation of iPS cells to cortical neurons

1. Pre-warm a Falcon tube with sterile water to 37 °C
2. Bring the cells to the cell culture on dry ice.
3. Place the cryovial in the pre-warmed water until all but a small fraction of the cells have thawed.
4. Spray the vial with ethanol.
5. Transfer the partially thawed cells to 10 ml of room-temperature neural maintenance medium.
6. Centrifuge the cells once at 400g (1800 rpm in large cell culture centrifuge) for 3 min and discard the supernatant
7. Gently resuspend the cells in 2 ml of neural maintenance medium supplemented with 20 ng/ml FGF2, and plate into laminin-coated wells (1 cryovial to 1 6-well).
8. Change to neural maintenance medium without FGF2 the following day and thereafter every other day.

A.4 Freezing of glial cells during differentiation of iPS cells to cortical neurons

Freeze media: Glial expansion media supplemented with 10% DMSO).

1. Start with confluent 6-wells (or the cells will not grow properly after thawing).
2. Prepare 1 ml 1x Freezing media/6-well (= GEM with 10% DMSO) and keep in the fridge. Place a CoolCell freezing container with pre-marked cryotubes in the fridge.
3. Wash the cells once with PBS.
4. Add 0.5 ml Accutase to each 6-well. Incubate at 37° C for 5 min.
5. Dissociate the cells by flushing them with the Accutase solution and transfer to a Falcon tube containing 10 ml DMEM/F12 media (wash media).
6. Centrifuge at 400 g (1800 rpm in large cell culture centrifuge) for 5 min.
7. Repeat the wash step once.
8. Discard the supernatant and dissolve the pellet in 0.8 ml cold Freezing media.
9. Quickly transfer the cell suspension from one tube to each cryovial. Place the vials in a pre-cooled CoolCell freezing container. Place the CoolCell freezing container in –80 °C overnight.
10. Transfer the cryovials to the nitrogen tank after 24h for long-term storage.

A.5 Coating of cell culture for iPS cell differentiation

A.5.1 With Matrigel

Corning™ Matrigel™ hESC-Qualified Matrix, Fisher Scientific: 11573560 Thaw in fridge and aliquot on ice. Store at -20°C and thaw aliquots in fridge. Keep the Matrigel cold at all times to avoid polymerization.

1. Add 15 l of Matrigel to each 1 ml of cold DMEM media. See table below for volumes required per well. Add an appropriate volume of Matrigel/DMEM to cover the total surface area of the well.

Culture vessel	Volume diluted Matrigel
6 well	1 ml
12 well	600 µl
24 well	300 µl
96 well	50 µl
8 well ibidi slide	300 µl
35 mm ibidi dish	400 µl

2. Incubate for at least 1 hour at 37° C (or room temperature).
3. Remove the Matrigel solution before adding cell suspension (no need to wash).

A.5.2 With Laminin 2020 (L2020)

Laminin (mouse) 1 mg/ml SigmaAldrich L2020. 1-2 µg/cm^2 is recommended for coating.

1. Thaw laminin in fridge to avoid polymerization.
2. Dilute laminin stock in PBS +/+ Dilute the amount of laminin used from the table below

Type of plate	Volume/well	Laminin 1 µg/cm^2 (µl/ml PBS +/+)
6 well	1 ml	10 µl (10 µl/ml)
12 well	500 µl	5 (10 µl/ml)
24 well	400 µl	2 µl (5µl/ml)
96 well	50 µl	0.5µl (10µl/ml)
8 well ibidi slide	300 µl	1.5µl (5µl/ml)
35 mm ibidi dish	400 µl	4µl (10µl/ml)
T25cm^2 flask	2 ml	25 µl (12.5µl/ml)
T75cm^2 flask	6 ml	75µl (12.5µl/ml)

3. Add desired volume to each well to be coated.
4. Incubate for at least 4 hours (the longer the better), or overnight at 37° C (Make sure that the wells do not dry out).
5. Remove laminin before adding cell suspension (no need to wash).

A.5.3 With Laminin 521 (LN521)

Laminin 521 (human) 100 µg/ml BioLamina. 0.5 µg/cm^2 is recommended for coating.

1. Thaw laminin in fridge to avoid polymerization.
2. Diltute laminin stock in PBS +/+. Calculate the amount of laminin used from the table below

Type of plate	Volume/well	Laminin 0.5 µg/cm^2 (µl/ml PBS +/+)
6 well	1 ml	50 µl (50 µl/ml)
12 well	500 µl	25 (50 µl/ml)
24 well	400 µl	10 µl (25µl/ml)
96 well	70 µl	3.5µl (50µl/ml)
8 well ibidi slide	300 µl	5µl (16.7µl/ml)
35 mm ibidi dish	400 µl	20µl (50µl/ml)
T25cm^2 flask	3 ml	150 µl (50µl/ml)
T75cm^2 flask	8 ml	400µl (50µl/ml)

3. Add desired volume to each well to be coated.
4. Incubate for 2 hours at 37° C or overnight in fridge, sealed in parafilm.
5. Remove laminin soultion before adding cell suspension (no need to wash)

Note: Coated plates can be kept in fridge for up to 4 weeks. Make sure that the wells do not dry out.

A.5.4 With poly-L-ornithine

Poly-L-ornithine 0.01% SigmaAldrich, P4957.

1. Add poly-L-ornithine to each well accordning to table below

Type of plate	Volume/well	Dilute before?
6 well	1 ml	No
24 well	400 µl	Yes 1:2
96 well	50 µl	No
8 well ibidi slide	300 µl	Yes 1:2
35 mm ibidi dish	400 µl	No

2. Incubate for at least 4 hours (or overnight)
3. Aspirate poly-L-ornithine solution and add laminin solution.

A.6 Passaging of iPS cells cultured on Matrigel in mTeSR+ with EDTA

Prior to passaging, pre-warm mTeSR+ to room temperature. If Matrigel coated plates were kept in the fridge, pre-warm them to room temperature.

Method

1. Aspirate the used medium from the cell-containing well.
2. Rinse the cells once with 0.5 mM 1 ml EDTA (alternatively 2 ml PBS -/-).
3. Add 1 ml 0.5 mM EDTA per 6-well.
4. Incubate at 37° C for 3-5 minutes (depending on iPS cell line). Check cells under microscope.
5. Remove Matrigel from the culture plates. Add required amount of mTeSR+ media to achieve 2 ml/well after adding cells.
6. When the cells start to round up and holes can be spotted in the cell mat, carefully remove EDTA and add 1 ml fresh, room-tempered mTeSR+ media. Gently detach the cells by rinsing the well with the added media using a 1 ml pipette. Do not pipette the cells up and down more than three times. Instead, transfer cells to a tuube and rinse remaining cells with a fresh ml of media. NOTE! The cells re-attach quickly after adding medium. Work fast!

7. Take the required amount of cell suspension and add to the new well with fresh mTeSR+ media. A passage of 1:4 to 1:10 can be performed depending on how the cells grow.
8. Move the plates in quick figure-eight motions to spread out the cells.
9. Change to fresh mTeSR+ medi after 24 h. Then change media daily.

A.7 Passaging of neuroepithelial cells with dispase

Preparations: Coat plates with laminin. Prepare media to seed cells in. Thaw dispase Aliquot wash media DMEM/F12+Glutamax
Method

1. Add 100 µl of dispase stock solution per ml (200 µl for a 6 well plate) directly into the well containing a confluent neuroepithelial layer or rosette containing sheet.
2. Incubate for 5 min at 37° C (more time, up to 30 min, may be necessary if the cells does not detach)
3. Use a transfer pipette to carefully detach the cell mat. Pipette up and down 2-3 times to break cells into smaller pieces (but not too small).
4. Transfer the cell colonies from each well to a 15 ml falcon tube containing 10 ml pre warmed (37° C) wash media (wash each well separately).
5. Let cells sink to the bottom of the tube for 3 minutes in room temperature (or 37° C heat block)
6. Gently remove supernatant. Make sure to leave some wah media to not lose cells.
7. Resuspend cells in 10 ml wash media.
8. Repeat the wash (step 5-7) twice. (However the third time let the cells sink for 5 min. Also remove as mush wash media as possible)
9. Resupend cells in NMM + FGF_2 (for neuron differentiation) or GEM (for astrocyte differentiation). 2 ml per well of chosen should be used.
10. Remove laminin from coated wells.
11. Add 2 ml of cell suspension to wells (aim to get cell clumps to approximately 200 µm in size).
12. Incubate overnight to let cells reattach,
13. Change media next day. Then every second day.

A.8 Passaging of neural progenitor cells with accutase

Preparations: Coat wells with laminin. Aliquot sterile PBS -/- for washing, 2 ml/6 well, at room temperature, accutase (A11105-01), 0.5 ml/6 well, at room temperature, media for plating (NMM or GEM), 2 ml/6 well, at room temperature and wash media (DMEM/F12+Glutamax), 20 ml/6 well, at 37° C.
Method:

1. Remove old media from well/wells.
2. Add 2 ml PBS -/- to each well.
3. Remove PBS -/-.
4. Add 0.5 ml accutase to each well.
5. Incubate at 37° C for 5 min.
6. Pipette the cells up and down 3-4 times in the accutase solution to dissociate cells into single cells.
7. Transfer accutase cell solution to a 15 ml falcon tube containing 10 ml wash media.
8. Centrifuge the cell suspension at 400g (1800 rpm) for 5 min.
9. Discard supernatant.

10. Repeat wash step once (step 6-8).
11. Resuspend cells in 2 ml of plating media.
12. Remove laminin from coated well and plate the cell suspension.
13. Incubate overnight.
14. Change media the day after and then every second day.

A.9 Manually picking of glial- and neuroprogenitor rosettes

1. Remove laminin from well.
2. Add 2 ml of media (GEM or NMM depending on cell type).
3. Cut out rosettes with a canula under microscope.
4. Transfer the rosettes to a well using a pipette.
5. Incubate overnight.
6. Change media the day after and then every second day.

A.10 Immunocytochemistry

A.10.1 Fixation of cells

1. Remove medium.
2. Wash cells with PBS -/-.
3. Add histofix and incubate in room temperature for 15 min.
4. Remove histofix.
5. Wash with PBS -/-.
6. Add fresh PBS -/-.
7. Civer with parafilm and store in fridge until use.

A.10.2 Add primary antibodies

1. Wash cells 3x5 min with TBS.
2. Permeabilize cells using 0.3% Triton X-100 in TBS for 15 min in room temperature.
3. Block cells using 5% goat serum in 0.3% TritonTriton X-100 - TBS 1 hour in room temperature.
4. Incubate cells in fridge with primary antibody in blocking buffer.

A.10.3 Add secondary antibodies

1. Wash cells 3X5 min in TBS.
2. Add secondary antibodies in block buffer, incubate for 1-2 hours in room temperature (dark).
3. Wash cells 3X with TBS. If needed, add DAPI in the second TBS wash, incubate for 5 min.
4. Wash once with MilliQ H_2O.
5. Mount coverslips using pro long gold antifade mounting media.

A.11 Sample collection

A.11.1 Collect media

1. Collect the media in a 15 ml Falcon tube (for 6-wells) or a 1.5 ml Eppendorf tube (for 24- and 96-wells)
2. Add 1 ml (6-well) or 500 µl (24-well) room temperatured PBS-/- to the cells
3. Spin down the media at 400g for 5 min to get rid of cell debris
4. Transfer the supernatant to Eppendorf tubes and discard the pellet
5. Freeze in -80°C for later analysis

A.11.2 Collect cells for total RNA extraction

1. Remove PBS from the well
2. Add 350 µl (or 600 µl) RLT buffer with DTT added
3. Pipet up and down to resuspend
4. Transfer the sample to a 2 ml Eppendorf tube
5. Freeze in -80°C for later analysis

A.11.3 Collect cells for protein extraction

1. Detach cells in the well using a cell scraper
2. Pipet to resuspend cells
3. Transfer 50 µl cell suspension to 150 µl PBS for cell counting (1:4 dilution)
4. Transfer remaining 450 (or 950) µl of cell suspension to a 1.5 ml Eppendorf tube
5. Spin down at 4000 rpm for 5 min
6. Remove supernatant
7. Freeze in -80°C for later analysis

A.12 RIPA lysis for Western blot

1. Dissolve 1 tablet of protease inhibitor cocktail in 10 ml of RIPA buffer.
2. Add desired amount (depends on number of cells to be lyzed) of RIPA buffer with cocktail to each sample and pipet to dissolve cells.
3. Sonicate the samples for 10 min.
4. Incubate on ice for 20 min.
5. Centrifuge at 4000g for 5 min at 4°C.
6. Transfer the supernatant to a new tube.

A.13 Protein measurment using PIERCE BCA protein assay

1. Thaw prepared standards in RT.
2. Add 10 µl of each standard (do not forget the blank) from 2 mg/ml to 0.125 mg/ml to a 96-well plate, in duplicates or triplicates.
3. Add 10 µl of each sample to a 96-well plate, in duplicates (dilute samples in RIPA buffer if necessary).

4. Mix 50 parts of BCA reagent A with 1 part of BCA reagent B to make up a working solution.
5. Add 200 µl to each well containing standards and samples.
6. Incubate for 30 min at 37°C.
7. Measure absorbance at 560 nm using a plate reader.
8. Calculate the concentration of each sample using the standard curve and linear regression.

A.14 Western blot

1. Prepare protein samples by lysing them in RIPA buffer according to protocol A.12.
2. Measure protein concentration using the BCA protein assay.
3. Dilute all samples to the same protein concentration in RIPA buffer with protease inhibitor cocktail, 15-30 µl total volume.
4. Mix the samples with 4x sample buffer (1/4 of total volume) and DTT (1 µl of 1M stock to 20 µl total sample volume).
5. Boil the samples for 2-5 min at 95°C.
6. Prepare a Novex bis-tris precast gel
   (a) Remove the comb
   (b) Remove the white tape at the bottom
   (c) Place it in the container (2 gels or 1 gel and one glass/plastic piece), with the low edge facing inwards.
   (d) Add running buffer (MES or MOPS 1x) between the gels, enough to cover the wells.
   (e) Add buffer on the outside to about 2/3 of the container height.
7. Load the samples using long sift tips.
8. Add 10 µl of protein ladder, Novex Blue plus 2 standard, into one well.
9. Run the gel, starting at 120 V. The voltage can be increased to 200 V when the protein has entered the gel. Run for 45 min to 1 h depending on buffer and gel concentration.

A.14.1 Blotting

1. Place filter pads and membrane (nitrocellulose .2 µm) in TGM buffer.
2. Remove gel from cask and cut off comb and bottom part.
3. Place gel in TGM buffer to equilibrate,
4. In the semi dry blot, place in the following order: filter pad - membrane - gel - filter pad.
5. Roll in between to remove air bubbles.
6. Run at 15 V for 30 min,

A.14.2 Incubation with antibodies

1. Block the membrane with 5% milk or 3-5% BSA in PBS-tween/TBS-tween for at least 1 hour.
2. Dilute primary antibodies in blocking solution.
3. Place the membrane in a plastic pouch, seal 3 edges, add the antibody solution and seal the last edge.
4. Incubate overnight at 4°C.
5. Wash the membrane 3x5min in PBS-tween/TBS-tween.
6. Dilute and add the secondary antibody in blocking solution.
7. Incubate for 45-60 min at room temperature.
8. Wash the membrane 3x5min in PBS-tween/TBS-tween.

9. Develop using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher) 2+2 ml, incubate for 5 min, put in thin plastic film and develop in the Chemidoc.

A.14.3 Restore membrane using stripping buffer

1. Add Restore PLUS Western Blot Stripping Buffer to the membrane for 7 min.
2. Wash once with TBS-tween.
3. Block in 5% milk for 30 min.
4. Add GAPDH (1:20000) diluted in milk and incubate for 45 min.
5. Wash 3x10 min in TBS-tween.

A.15 Calcium imaging

1. Wash astrocytes once with HBSS.
2. Add Flou-4 (2µM, Life Technologies) diluted in DMEM to the astrocytes and incubate for 15 min in 37°C.
3. Wash astrocytes again with HBSS and image immediately.
4. Perform live imaging with inverted microscope Axiovision Observer.Z1.
5. Image cells for 120s after ATP (3 µM) addition and take one picture every 683 ms.

B

Appendix - Results

In this section, results from the PIERCE BCA protein assay and PCM images are presented.

Table B.1: Experimental results from PIERCE BCA protein assay, for western blot concentration calculations

Sample	Plate & Well	Conc. (µg/ml)	Mean Conc. (µg/ml)
1	N+A NMM P1	156.152 174.768	165,46
2	N+A NMM P2	99.838 97.51	98.674
3	N+A NMM P3	195.246 196.177	197.108
4	N+A BPM P1	214.792 202.692	208.742
5	N+A BPM P2	12.342 38.87	25.606
6	N+A BPM P3	100.768 94.252	97.51
7	N BPM P1	70.052 69.12	69.586
8	N BPM P2	93.322 85.41	89.366
9	N BPM P3	105.422 59.812	82.617
10	N NMM P1	107.75 119.85	113.8
11	N NMM P2	85.41 97.046	91.228
12	N NMM P3	93.788 69.12	81.454
13	A AM P1	-7.672 -9.532	-8.602
14	A AM P2	268.78 254.352	261.566
15	A AM P3	183.146 190.126	186.636
16	A NMM P1	200.83 188.264	194.547
17	A NMM P2	6.756 18.856	12.806

Continued			
Sample	**Plate & Well**	**Conc. (µg/ml)**	**Mean Conc. (µg/ml)**
18	A NMM P3	338.59 348.364	343.477
19	A BPM P1	83.548 78.894	81.221
20	A BPM P2	106.818 119.85	113.33A
21	A BPM P3	74.24 59.348	66.794

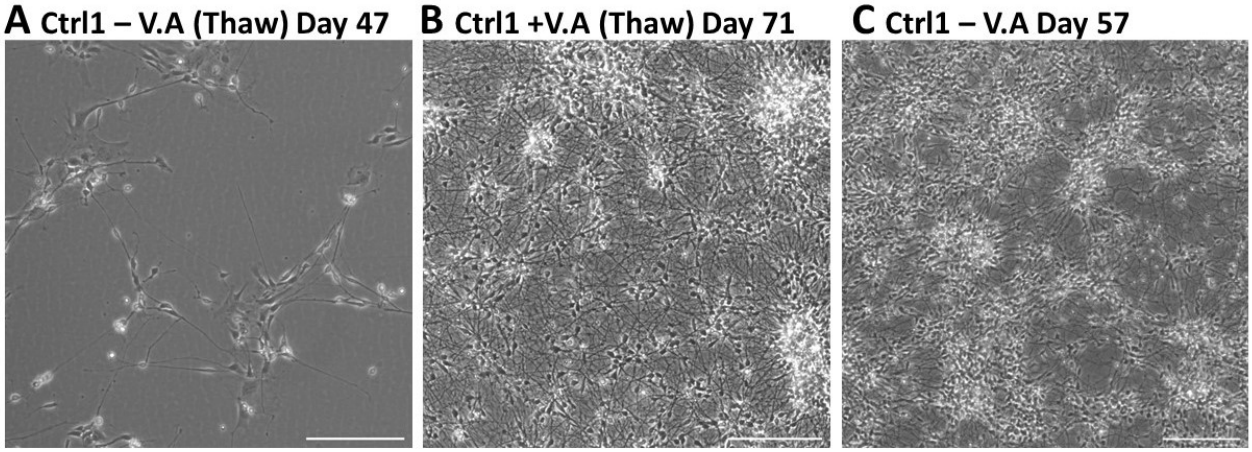

Figure B.1: Ctrl1 cell lines shows neuronal like differentiation instead of astrocyte. PCM images are around the days when discarding occurred. **A** Ctrl1 -vit. A thawed, day 47, **B** Ctrl1 +vit. A thawed, day 71 and **A** Ctrl1 - vit. A day 57.
Scale bar: 200µm

C
Appendix - Code

In this appendix macros that were used in ImageJ for quantification of ICC images are presented. The first macro is for counting cells, the second for measuring intensity and the third for measuring neurite length.

Macro for counting cells

```
run("Slice Remover", "first=2 last=3 increment=1");
setAutoThreshold("Default dark");
//run("Threshold...");
setAutoThreshold("MinError dark");
setOption("BlackBackground", false);
run("Convert to Mask");
run("Subtract Background...", "rolling=7 light create disable");
setAutoThreshold("MinError");
//run("Threshold...");
//setThreshold(240, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", " show=[Outlines] display clear summarize");
```

Macro for measuring integrated density

```
run("Delete Slice", "delete=channel");
setAutoThreshold("Default dark");
//run("Threshold...");
setThreshold(1300, 65535, "raw");
run("Set Measurements...", "area mean standard integrated limit
redirect=None decimal=3");
run("Measure");
```

Macro for measuring neurite length

```
run("Delete Slice", "delete=channel");
run("Delete Slice", "delete=channel");
rename("original");
run("Duplicate...", "title=neurites");
selectWindow("neurites");
run("Threshold...");
waitForUser("select a threshold and click ok");
run("Create Mask");
```

```
rename("neuritelength");
selectWindow("neuritelength");
run("Subtract Background...", "rolling=1 create disable");
selectWindow("neuritelength");
setThreshold(2, 255, "raw");
run("Convert to Mask");
run("Skeletonize");
run("Analyze Particles...", " show=Masks summarize");
selectWindow("neurites");
run("Convert to Mask");
run("Subtract Background...", "rolling=1 create disable");
setThreshold(2, 255, "raw");
run("Analyze Particles...", " show=Masks summarize");
selectWindow("neuritelength");
close();
selectWindow("neurites");
close();run("Delete Slice", "delete=channel");
run("Delete Slice", "delete=channel");
rename("original");
run("Duplicate...", "title=neurites");
selectWindow("neurites");
run("Threshold...");
waitForUser("select a threshold and click ok");
run("Create Mask");
rename("neuritelength");
selectWindow("neuritelength");
run("Subtract Background...", "rolling=1 create disable");
selectWindow("neuritelength");
setThreshold(2, 255, "raw");
run("Convert to Mask");
run("Skeletonize");
run("Analyze Particles...", " show=Masks summarize");
selectWindow("neurites");
run("Convert to Mask");
run("Subtract Background...", "rolling=1 create disable");
setThreshold(2, 255, "raw"); r
un("Analyze Particles...", " show=Masks summarize");
selectWindow("neuritelength");
close();
selectWindow("neurites");
close();
```

D

Appendix - Ethical Approval

Regionala etikprövningsnämnden i Göteborg

Projektansvarig:
Henrik Zetterberg
Neurokemi, V-huset
SU/Mölndals sjukhus
431 80 Mölndal

Dnr:
731-14

Exp. 2014-12-04
20150119

Forskningshuvudman: Göteborgs universitet

Granskad och godkänd som sekreterarärende
Göteborg 2015-01-16
Lennart Andrén, [illegible] sekr
Med avd 1
Regionala etikprövningsnämnden i Göteborg

Närvarande beslutande:
Marika Estreen, *ordförande*
Lennart Andrén, *vetenskaplig sekreterare*
Ledamöter med vetenskaplig kompetens:
Rolf Axelsson
Jane Carlsson
Kecke Elmroth
Gunnar Göthberg
Margareta Hellgren Wångdal, *deltog 860-14, 861-14, 866-14, 867-14, 868-14, 873-14 och 874-14*
Per-Anders Jansson
Margareta Kreuter
Ann Nachemson, *deltog ej i ärenden 855-14, 857-14, 860-14, 861-14, 864-14, 866-14, 867-14, 868-14, 873-14 och 874-14*
Frode Slinde, *deltog ej i ärende 864-14 p g a jäv*
Ledamöter som företräder allmänna intressen
Bengt Andersson
Jane Bredin
Berit Frändås
Heléna Okomdal Holmgren
John Sjöbohm

Projekttitel: Studie av neurodegenerativa sjukdomar i patientspecifika hjärnceller framtagna ur somatiska celler via stamceller
Projekt ID: Neuro 1, **Version:** 1

Beslutsprotokoll från sammanträde med Regionala etikprövningsnämnden i Göteborg, Medicinska avdelningen (M 1), den 1 december 2014

Föredragande: Rolf Axelsson

Sekreterarärende efter komplettering

Nämnden ifrågasätter om det inte bör vara Västra Götalandsregionen som är huvudman. Ansökan samt resursintyg ska därvid undertecknas av verksamhetschefen inom berörd klinisk verksamhet. Korrektion av punkterna 1:1 och 1:2 emotses.

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Nämnden anser att den etiska diskussionen behöver utvecklas och anser att det är olämpligt att engagera anhöriga som kontroller.

Patientinformationen behöver kompletteras och biobanksavsnittet bör utvidgas och lämpligen kan följande formulering användas: "De prover (vävnads- blodprover) som tas i samband med den undersökning/forskning du ska genomgå kommer att lagras i en biobank. Proverna kommer att förvaras kodade, vilket innebär att de inte direkt kan härledas till dig som person. Proverna liksom en tillhörande identifieringslista (kodnyckel) förvaras på ett säkert sätt och åtskilda. Proverna får endast användas på det sätt som du gett ditt samtycke till. De kan endast bli aktuella för ett nytt forskningsprojekt efter det att du lämnat ett nytt samtycke och/eller godkännande skett av Etikprövningsnämnden. Du har full rätt att utan närmare förklaring begära att dina prover ska förstöras eller avidentifieras (dvs. de kan inte spåras till din person). Biobankslagen (SFS 2002:297)."

Eftersom en del deltagare har kognitiv störning, så bör patientinformationen förenklas och skrivas med ett mera lättillgängligt språk. I informationen ska det framgå att man kan avbryta deltagande i studien utan att ange skäl för avbrytande. En mening som specifikt lyder "Ingen obehörig kommer att få tillgång till dina data" ska adderas.

De specifika kompletteringar som görs bör anges i ett separat följebrev. Textavsnitt som ändras i ansökningshandlingarna/forskningspersonsinformationen bör tydligt markeras.

Komplettering av ärendet (1 ex) ska ha kommit in till Etikprövningsnämnden inom tre månader från beslutsdatum då ärendet tas upp på nytt. Om komplettering ej inkommit kan ärendet komma att avgöras i befintligt skick.

Att denna avskrift i transumt överensstämmer med originalet intygar

Barbro Morsing, administrativ sekreterare

INKOM
2018 -05- 07
Etikprövningsnämnden i Göteborg

GÖTEBORGS UNIVERSITET
SAHLGRENSKA AKADEMIN

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2018 05 04 1 / 1

Till Regionala etikprövningsnämnden
Göteborg

Dnr T417-18
Ad 731-14
Betalt 2018-06-11
Exp 2018-06-11

Inför inklusionsstart av patienter till studien Neuro1 med diarienummer 731-14 har vi gjort några smärre förändringar i ansökan. Dessa beskrives nedan:

I själva ansökan har vi under punkt 1:4 (andra medverkande) tagit bort:
Anders Wallin, Minnesmottagningen, Mölndal
Björn Holmberg, Neurologen, SU/Sahlgrenska
Fredrik Asztely, Neurologen, SU/Sahlgrenska och Kungsbacka

Och istället lagt till:
Dzeneta Vizlin Hodzic, Klinisk patologi och cytologi, SU/Sahlgrenska sjukhuset, Göteborg
Silke Kern, Minnesmottagningen, SU/Mölndal, Göteborg
Radu Constantinescu, Centrum för Huntingtons sjukdom, SU/Mölndal, Göteborg
Camilla Palmqvist, Minnesmottagningen SU/Mölndal, Göteborg

Detta på grund av att tidigare ansvariga personer inte längre är involverade i projektet.

I samtyckesformuläret har vi lagt till plats för underskrift av ansvarig läkare, kontaktuppgifter till mottagningen samt en fråga om patienten kan tänka sig att bli kontaktad i framtiden för eventuella nya studier.

Både de gamla och de nya versionerna av dessa dokument bifogas.

Henrik Zetterberg, Md. PhD, professor, sektionschef

Granskad och godkänd som sekreterarärende
Göteborg
7/5-18
Gunnar Göthberg, docent, vetenskapl sekr
Med avd 1
Regionala Etikprövningsnämnden i Göteborg