iPSC derived cerebral organoids as a model of tauopathy

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2021

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Declaration

I, Christopher Edward James Lovejoy, confirm that the work presented in this thesis is my own. Where information has been derived from other sources or work has been produced through collaboration, this has been indicated in the text. This includes:

2D neuronal cultures generated by Dr Charlie Arber and Argyro Alatza.

Aβ assays conducted by Dr Charlie Arber and Dr Jamie Toombs.

Forebrain organoids generated by Argyro Alatza.

Forebrain PCR and forebrain immunoblot in section 5 generated by Argyro Alatza.

This thesis is not substantially the same as any other that may have been submitted at any other university or similar institution.
Acknowledgments

First and foremost I would like to thank Professor Selina Wray for the amazing opportunity to not only work with her and her lab, but also work on an incredible, cutting edge, and fascinating project. For letting me develop ideas, both good and bad, and listening to them, as well as the support for my scientific writing, which has been most appreciated. Also her valuable career advice which has helped me path out my future endeavours.

Dr Charlie Arber has been instrumental in my journey, answering numerous questions and helping in many a protocol, without which a lot of the work in this thesis would not be possible.

I would also like to thank all members of the Wray lab, both past and present, for help they have provided during my project, as well as the fun and laughs throughout.

My friends in London, Wales, and around the world for their support. Always willing to listen to me babble on about some cool new results, a beautiful ICC image I’ve taken, or of course complain about my one billionth day in tissue culture. All this social support really meant a lot to me, especially in 2020.

Finally my family who always support my efforts and decisions. Especially my grandparents, who were very proud of my PhD studies, but unfortunately never got to see the fruits of that. I know you would be very proud.

Finally I would like to thank Phil Bunker, the donor of the Joy Bunker scholarship and subsequently now good friend. Without Phil’s generous donation and this scholarship, this work would not have been possible. Not only has Phil allowed this exciting work to be carried out, he has allowed me to continue my studies and gaining experience. I will be forever grateful of this, and thank him deeply for the opportunity.
Papers arising from this thesis


Stem cell models of Alzheimer’s disease: progress and challenges – Charlie Arber, Christopher Lovejoy, & Selina Wray. In press at Alzheimer’s Research and Therapy, 2017

Full text available in Appendix.
Abstract

Insoluble, hyper-phosphorylated aggregates of tau are a pathological hallmark of a range of clinically diverse neurodegenerative diseases termed tauopathies, of which Alzheimer’s disease (AD) is the most common. The mechanisms linking neuronal death and tau dysfunction are not fully understood, but mutations uncovered in microtubule associated protein tau (MAPT) that cause aggressive frontotemporal dementia confirm a causative relationship between tau dysfunction and neurodegeneration.

The tau protein exists as multiple protein isoforms in the adult human central nervous system (CNS), generated by alternative splicing of the MAPT gene. Disruptions to tau splicing are associated with a number of tauopathies, however, in vitro and in vivo models to understand the consequences of disrupted tau splicing have been lacking, due in part to species differences in tau splicing and the developmental regulation of tau in human neurons. Recently, the development of induced pluripotent stem cells (iPSC) has enabled the derivation of limitless numbers of human neurons with disease associated mutations of interest. The use of this system to model tauopathy has been challenging, in part due to the developmental regulation of tau splicing, with extended culture periods required for mature tau expression in iPSC derived neurons.

Cerebral organoids are 3D based iPSC derived neuronal cultures, which help capture the heterogeneity and key aspects of architecture of the developing brain, such as distinct progenitor zones and lamination of neurons into distinct layers. We hypothesised that this may allow neurons to mature at a faster rate, resulting in earlier expression of all 6 isoforms of tau without extensive culture times.

We investigated the utility of iPSC-derived cerebral organoids to model key aspects of tau biology. Cerebral organoids showed high variability in neuronal content and tau expression. To reduce this heterogeneity, we generated engineered cerebral organoids (enCORs), which utilise a floating scaffold to increase the efficiency of neural induction and reduce heterogeneity. We show that enCORs provide a robust and reproducible in vitro system for the analysis of tau expression and splicing in a 3D model. To investigate the effect of tau mutations, we generated enCORs from an isogenic series of iPSC with the MAPT 10+16 and P301S mutations. The presence of tau splicing mutations results in disease-associated alterations in tau expression, specifically a dose-dependent increase in 4R tau isoforms in the presence of the MAPT 10+16 variant. While the developmental regulation of tau splicing is conserved, maturation of tau splicing is accelerated in 3D cultures compared to 2D cultures. Finally, enCORs with coding mutations in MAPT are able to produce seed-
competent tau species, suggesting enCORs recapitulate early features of tau pathology. In summary, enCORs provide a novel, robust in vitro system for the study of tau in development and disease.
Impact Statement

Affecting 50 million people globally, dementia is one of the most serious health issues facing our aging world. With dementia suffers predicted to hit 152 million worldwide by 2050, marking a 204% increase, this health crisis is set to get worse without treatment options. Modelling complex disorders of the human brain is challenging, with culminating research in additional models needed to investigate mechanisms of disease. Although extremely valuable, current 2D human iPSC derived models of tauopathies are arguably reductionist in approach and struggle to recapitulate MAPT expression, an essential part of tau aggregation disorders. 3D human iPSC derived neuronal cultures potentially offer a solution to this problem. These 3D models also help recapitulate simplified neuronal architecture of the human brain, increasing cell to cell interaction and neuronal maturity.

Within this project we show how Aβ processing can be investigated using both 2D and 3D iPSC models, revealing mutation specific Aβ species secretion. With paired patient CSF showing similar Aβ profiles. This type of iPSC based patient matched research allows investigation of possible mutation or Aβ species specific treatments to be trialled in these models.

With identification of mature MAPT splicing within 3D cultures, and favourable comparison to human brain samples, research in to tau aggregation utilising all tau isoforms could illuminate tau aggregation mechanics. 3D analysis may even allow pinpointing the origin of tau isoform expression over time, potentially even cell type specific tau isoform expression. This kind of data could again be utilised for drug and antibody screening in similar ways to anti-Aβ antibody trials, with potentially MAPT mutation specific therapies.

Further to this, other groups are expanding on 3D organoid research, utilising assembloids for multi-brain region models, investigating neuronal migration and circuit development. Other organoid avenues include investigating blood brain barrier mechanics, drug accessibility with the human brain, and CSF production in choroid plexus organoids. Also with refinement of 3D organoid models, high throughput imaging and drug screening can be utilised for further results in a 3D neuronal model.

Through continued development of 3D brain models complimenting other model data, for example animals, further discoveries about tau aggregation and dynamics are possible.
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**List of abbreviations**

3R tau – Tau with 3 microtubule binding repeats  
4R tau – Tau with 4 microtubule binding repeats  
0N tau – Tau with 0 N-terminal insert  
1N tau - Tau with 1 N-terminal insert  
2N tau - Tau with 2 N-terminal insert  
2D – Two dimensional  
3D – Three dimensional  
10+16m – 10+16 monoallelic  
10+16bi - 10+16 biallelic  
10+16bi/P301Sbi - 10+16biallelic/P301Sbiallelic  
AD – Alzheimer’s disease  
APP – Amyloid precursor protein  
B27 – B27 supplement  
bFGF – Basic fibroblast growth factor  
BRN2 - POU class 3 homeobox 2  
BSA - Bovine serum albumin  
CO – Cerebral organoid  
CBD – Corticobasal degeneration  
cDNA - Complementary DNA  
CFP – Cyan fluorescent protein  
CHIR - CHIR99021  
cm - Centimetre  
CNS – Central nervous system  
CODM – Cerebral organoid differentiation media  
CTIP2 - Bcl11b  
DAPI - 4’,6-diamidino-2-phenylindole  
DIV – Days *in vitro*  
DTT - Dithiothreitol  
DMEM - Dulbecco’s modified eagle medium
DM - Dorsomorphin
DNA – Deoxyribonucleic acid
dNTP - Deoxynucleotide
DSO – Dual-SMAD organoid
E8 – Essential 8 media
EB – Embryoid body
EDTA - Ethylenediaminetetraacetic acid
EGTA - Ethylene glycol tetraacetic acid
EM – Electron microscopy
enCOR – Engineered cerebral organoid
ESC-Quality FBS - ESC-Quality FBS
FBS – Fetal bovine serum
FOXG1 - Forkhead box G1
FRET - Förster resonance energy transfer
FTD - Frontotemporal dementia
FTDP17T - Frontotemporal dementia and parkinsonism linked to chromosome 17
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GFAP - Glial fibrillary acidic protein
h - Hour
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICC – Immunocytochemistry
IDM-A – Improved differentiation media minus vitamin A
IDM+vA - Improved Differentiation Media plus Vitamin A & C + HEPES
iPSC – Induced pluripotent stem cells
Ki67 - Antigen KI-67
LDS - Laemmlie sample buffer
MAP2 - Microtubule associated protein 2
MAPT - Microtubule associated protein tau
min – Minute
MTBR – Microtubule binding repeat
N2 – N2 supplement
N2B27 - Neuronal maintenance media
NEAA – Non-essential amino acids
NPC – Neural precursor cell
OCT - Optimum cutting temperature compound
PAX6 - Paired box protein Pax-6
PBS - Phosphate buffered saline
PBS-T - Phosphate buffered saline + triton X-100
PCR – Polymerase chain reaction
PFA - Paraformaldehyde
PLGA - poly(lactic-co-glycolic acid)
PMP - Protein metallo phosphatases buffer
PSEN1 - Presenilin-1
PSP - Progressive supranuclear palsy
qPCR – Quantitative polymerase chain reaction
rpm – Revolutions per minute
RNA - Ribonucleic acid
RT – Room temperature
RT-PCR – Reverse transcription polymerase chain reaction
SB - SB431542
SSIV – Superscript IV
TBR1 - T-box brain transcription factor 1
V - Volts
VGULT - Glutamate vesicular transporter
WT – Wild type
YFP – Yellow fluorescent protein
1 Introduction

1.1 MAPT expression and splicing

The microtubule-associated protein tau protein was first discovered in 1975 by Weingarten et al (Weingarten et al., 1975). Tau is encoded by the MAPT gene located on the long arm (q) of chromosome 17, position 21.31 (Neve et al., 1986; Hefti et al., 2018). The tau gene consists of 16 exons, with exon 1 encoding the 5’ untranslated region and the final exon, exon 14, encoding the 3’ untranslated region (Goedert, Spillantini, Jakes, et al., 1989; Guo, Noble and Hanger, 2017). Exons 4a and exon 6 are only expressed in the peripheral nervous system and the spinal cord and skeletal muscle respectively (Caillet-Boudin et al., 2015; Guo, Noble and Hanger, 2017). Alternative splicing of MAPT generates six protein isoforms of tau that are expressed within adult human brain, ranging from 352 to 441 amino acids in length (Figure 1) (Goedert, Spillantini, Jakes, et al., 1989). Exclusion or inclusion of exon 2, or exons 2 and 3 leads to the presence of either 0, 1, or 2 inserts at the N terminus of the protein. Exon 3 is never included independently of exon 2 (Goedert, Spillantini, Potier, et al., 1989). Additionally, alternative splicing of exon 10 leads to the presence of either 3 (3R) or 4 (4R) microtubule binding domain repeats (MTBR) at the C-terminus. Due to exon 3 never being included independently of exon 2, six tau isoforms are present in the human adult brain: 0N3R, 0N4R, 1N3R, 1N4R, 2N3R and 2N4R (Figure 1) (Goedert, Spillantini, Jakes, et al., 1989). Between the C and N terminal domains of MAPT is the proline-rich region, which enhances binding to microtubules (MT) and microtubule construction, with a C-terminus tail at the end of the molecule (Kadavath et al., 2015).
Figure 1. Alternative splicing of MAPT produces six tau isoforms.

The MAPT gene is alternatively spliced to generate six tau isoforms. The inclusion or exclusion of exons 2 (1N in blue) or 3 (2N in orange) leads to the generation of 0N, 1N, and 2N tau. The inclusion or exclusion of exon 10 (R2 in yellow) leads to 3R or 4R tau, referring to the number of microtubule binding repeats. Tau kDa varies from 441 kDa to 325 kDa.

In the healthy adult human central nervous system (CNS), the levels of 0N, 1N, and 2N tau are present as around ~36.8%, ~53.7%, and ~9.5%, of total tau respectively (Hong et al., 1998; Liu and Gong, 2008). The levels of 3R and 4R tau isoforms are expressed in an equimolar ratio (Hong et al., 1998; Wang and Liu, 2008).

MAPT expression within neurons is high, with tau protein being primarily located within the axon (Trojanowski et al., 1989; Lee, Goedert and Trojanowski, 2001). Astrocytes have shown low or undetectable levels of tau, with upregulation and hyperphosphorylation during disease (Kovacs, 2020). Few studies examine MAPT expression within astrocytes, however groups have shown evidence of tau staining within normal brain and astrocytic tumour cells, complimented by mRNA expression within the same astrocytic tumours (Shin et al., 1991; Miyazono et al., 1993). Similar studies need to be repeated but with current methodology. MAPT splicing is heavily developmentally regulated, with only the smallest isoform (0N3R) expressed during fetal brain development (Goedert and Jakes, 1990; Wang and Mandelkow, 2015). The reasons for this developmental regulation are not fully understood, although the current consensus revolves around 0N3R tau having a lower affinity for
microtubule binding thus allowing enhanced cytoskeletal plasticity, something required for guiding and growing immature neurons during development (Jovanov-Milošević et al., 2012; Qiang et al., 2018).

This developmental regulation is also observed in other species, although with species specific differences in which isoforms are expressed in adult brain tissue (Takuma, Arawaka and Mori, 2003). Mice and rats express only 3R tau during development, with a switch to only 4R tau isoforms expressed postnatally (0N4R, 1N4R, 2N4R). Decreased levels of 3R tau and increased levels of 4R tau were seen at post-natal day 5 (P5) with the switch over almost complete by P18 (Tuerde et al., 2018). Recent examination of MAPT expression in marmosets was expected to reflect human tau isoforms and ratios, however these nonhuman primates were shown to express tau in a similar manner to mice (Sharma et al., 2019).

Holzer et al, 2004, examined primate MAPT splicing, again hypothesising it would be similar to human MAPT splicing and expression. Interestingly, they report splicing and expression within gorilla, gibbon, and chimpanzee to be very similar to humans, specifically expression of six MAPT isoforms (Holzer et al., 2004). However, for obvious practical, ethical, and monetary reasons, primates are not a model that can be widely used in tauopathy research. Interestingly, chickens have been shown to have 5 tau isoforms expressed in their adult brain tissue with two of these isoforms containing 5 C-terminus MTBD repeats (Yoshida and Goedert, 2002).

Correct tau splicing appears to be critical for neuronal health, as disrupted tau splicing is observed in a number of tauopathies, discussed in more detail in section 1.4 (Hutton et al., 1998). Tau is predominantly expressed within the CNS, but has also been detected at low levels in other tissue including the liver (Dugger et al., 2016). Interestingly regional differences in tau expression have been documented in the brain, including lower levels of 0N3R within the cerebellum and increased 4R tau isoform expression in the globus pallidus (Allal Boutajangout et al., 2004; McMillan et al., 2008). In a similar manner, granule cells in the hippocampus have been shown to express only mRNA for 3R tau isoforms instead of the full complement of six (Goedert, Spillantini, Potier, et al., 1989). This region of the brain is highly resistant to tauopathies and it has been suggested that the expression of 3R tau may account for this (Hof et al., 1994; Trabzuni et al., 2012; Caillet-Boudin et al., 2015).

In addition to the CNS tau isoforms, a larger isoform of MAPT known as big tau has been described, which is a high molecular weight protein at 110 kDa compared to the 45-65 kDa of the other 6 tau isoforms (Fischer and Baas, 2020). This isoform is generated by the inclusion of an additional large exon (E4a) and is expressed primarily by neurons in the peripheral nervous system (PNS), although very low expression is found in the CNS (Boyne et al., 1995). Big tau function is not entirely understood,
however early studies showed low molecular weight tau as well as big tau expressed in developing rat dorsal root ganglia, with big tau being predominant by postnatal day 7 (Oblinger et al., 1991). Suggesting the need for both tau as DRG axons grown, and big tau for the maintenance of these exceptionally large axons. Despite not being intensely studied since its discovery 25 years ago, there is a resurgence in interest in this large and mysterious tau isoform (Goedert, Spillantini and Crowther, 1992; Fischer and Baas, 2020).

1.2 Tau Protein

The tau protein can be divided into four domains each with different functions.

The most well-characterised protein function of tau is that of a microtubule-stabilising protein, mainly utilising the microtubule binding repeats (MTBR) encoded by exons 9, 10, 11, and 12 at the C-terminus of the protein (Lee, Neve and Kosik, 1989). Microtubules are essential for many functions including cell mobility, transport and division, and are composed of repeating α/β-tubulin heterodimers (Nogales, 2000). Other work has indicated that tau is important in many additional processes including cell signalling, synaptic plasticity, and regulation of genomic stability (Guo, Noble and Hanger, 2017). Furthermore, recent findings have challenged the primary role of tau as a microtubule stabilising protein and instead has suggested that tau allows microtubules to have long flexible domains, desirable in neurons and synapses (Qiang et al., 2018). It is hypothesised that these labile domains would allow flexible assembly of microtubule structures, specifically during important axonal growth and development.

The N-terminal domain is acidic and contains the alternatively spliced N inserts encoded by exon 2 and 3. In the largest 2N4R isoform, this N-terminal domain runs from 1 to 150 amino acids (Fichou et al., 2019). The function of the N-terminal repeats is not fully understood although several studies have investigated potential roles for this domain. The formation of MT bundles has been shown to be influenced by N-terminal repeats, which is potentially important during development of long and more permanent axons and dendrites, which are also the subcellular compartments of neurons where tau is most prominent (Rosenberg et al., 2008). This could also explain why fetal tau expression during development is lacking exon 2 and 3 which encode N-terminal domains, with plasticity needed in young developing and growing neurons. Another function of the N-terminal repeats involved spacing between microtubules within axons and dendrites, again another important function within long more permanent axons (Chen et al., 1992).
The N-terminal region may also be important for the regulation of tau localisation; deletion of the N-terminus, the first 150 residues, causes extreme localisation of this truncated tau to the nucleus (Paholikova et al., 2015). This supports a role for the N-terminus in regulating tau localisation to the axons and dendrites. Recent work using co-immunoprecipitation has shown N-terminus inserts to be involved with cell-to-cell signalling and interaction proteins (Liu et al., 2016). For example β-synuclein was shown to bind preferentially to 0N tau, while apoA1 bound to 2N tau over 5 times more readily than other tau isoforms. On reverse immunoprecipitation utilising apoA1, Liu et al was only able to detect 2N tau isoforms. This suggests different N-terminus isoforms to have specific roles that utilise specific protein partners.

The extreme N-terminus has also been linked to microtubule transport. Work by Kanaan et al, (2011), has highlighted residues 2-18, which are outside alternatively spliced regions and present in all tau isoforms, as important within the kinesin-based fast axonal transport (FAT) pathway in squid giant axons (Kanaan et al., 2011).

Tau in solution has been shown to natively fold with itself, with the N-terminus folding back on itself to interact with the C-terminus. This structure, coined the ‘hairpin’ or sometimes ‘paperclip’ structure, is achieved by the C-terminus folding over the MTBD, and the N-terminal repeats folding back over the C-terminus (Figure 2) (Jeganathan et al., 2006). Figure 2 also shows how the N-terminus projects away from the microtubules when bound, facilitating the spacing of microtubules as mentioned before.
Figure 2. Structure of tau in relation to microtubules and cytoplasm.

Microtuble (in green) showing both bound and unbound tau. Tau structure when bound to microtubules exhibits a more open protein structure, exposing the MTBR. The hairpin tau structure, where the C and N-terminus of the protein fold over one another when free within the cytoplasm. Diagram adapted from Guo et al, 2017.

Amino acids 151 to 243 of tau contain the proline rich domain, containing seven Pro-X-X-Pro (PXXP) motifs. This region shows homology with recognition sites for the SRC family of protein kinases, a potential important interaction for a highly phosphorylated protein (Morris et al., 2011). Similarly to other regions of tau, the proline-rich domain has been implicated in the regulation of microtubule assembly, but also actin binding (Eidenmüller et al., 2001; He et al., 2009). These interactions indicate functions in unison with the C-terminus as well as functions for actin mediated cytoskeletal maintenance. This region has also been implicated in facilitating both RNA and actin binding (Wang et al., 2006; He et al., 2009).

The third domain of tau at the C-terminus of the protein is the microtubule binding region. This contains either three or four microtubule binding repeats (MTBR), depending on alternate splicing of exon ten, as described earlier. These imperfect repeats enable the binding of tau to microtubules, each separated by small flanking regions (Trinczek et al., 1995). 4R tau has been shown to associate with microtubules more strongly, as well as assemble microtubules up to 3 times faster than 3R tau (Goedert and Jakes, 1990). Although both isoforms stimulate microtubule polymerization to similar levels, 4R tau stabilises microtubules significantly more strongly than 3R tau (Panda et al., 2003; Bunker et al., 2004). The second and third of the MTBR show a tendency to form β-sheet structures (Mukrasch et al., 2009).
Additional proteins that have been shown to interact with the MTBD include, but are not limited to, F-actin, α-synuclein, histone deacetylase 6, apolipoprotein E, and presenilin 1 (Guo, Noble and Hanger, 2017). Actin is able to bind to a minimum of two MTBR, enabling it to link actin and microtubules, providing stability between actin and microtubule cytoskeletons (Elie et al., 2015). This relationship between tau MTBR, actin, and microtubules may be involved in the upkeep of the cytoskeleton, maintenance of healthy synapses and therefore critical during dynamic neuronal development stages. These processes have also been shown to be interrupted by tau phosphorylation (section 1.3), potentially contributing to the tau and microtubule dysregulation observed in the tauopathies (Fulga et al., 2007; Gallo, 2007).

The final domain, between amino acids 370 and 441, is the C-terminal tail of the tau protein. The definitive functions of this domain along with any proteins it is associated with are not well established. As mentioned earlier, the final C-terminal tail is most likely utilised within structural changes, especially the cytoplasmic ‘hairpin’ structure (Jeganathan et al., 2006).

The C-terminal tail domain can form filamentous structures, however this was with recombinant protein in vitro rather than in cells (Yanagawa et al., 1998). Another study showed that truncations as small as 12 and as large as 121 amino acids from the C terminal tail of tau greatly increased the rate of recombinant tau polymerization. Interestingly additional deletion of a key region of 7 amino acids between 314 and 320 (within the 3rd MTBD) resulted in tau losing its ability to polymerise in vitro (Abraha et al., 2000).

Several tau KO mice have been generated which show no or mild phenotypes (Harada et al., 1994; Dawson et al., 2001). This suggests functional redundancy between tau and other MT binding proteins if knocked out in embryonic stages. Some mild behavioural changes have been reported, but interesting most relate to muscle weakness rather than neurodegeneration (Ikegami, Harada and Hirokawa, 2000; Ke et al., 2012). At the time of writing this thesis, no conditional tau KO exists, which could provide insight into the consequences of loss of tau in the absence of compensation from other MT binding proteins.

An intriguing finding by Violet et al, 2014, suggested that tau may play a role in DNA and RNA protection (Violet et al., 2014). WT and tau KO mice were exposed to heat stress, with significantly more DNA breaks reported within the tau KO mice. Similar findings of tau being involved within stress response pathways and DNA protection have been reported (Maina et al., 2018). These findings of DNA protection within the nucleus are curious as tau is usually localised within the axons, dendrites and to a lesser extent in the cytoplasm, however reports have confirmed some nucleus localisation.
In vitro and in vivo models of tau function and dysfunction are discussed further in section 1.8.

1.3 Post-translational modifications to tau

In addition to alternative splicing, tau is also subject to extensive post-translational modification (PTM). Tau can be modified by numerous modifications, including but not limited to, phosphorylation, acetylation, glycosylation, glycation, prolyl-isomerization, nitration, polyamination, ubiquitination, and sumoylation, with cleavage or truncation adding to this PTM diversity (Gong et al., 2005).

The importance of tau acetylation has become more apparent thanks to recent research. Early work suggested tau binding was increased and decreased via acetylation, as well as its aggregation being promoted by this specific PTM (Cohen et al., 2011). Impressive work from the same group showed that tau has intrinsic acetyltransferase activity enabled by two cysteines within MTBD R2 and R3 (C291 and C322), which regulate acetylation at K280 (Cohen et al., 2013). Further work was able to show 4R tau, due to MTBR R2 being present in this isoform, displaying higher levels of auto-acetylation than 3R tau (Cohen et al., 2016). Recent unpublished work has demonstrated the potential of immunotherapy for K280 acetylated tau, which improved behavioural phenotypes in addition to reducing pathology within tau transgenic mice (Kim et al., 2020).

High levels of tau glycosylation of tau have been observed in tau from Alzheimer’s disease (Wang, Grundke-Iqbal and Iqbal, 1996). Enzymatic deglycosylation of tau extracted from AD brain does not restore tau function, nor disassociate the fibrils, however remarkably it “untwists” the paired helical filament tau into bundles of straight filaments, suggesting this particular tau PTM may have an important influence on the structure of filamentous tau.

Tau phosphorylation is the most extensively studied modification to tau, and phosphophorylation of tau is important to regulate its function as well as being implicated in disease (Trushina et al., 2019). 2N4R tau, the largest tau isoform, has 80 serine or threonine residues and 5 tyrosine residues, allowing 85 possible phosphorylation sites. This means almost 20% of the largest tau molecule has the potential to be phosphorylated (Goedert, Spillantini, Jakes, et al., 1989). Tau phosphorylation favours its detachment from microtubules, with early tau research showing that non-phosphorylated tau promoted significantly more rapid and more extensive polymerization of microtubules in vitro (Lindwall and Cole, 1984). Other phospho-sites have been shown to regulate the localisation of tau. Kobayashi et al showed AT8 positive tau (a phospho-specific antibody which recognises tau...
phosphorylated at S205 and T205) accumulation within dendritic spines, with increased accumulation on application of glutamate (Mandell and Banker, 1996; Kobayashi et al., 2017).

Tau phosphorylation is developmentally regulated, with higher levels of tau phosphorylation within developing brain tissue than in adult control tissue (Yu et al., 2009). In WT mice, it was found that the switch from highly phosphorylated 0N3R tau to less phosphorylated adult tau isoforms occurs simultaneously (Tuerde et al., 2018).

Multiple kinases that are able to phosphorylate tau have been identified. Glycogen synthase kinase-3 (GSK3), or more specifically isoform GSKβ, has been implicated in a myriad of functions including but not limited to neuronal function, embryonic development, and apoptosis, but also tau phosphorylation (Woodgett, 1990; Hooper, Killick and Lovestone, 2008). GSKβ also exists as two isoforms, β1 and β2, with the former being located primarily in the neuronal soma and with latter found in both the neuronal soma and axons (Mukai et al., 2002).

Tau can be phosphorylated at 42 sites by GSK3β kinases using recombinant proteins in in vitro phosphorylation assays, and 29 of these sites have also been found to be phosphorylated in AD brains (Morishima-Kawashima et al., 1995; Yoshida and Goedert, 2006; Hanger, Anderton and Noble, 2009). Overexpression of GSK3β in mice induces tau hyperphosphorylation and reported neurodegeneration and neuronal death (Hernández et al., 2002). However interestingly no neurofibrillary tangles (NFTs) were reported within this overexpression model. GSK3β has also linked Aβ to tau pathology and hyperphosphorylated tau. Inhibition of GSK3β via lithium treatment has been shown to reduce tauopathy and degeneration in murine models over expressing mutated human MAPT (Noble et al., 2005).

Other studies utilising rat primary cultures showed exposure to Aβ increased specifically GSKβ activity, tau phosphorylation, as well as increasing apoptotic neuronal death (Takashima et al., 1996)

Cyclin-dependent-like kinase 5 (CDK5) is another important kinase, involved in neuronal development, CNS development, and regulation of the cytoskeleton (Hallows et al., 2003). Initially CDK5 requires calpain to cleave p35 into p25 in order to become active. This CDK5/p25 complex is able to increase tau phosphorylation and even promote neurodegeneration in primary cultures (Patrick et al., 1999). In vitro, CDK5 is able to phosphorylate tau at 11 residues, all of which are shown to be present in AD patient brain (Patrick et al., 1999). P301L mice over expressing the CDK5 activator P25 have shown NFT formation, with extended activation within mice of 27 weeks also showing NFT formation (Cruz et al., 2003; Noble et al., 2003). Remarkably, silencing of CDK5 within these mice reduced NFT formation, showing the important role of kinases within the puzzle of NDD (Piedrahita et al., 2010).
Although tau phosphorylation is essential for the physiological function of tau, tau hyperphosphorylation is observed in disease and may contribute to tau aggregation and toxicity [26]. Interestingly, some frequently phosphorylated sites are the same in both developmental phosphorylation, tau phosphorylation in control biopsies and disease related hyperphosphorylation (Brion et al., 1993). Many sites of tau phosphorylation and candidate kinases have been directly identified by mass spectrometry (Hanger et al., 2007; Barthélemy et al., 2019; Dujardin et al., 2020) and are comprehensively detailed here: (Diane Hanger, 2020)

Recent high resolution examination of PTMs in AD patient tau identified a progressive modification of tau which reflected disease progression, potentially highlighting a way to slow the disease (Wesseling et al., 2020). Classic AD related epitopes such as AT8 (S202) and AT180 (T231) correlated significantly with AD progression. However, lesser known epitopes such as ubiquitination at K311 and K317, and acetylation at K369 were also reported in high frequencies within AD samples.

This is corroborated with work by Arakhamia et al, (2020), highlighting that tau filament structure is impacted by PTMs, showing tau fibril diversity can be mediated by ubiquitination (Arakhamia et al., 2020).

### 1.4 Tau in neurodegenerative disease

Hyperphosphorylated, insoluble aggregates composed of the microtubule associated protein tau are a defining pathological feature of multiple neurodegenerative diseases (NDD), commonly referred to as the tauopathies.

Tauopathies are classed as either a primary or secondary. Primary tauopathies are defined as such because the presence of tau pathology is the main protein aggregation present within patients brains at post-mortem and therefore the defining feature of the disease (Iqbal et al., 2010; Domise and Vingtdeux, 2016). The primary tauopathies include progressive supranuclear palsy (PSP), Pick’s disease (PiD), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), frontotemporal lobar degeneration with tau pathology (FTLD-tau), and globular glial tauopathy (GGT) (Ferrer et al., 2014).

Alzheimer’s Disease (AD) is the most common tauopathy, where tau pathology in the form of neurofibrillary tangles (NFTs) exists alongside extracellular plaques composed of amyloid beta (Aβ) (Irwin, 2016). Although tau pathology is closely correlated with disease severity, the identification of mutations linked to Aβ processing that are causative of familial AD suggest tau is downstream of Aβ, and is therefore a secondary tauopathy (Hardy and Higgins, 1992; Goate and Hardy, 2012).
Each of the tauopathies are associated with the deposition of specific tau isoforms. For example, tau pathology in AD is formed of paired helical filaments assembled into NFTs, which are comprised of all tau isoforms (Maccioni, Muñoz and Barbeito, 2001). In contrast, PSP, CBD and GGT are characterised by the deposition of predominantly 4R tau isoforms in straight filaments (SFs) which are found in both neurons and glia (Dickson, 1999; Shiarli et al., 2006; Williams et al., 2007). PiD exhibits only 3R tau, within spherical Pick body inclusions rather than tangle structures (Rasool and Selkoe, 1985; Delacourte et al., 1998; Falcon, Zhang, Murzin, et al., 2018). Finally, the recently described primary age-related tauopathy (PART) is characterised by NFTs similar to those in AD, but very limited amyloid pathology (Crary, 2016). FTLD-tau is caused by mutations in the MAPT gene, and the pathology depends on the specific mutation. The role of tau pathology in AD and FTLD-tau caused by MAPT mutations are discussed in more detail below (section 1.6).

The disruption of the 3R/4R tau ratio has also been shown to cause NDD. Mutations that affect splicing of MAPT were reported over 20 years ago, generally causing a 3-5 fold excess of 4R tau to be expressed, (Hutton et al., 1998), something which is also observed in the sporadic tauopathies PSP and CBD. This shows that proper splicing of tau is crucial within the adult human brain, with alterations in the 3R/4R ratio of tau causing NDD. Splicing mutations in MAPT are explained in more detail in section 1.6.

1.5 **Tau pathology in Alzheimer’s disease**

Alzheimer’s disease (AD) is the most common cause of dementia affecting 50 million people globally. This is expected to reach 152 million by 2050, and therefore represents a significant public healthcare issue due to the absence of disease modifying therapeutics (Prince et al., 2016). Stats from Alzheimer’s Research UK (Prince et al., 2016; AR UK, 2018).

AD is a NDD which is primarily characterised by atrophy of the brain through neuronal death, specifically the neurons within the hippocampus and cerebral neocortex (Fjell et al., 2014). This leads to symptoms such as confusion, memory impairment, cognitive impairment, inability to learn new things, and individuals eventually requiring full time care (Neugroschl and Wang, 2011).

The two major pathological hallmarks of AD are extracellular amyloid beta (Aβ) plaques and intracellular NFTs Figure 3 (Deture and Dickson, 2019). This pathology is accompanied by widespread neuroinflammation and recruitment of microglia around Aβ plaques (Heneka et al., 2015).
Figure 3. Histopathology of Alzheimer’s disease.
A) Amyloid Beta plaque (black arrow) within the cortex visualised via anti-amyloid antibodies and IHC.
B) Antibodies against tau (black arrow) label PHF-containing neurites. Figure created from images under a creative commons license.

Development of AD pathology is complex and the mechanisms underlying the disease are still not fully understood. Research suggests involvement of irregular amyloid-β (Aβ) metabolism/production (O’Brien and Wong, 2011), tau hyper phosphorylation, reactive oxygen species production (Oksanen et al., 2017) as well as astrocytic and microglial activation (Stelzmann, Schnitzlein and Murtagh, 1995; Graeber et al., 1997; Park et al., 2018) all contribute to neurodegeneration. Although the majority of cases of AD are sporadic, rare cases of familial Alzheimer’s disease (fAD) have enhanced our understanding of AD disease mechanisms. Mutations in amyloid precursor protein (APP), and presenilin 1 and 2 (PSEN1, PSEN2) are sufficient to cause AD (Kang et al., 1987; Levy-Lahad et al., 1995; Sherrington et al., 1995; Bekris et al., 2010). Successive proteolytic cleavage of APP leads to the production of Aβ, and presenilin 1 and 2 form the catalytic core of γ-secretase, which is responsible for the final cleavage step in this process (O’Brien and Wong, 2011; De Strooper, Iwatsubo and Wolfe, 2012). These genetic findings led to the development of the Amyloid Cascade Hypothesis (ACH), first described by Hardy and Higgins (1992), which postulates that the aberrant Aβ production and the development of amyloid plaques is upstream of the development of tau pathology (Hardy and Higgins, 1992). Thus, tau is a secondary tauopathy as the tangles lie downstream of Aβ.

However, despite the fact AD is classed as a secondary tauopathy, there is substantial evidence that tau is a driver of neurodegeneration in AD. Cognitively-normal, aged patients can exhibit amyloid pathology in the absence of neurodegeneration, with one study showing Aβ load increasing between age 50 to 90 years from 10% to 44% (Author et al., 2015). In contrast, the extent of tau pathology in AD correlates strongly with symptom severity and neuronal death (Braak et al., 2006; Furman et al.,
Early stages of Alzheimer’s disease have shown tau oligomer accumulation before the formation of tau tangles and neuronal death (Lasagna-Reeves et al., 2012). With growing interest in targeting early oligomers for prevention of NFT formation and NDD (A. Lasagna-Reeves et al., 2011).

Figure 4. The Amyloid Cascade Hypothesis

Diagram showing the amyloid cascade hypothesis theorised by John Hardy. Membrane bound APP processing by β and γ secretase enzymes, leading to the release of Aβ peptides, which include amyloidogenic species such as A642. Pathogenic monomeric Aβ species then undergo conformational changes, assembling into oligomers (2-100 units) and then protofibrils (>100 kDa), which are assumed neurotoxic, and deposit as plaques outside of the cell. Original figure created via SMART Servier Medical Art, (LesLaboratories Servier SAS, 2020).

The tight correlation between tau pathology and extent of AD was demonstrated further by the development of Braak staging of AD progression in 1991 (Braak and Braak, 1991). Braak used methodical tau and NFT staining utilising both Gallyas silver staining and the phosphotau antibody AT8, to correlate neuropathology with symptom severity (Braak et al., 1988, 2006; Braak and Braak, 1991).
Figure 5. Braak staging of tau pathology within Alzheimer’s disease.

Representative image of tau pathology with diagrams showing progression of hyper-phosphorylated tau tangles within the human brain. Stages I and II show early NFT presence within the transentorhinal region, stages III and IV the pathology spreads to the limbic regions and hippocampus. Stage V and VI involves broad cortical spread of pathology. Figure adapted from Marie Jouanne et al (Jouanne, Rault and Voisin-Chiret, 2017)

The different Braak stages are summarised in Figure 5. The entorhinal cortex (EC) of the brain is the first area that NFTs accumulate within, corresponding to Braak’s stages I and II. At this early stage patients are largely pre-symptomatic although may present with mild cognitive impairment (MCI). NFT pathology then spreads to the limbic areas and hippocampus, corresponding to Braak stages III and IV, at which stage cognitive impairment is noticeable with memory issues also becoming more apparent. Finally spreading to the neocortical region corresponding to Braak stages V and VI, in which severe cognitive and memory deficits are severe, eventually leading to loss of self and eventually full time care (Braak and Braak, 1991; Jouanne, Rault and Voisin-Chiret, 2017). Within damaged regions of AD patient brains, like the hippocampus, there is an inverse relationship between the number of extracellular tangles and the number of surviving cells (Gómez-Ramos et al., 2006).

Due to the spread of tau correlating with AD progression, tau has been hypothesised as the neurotoxic element in AD and other NDD (Sebastián-Serrano, Diego-García and Díaz-Hernández, 2018). Further evidence for a causal role in AD comes from experimental models, where tau KO is protective against amyloid toxicity (Ittner et al., 2010; K. Vossel et al., 2010). Studies using tau knockout mice crossed with human APP models of amyloid have demonstrated that tau is necessary for amyloid toxicity, and tau reduction is protective. However, tau reduction did not change Aβ plaque deposition (Rapoport et al., 2002; Roberson et al., 2007; K. A. Vossel et al., 2010). With this complex interplay between Aβ and tau, more groups are suggesting the combination of Aβ and tau may be required to cause neuronal dysfunction and eventually death (Mastroberardino et al., 2015). The high profile failure of recent clinical trials targeting amyloid has supported the idea that therapies which target both amyloid and
tau may be necessary for the successful treatment of AD (Panza et al., 2019). Although targeting these proteins once neurodegeneration has begun may be too late, requiring pre-emptive treatment.

Figure 6. The cryo-EM structure of tau filaments from AD brain
A) Diagram of the largest isoform of tau showing the regions involved with the core and the fuzzy coat of AD filaments. B) Diagram of AD filaments stacked upon one another, colour coded to show areas of tau involved with each region. C) Cross section of a Paired helical filament. D) Cross section of a straight filament. Figure adapted from Fitzpatrick et al, 2017.

Recent work has allowed visualisation of the structure of AD NFTs at high resolution. Fitzpatrick et al, (2017), conducted cryo-electron microscopy (cyro-EM) on tau fibrils retrieved from an AD patient’s brain tissue, revealing that the filament cores of both PHF and straight filaments (SF) consist of two identical proto-filaments consisting of residues 306 to 378 of tau (Fitzpatrick et al., 2017). This core encompasses most of MTBR 3, all of MTBR 4, and some of the C-terminal tail of tau (Figure 6). The rest of the tau molecule, including all N terminal inserts (N1 and N2) comprise the ‘fuzzy coat’ around the filament. These cores create a combined cross β/β-helix structure and polymerise / stack on top of one another (Figure 6.C & D), creating insoluble PHF or SF (Falcon, Zhang, Schweighauser, et al., 2018).

Since this initial breakthrough, the structures of tau filaments from multiple tauopathies have been solved. The deposition of specific tau isoforms also results in different filamentous structures across the tauopathies (Lee and Leugers, 2012). Cryo EM structures have now been resolved for tau filaments
in PiD, CBD and CTE (Irwin et al., 2016). In PiD, tau residues 254 to 378 form the core of the filament, with the remaining domains creating the fuzzy coat (Falcon, Zhang, Murzin, et al., 2018) (Figure 7.C).

Due to PiD tau folds utilising additional tau protein residues compared to the AD tau folds, this demonstrates different conformers being generated depending on the regions involved within filaments core. The residues involved with PiD and the structure of the fold allow only 3R tau to be utilised in the growing filaments.

A number of studies from Falcon and other groups characterised both the CTE and CBD tau folds, both of which again had distinct morphologies (Figure 7. B & D) (Falcon et al., 2019; Zhang et al., 2020). CTE is a tauopathy that is usually associated with repeated blows to the head (Inserra and DeVriese, 2020), although some recent evidence has pointed to variation in TMEM106B potentially altering CTE severity (Cherry et al., 2018). Interestingly CTE tau folds, a condition not yet associated with a definitive causal mutation and more associated with environmental factors, show unique morphology, at least when compared to AD, PiD and CBD folds (Scheres et al., 2020).
Figure 7. Cryo-EM structures of tau filaments from multiple tauopathies.
A) AD tau fold, the structure allows incorporation of both 3R and 4R tau. B) Chronic traumatic encephalopathy (CTE) tau fold structure, also allows both 3 and 4R tau to polymerise. C) PiD tau fold structure, only allows 3R tau to polymerise to form filaments. D) CBD tau fold structure, only allows 4R tau to polymerise to form filaments. Figure from Scheres et al., 2020.

Many groups have used recombinant tau, aggregated in the presence of heparin, for in vitro experiments to determine mechanisms of tau toxicity (Avila, 2010). Interestingly, work from Zhang et al., 2019, showed that heparin induced tau filaments differed from other tauopathy filaments. These in vitro filaments had larger cores with different repeat regions, potentially pointing to different cellular mechanisms influencing the creation of different tau structures (Zhang et al., 2019). Thus, it is important to interpret experimental findings using recombinant tau with caution, given the structural differences when compared to filaments derived from tauopathy brain.
1.6 Tau in FTD

Although AD is the most common (secondary) tauopathy, interestingly genetic mutations in MAPT do not lead to early onset Alzheimer’s disease (EOAD) or Familial Alzheimer’s disease (FAD), but instead cause autosomal dominant FTLD with tau pathology, in the absence of Aβ plaques (Hutton et al., 1998; Poorkaj et al., 1998). The discovery of MAPT mutations confirmed a causative link between tau dysfunction and neurodegeneration.

![Diagram showing common mutations grouped around the microtubule binding repeat domains.](image)

**Figure 8.** FTD linked mutations in MAPT

A) **Diagram showing common mutations grouped around the microtubule binding repeat domains.**

*Mutations here are protein coding mutations. Few mutations have been discovered that affect the N-terminus of the protein.*

B) **10+x mutations in the splicing stem loop structure, causing disruption of developmental regulation of MAPT isoforms. Mutations here are splicing mutations.**

The majority of known MAPT mutations occur within the C terminus, and can either alter the amino acid sequence of tau or the splicing of the tau gene (Hutton et al., 1998; Makrides et al., 2003; Ghetti et al., 2015). Coding region mutations fall mostly in the microtubule binding repeat domains, and can affect both the microtubule binding capacity of tau, as well as increase its propensity to polymerise, forming insoluble aggregates (Dayanandan et al., 1999). The pathologies associated with MAPT
mutations are mutation specific and dependent on which isoforms are impacted by a specific mutation, some examples of this are given below.

Two of the most well studied tau mutations are the P301L and P301S MAPT mutations, located within exon 10 and therefore only affecting 4R tau isoforms. Discovered in 1999, these mutations are clinically aggressive, leading to MCI and memory loss as well as personality related symptoms such as disinhibition and inappropriate behaviour, all before 40 years of age in patients (Bugiani et al., 1999; Lossos et al., 2003). Pathologically, patients display significant frontotemporal atrophy, with widespread inclusions of hyperphosphorylated tau in neurons and glia (Bugiani et al., 1999). The clinically aggressive nature of these mutations means they have formed the basis of many in vitro and in vivo models of tauopathies, which will be discussed further in section 1.8.

Very few mutations have been discovered in the N-terminal region of MAPT. One such mutation, the R5L mutation, was discovered in 2002 and is thought to interrupt the paperclip structure of soluble tau (Poorkaj et al., 2002). This mutation has been shown to not affect cognitive ability, although movement was severely impacted, starting with stiffness in neck and leg muscles, ultimately leading to the patient being bedbound (Poorkaj et al., 2002). Interestingly this mutation lead to a more PSP-like pathology with predominantly 0N4R and 1N4R aggregated tau in subcortical areas, although cortical regions were shown to have 1N3R tau aggregates. This mutation has a later age of onset than C-terminal MAPT mutations, of around 60 years of age (Poorkaj et al., 2002; Chang et al., 2008).

Intronic MAPT mutations are mainly grouped in the 5′-splice site of the intron following exon 10. These mutations disrupt tau splicing, generally favouring the inclusion of exon 10 which causes an imbalance in the 3R/4R tau ratio in the brain (Grover et al., 1999). The most common MAPT mutation in the UK is the 10+16 mutation, leading to frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (Hutton et al., 1998). This mutation was thought to arise in North Wales and spread through the UK, particular the north west, during the industrial revolution (Pickering-Brown et al., 2004). This is a non-coding intronic point mutation located 16 bases after exon 10 which causes a codon change from a cytosine to a thymine (C>T). This non-coding mutation results in the production of an 2 - 6 fold increase of 4R tau isoform mRNA, which is believed to drive the FTD phenotype and neuronal degeneration observed in patents with this mutation (Hutton et al., 1998; Grover et al., 1999; Connell et al., 2005). The stem-loop structure during MAPT expression is important in regulating alternate splicing, however the 10+16 mutation destabilises this stem-loop structure, causing exon 10 to be included in the transcript more frequently (Hutton et al., 1998). This mutation can result in a variety of clinical phenotypes, with patients exhibiting clinical features of PSP and AD (Morris et al., 2003; Doran et al., 2007). PSP patients more exhibit movement based phenotypes
including gait abnormalities with postural instability, although cognitive impairment is not unusual (Litvan et al., 1996). Pathological hallmarks include neuronal loss, ballooned neurons in the cortex and tau aggregate positive neurons. Tau aggregates form NFTs that are categorised as straight type filaments and also primarily composed of only 4R tau (Goedert et al., 1999; Pickering-Brown et al., 2002).

MAPT mutations have also been shown to result in different atrophy patterns. Splicing mutations (specifically 10+16, 10+3, N279K, and S305N) show more grey matter loss in the anterior temporal lobes, with varying atrophy within frontal and parietal lobes. In contrast, coding mutations (P301L or V337M) showed increased grey matter loss in the lateral temporal lobes (Whitwell et al., 2009).

Tau is central to the pathogenesis of a range of clinically and pathologically diverse NDDs. Understanding the molecular mechanisms linking tau dysfunction to neuronal death and developing in vitro and in vivo models to recreate key features of tau dysfunction is an important area of research.

### 1.7 Sporadic tauopathies and MAPT haplotypes

Along with familial tauopathies where a mutation can be identified to be the cause of the neurodegenerative disease, there are greater volumes of sporadic tauopathies, the largest being AD, where no causal gene can be clearly identified.

Sporadic disease has been linked to MAPT and the two MAPT haplotypes present in some populations. This is especially true within European populations, but interestingly absent from the certain populations, including the Japanese (Conrad et al., 1998).

These two haplotypes, identified as H1 and H2, are due to numerous single nucleotide polymorphisms (SNPs) as well as a 900 kB inversion which includes the MAPT gene (De Silva et al., 2001; Pittman et al., 2004). These haplotypes have been associated with neurodegenerative disease, especially PSP and CBD (De Silva et al., 2001; Pittman et al., 2005). Analysis of PSP patients revealed 94% possessed haplotype H1 when compared to 78% in non-demented controls (Baker et al., 1999). Further to this haplotypes were broken down in to additional sub-haplotypes depending on unique SNPs, with haplotype H1C being over represented within PSP patients when compared to controls (Pittman et al., 2005).

PSP patients were shown to over express 4R tau, especially within highly affected regions such as the brain stem, where a significant increase was detected (Chambers et al., 1999). When pathology free tissue was examined, the H1 chromosomes expressed significantly more 4R tau isoforms than the H2
chromosomes, both in the frontal cortex and the globus pallidus (Caffrey et al., 2006). This was validated and refined via Myers et al. 2007, with up to 25% more 4R transcripts being expressed in the globus pallidus in specifically H1C haplotype carrying patients (Myers et al., 2007).

1.8 Modelling tauopathy in vitro and in vivo

The discovery of causative mutations in MAPT stimulated the generation of in vitro and in vivo models of tauopathy, to enable investigation of mechanisms linking tau dysfunction to neurodegeneration. In the interest of brevity, this thesis will review only human and rodent in vitro models with a particular focus on tau splicing.

1.8.1 In vitro models

In vitro modelling has been hampered by the lack of availability of human neurons. Immortalised cell lines such as SH-SY5Y neuroblastoma cells (SH-SY5Y) have been used extensively, however these cells are not neurons or glia, and do not precisely recapitulate neuronal aspects such as cell morphology or functions (Shipley et al., 2017). Methods to differentiate these cells into more neuronal-like entities are available and have been shown to be useful for large scale testing due to the ease of differentiation and growth of this model (Forster et al., 2016).

Neuroblastoma lines also typically express only a single tau isoform, 0N3R, with 0N4R tau being detectable by PCR, but at extremely low levels (Uberti et al., 1997). Differentiation of these lines to neuronal-like cells did not change the tau isoform expression pattern after 25 DIV (Smith et al., 1995; Uberti et al., 1997). SK-N-SH, a parent cell line of SHSY5Y neuroblastoma cell line, was also shown to primarily express fetal tau (0N3R), with differentiation of this line over 25 days not changing tau isoform expression (Smith et al., 1995). The native mutations these cells carry is also another consideration when using this model, as well as these cells being highly proliferative in nature (Tweddle et al., 2001).

Exogenous expression of tau is therefore present in these cell lines (Bachmann et al., 2020). However, this does not guarantee the whole culture expresses the desired protein, expression is most likely not at physiological levels, and expressing all 6 isoforms using this method is not feasible. Overexpression of tau in stable cell lines has shown MT bundling defects as well as vesicular trafficking defects (Kanai et al., 1989; Ebneth et al., 1998)
Primary cultures of pre- or post-natal rat or mouse brain tissue are also routinely used in neuroscience research. However, as discussed earlier (section 1.11.8.2), there are species specific differences in tau splicing and thus primary rodent neurons fail to recapitulate tau isoform expression seen in the adult human brain. ON3R tau is present during gestation, however after birth tau expression consists solely of three isoforms of 4R tau (Janke et al., 1999). One report suggested that all 6 isoforms are present at the protein level in the adult rat brain, with 4R being dominantly expressed, but 3R tau being present at extremely low levels (Hanes et al., 2009). Ultimately though, the 3R and 4R ratio should be equal to recapitulate human brain tissue. Ratios of N-terminal splicing are shown to be different to the adult human brain, with equal amounts of ON, 1N, and 2N tau being expressed compared to ~36.8%, ~53.7%, and ~9.5% in adult human brain (Hanes et al., 2009; Götz, Bodea and Goedert, 2018; Rodriguez et al., 2020).

Primary human cortical samples have been cultured from 16 – 21 week old fetal brain tissue, after dissociation and plating, with neurons being cultured for up to 40 days (Deshpande, Win and Busciglio, 2008). 2D human cortical neuronal (HCN) cultures were shown to express 4R tau as well as multiple tau protein isoforms, with the 3R to 4R ratio becoming ~1 after 20 days in vitro. This technique is very sparsely used due to ethical implications as well as the difficulty in obtaining such tissue.

1.8.2 In vivo rodent models of tauopathy

The identification of multiple genes linked to fAD as well as MAPT mutations linked to FTD permitted the development of transgenic rodent models. MAPT knockout mice surprisingly exhibit no neurodegeneration or severe phenotypes (Harada et al., 1994; Dawson et al., 2001). This suggests functional redundancy of tau in development with a compensation affect from other microtubule binding proteins. Microtubule associated protein 1A and 1B (MAP1A and MAP1B) play roles in regulating the neuronal cytoskeleton and actin structures, with MAP1B important during neurogenesis and MAP1A being more active in mature neurons. Mice deficient of MAP1B also showing no neurodegeneration or severe phenotypes, suggesting possible compensatory mechanisms for microtubule management. Somewhat affirming this hypothesis, mice deficient in both MAPT and MAP1B show severe neuronal defects and perish within 4 weeks of birth (Takei et al., 2000).

Multiple APP, PSEN1 and PSEN2 transgenic mouse models have been generated which recapitulate amyloid pathology to a degree, and show some features of cognitive impairment, but these mice do not develop tau pathology (Hall and Roberson, 2012; Teich, Patel and Arancio, 2013).
The first mouse model to develop NFT pathology was generated by expressing the FTLD MAPT mutation P301L under the control of the mouse prion promoter (Lewis et al., 2000). In this model NFT and Pick-body-like lesions can be found in the amygdala, hypothalamus, midbrain, medulla, deep cerebellar nuclei but also the spinal cord, with tau-immunoreactive pre-tangles in the cortex, hippocampus and basal ganglia (Lewis et al., 2000). These mice display motor deficits, including holding their legs in crossed positions, hunched postures, clenched paws, and as the disease progresses the hind limbs of the P301L animals became dystonic (Lewis et al., 2000). Mice expressing 1N4R human tau with the FTD associated mutations G272V and P301S were shown to exhibit NFT pathology without any motor defects (Schindowski et al., 2006).

To address the species differences in tau splicing, humanised tau mice have been generated. WT murine tau overexpression in a mouse model does lead to tau pathology, although not until 15 – 18 months of age (Adams et al., 2009). Mice that overexpress express all 6 isoforms of human tau were produced, via P1 artificial chromosome (PAC) and bacterial artificial chromosome (BAC) vectors harbouring the tau cDNA transgene (Duff et al., 2000). Minor pathology within the spinal cord and motor defects was seen within these mice. All 6 isoforms of tau were detectable, however 3R tau was expressed at higher levels than 4R, unlike the 1:1 ratios within human adult brain.

Through this work, the BAC transgene mouse was crossed with an mouse tau knock out line, creating a murine model expressing only human tau, and all 6 isoforms (Andorfer et al., 2003). This model exhibited more extensive pathology within the CNS and hyperphosphorylated tau (Andorfer et al., 2003).

A mouse model of the 10+16 mutation has been developed using expression of a human tau transgene harbouring the 10+16 C>T mutation, which has been shown to express up to 11 times higher levels of 4R tau than WT mice (Roberson, 2012; Umeda et al., 2013). The 10 + 16 C>T model dominantly expresses 4R human tau and exhibits memory impairments at 6 months prior to abnormal tau phosphorylation, synapse loss and dysfunction, glial activation, neuronal loss in an age-dependent manner and importantly, tangle formation (Umeda et al., 2013). NFTs and neuronal loss are observed at 15 months in layer 2 and 3 of the entorhinal cortex (Umeda et al., 2015). However this model was achieved via an established cDNA transgene, meaning no splicing was needed (Yamashita et al., 2005). This lack of splicing also causes 2N4R human tau to be the dominantly expressed isoform, as well as mouse tau to be co-expressed.

As mentioned earlier (1.6), the ratio of tau isoforms is incredibly important within the adult human brain, with alterations in the 3R:4R balance tau causing disease. Excess 4R tau has been shown to lead to increased hyperphosphorylation in murine models (Barron et al., 2020).
For example, the humanised MAPT mice generated by Andorfer et al, 2003, expressed all 6 tau isoforms, however 3R tau was the predominant isoform. This mouse model showed hyperphosphorylated and insoluble tau aggregates (Andorfer et al., 2003). This was efficiently shown to be alleviated by Espíndola et al, 2018, with the treatment of 3R over expressing htau mice with application of a pre-trans-splicing tau mRNA molecule which led to an almost 1:1 ratio of 3R to 4R tau. Mice with tau balance partially restored showed less hyperphosphorylated and insoluble tau as well as cognitive improvements (Espíndola et al., 2018).

Several murine models of tauopathy have been engineered with severe pathology and phenotypes driven by high exogenous expression of tau. The rTg4510 mice attain 13-fold overexpression of mouse/human 0N4R tau with the P301L mutation, leading to early (1.5 – 4 month) neuronal loss, NFT-like lesions, cognitive impairment, and brain atrophy (Atwood et al., 2005; Ramsden et al., 2005). The P301S mutation, under a Thy1 promotor, exhibits brain but also spinal cord hyperphosphorylated tau. Additionally these animals exhibit motor defects, and electron microscopy indicates significant death of motor neurons (Allen et al., 2002). A criticism of this model, however, is the reliance on the overexpression of tau to non-physiological levels. However it is important to bear in mind the limits of over expression as a model. At a certain point any protein that is overexpressed has the potential to become toxic in its own right and contribute to an overall pathology and phenotype (Bolognesi and Lehner, 2018; Belbellaa et al., 2020).

Work from K. SantaCruz et al, (2005), showed that tau induced damage could potentially be reversed through creating an inducible tauopathy mouse model (Santacruz et al., 2005). Human tau harbouring the P301L mutation was expressed which included a tetracycline operon responsive element (TRE), allowing the gene to be suppressed via tetracycline. Suppression after formation of pathology and emergence of behavioural phenotypes resulted in memory improvement as well as neuronal numbers stabilising. Interestingly NFTs continued to form and accumulate despite tau suppression of transgenic tau, suggesting NFTs alone are not sufficient to cause neuronal death or cognitive decline. Hochgräfe et al, 2013, showed a similar result in which tau induced damage could potentially be reversed through creating inducible tauopathy mouse models (Hochgräfe, Sydow and Mandelkow, 2013). Transgenic mice with expression of pro-aggregation tau variants (truncated tau and full length hTau40/ΔK280) exhibited pre-tangle pathology and neuronal loss, with synaptic recovery after expression of tau was switched off. Both of these papers suggest the aggregated forms of tau protein are not the neurotoxic elements, and the oligomeric tau, before aggregation occurs, is potentially the toxic species.

Rodent models are invaluable within neuroscience research, but the translational value of findings from these studies needs to be validated as they lack the specific complexity and architecture present
in the human brain (Semple et al., 2013; Hodge et al., 2019). Furthermore, certain aspects of tau biology that are important for human neuronal health, notably its alternative splicing, are species-specific and experimental models do not recapitulate tau splicing patterns observed in the adult human CNS. For example, adult mice endogenously only express 4R tau isoforms, and although humanised mice express human tau, the protein is not present in the same ratios as adult human brain, either by design or likely owing to splicing by endogenous murine splicing machinery (Andorfer et al., 2003; Yoshiyama et al., 2007). Due to these differences, drug discovery within these murine models does not always recapitulate the same results when applied to human neurons and patients.

New human models are needed to reproduce both the neuronal environment within the human brain, but also pathology related to tauopathies that is currently difficult to replicate using animal systems (Neha et al., 2014).

1.9 Neuronal Development

Utilising stem cells and differentiating them into neurons tries to recapitulate neuronal development. This is due to the differentiation from stem cell to early neural precursor cell (NPC), through early neuronal architecture such as neuronal-tube like structures, to finally post-mitotic neuron (Shi, Kirwan and Livesey, 2012).

Other regions of the brain emerge from different areas of the developing neural tube. For example, the medial ganglionic eminence (MGE) and the caudal ganglionic eminence (CGE) mainly give rise to interneurons, a neuronal subtype that isn’t seen in most iPSC neuronal cultures. Other more hind-brain regions, such as the cerebellum develop from the caudal dorsal region of the neural tube, an area not recapitulated within cerebral iPSC derived neurons (Butts, Green and Wingate, 2014). However, groups have started patterning towards hindbrain, allowing cerebellar organoids to be produced via SDF1 and FGF19 induction (Muguruma et al., 2015; Ballabio et al., 2020). Patterning early neuronal identity further dorsally allows the creation of spinal cord organoids (Hor and Ng, 2020).

Initial steps in neuronal development of the forebrain and cortex involve neuroectoderm formation, one of the germ layers, which leads to a neural tube structure being formed. Development of the ventricular zone (VZ) and the subventricular zone (SVZ) commences at the apical surface. These areas in neuronal development contain radial glia and is the starting point for neuronal migration, allowing cells to differentiate as they migrate away from the SVZ. Within the more complex human development, an outer SVZ (oSVZ) is created, also containing radial glia. Interestingly the SVZ is also important in adult neurogenesis, as well as being implicated in neurodegenerative disease, therefore
retaining this structure in human 3D models could provide to be extremely valuable (Curtis, Faull and Eriksson, 2007).

Radial glia asymmetrically divide, with daughter cells migrating away from the SVZ, and oSVZ, towards the outer edge (basal surface) of the neural tube, differentiating into neurons. This allows the development of a cortical plate and marginal zone as the neurons arrive at the basal surface and organise into layers (M. A. Lancaster and Knoblich, 2014; Lancaster et al., 2017; García-Cabezas and Zikopoulou, 2019). Cortical neurons develop in a temporal manner, with layer VI containing deep layer neurons being formed first at the basal surface. Subsequent migrating neurons journey through previous formed layers to create layers V, IV, III, II, and I.

**Figure 9. Neuronal dorsal cortical organisation in the developing mouse and human brain.**

Mouse cerebral cortical development (A) showing apical radial glia (aRG) giving rise to intermediate progenitors (IPs), or to neurons directly, that propel themselves to the cortical plate (CP). However the developing human cerebral cortex is more complex (B), showing how the subventricular zone (SVZ) is divided into the inner SVZ (iSVZ) (a homolog to the mouse SVZ) and the outer subventricular zone (oSVZ). This oSVZ is colonised by outer basal radial glia (bRG) and basal intermediate progenitors (bIPs) which are highly proliferative and generate neurons, which migrate towards the cortical plate and marginal zone (MZ). Shading behind the different models indicate differences in the extracellular matrix (ECM) composition. CP - cortical plate; IFL - inner fibre layer; iSVZ - inner subventricular zone; IZ
This migration and subsequent layers give rise to the developing cortex as shown in Figure 9. This human cortical developmental process is more complex than in other traditionally used models like rodents (Molnár et al., 2006). Human neuronal development displays multiple distinct layers of specialised cells, with the marginal zone itself having distinct characteristics over other species, mainly morphological differences that would be more viable within 3D culture (Tkachenko et al., 2016). Being able to recapitulate human neuronal development in both 2D and 3D cultures allows deeper investigations into protein function as well as aggregation. These models become especially relevant as primary human neuronal cultures are difficult to obtain, although not impossible, and come with ethical issues (Deshpande, Win and Busciglio, 2008).

### 1.10 Induced Pluripotent Stem Cell models

#### 1.10.1 Induced pluripotent stem cells

Similarly to primary neuronal cultures, stem cell models of disease have been difficult to pursue in the past due to both the acquisition of embryonic stem cells and the ethics behind this model (Zakrzewski et al., 2019). Although the difficulty obtaining these embryonic cell lines is not as great as primary cultures, there are additional hurdles to overcome, for example the 14 day creation rule on embryos (Appleby and Bredenoord, 2018). iPSC allows almost limitless access to neuronal cultures, as well as other cell types, all derived from adult human patient somatic cells (Takahashi and Yamanaka, 2006).

In 2006, Yamanaka et al., demonstrated it was possible to reprogram somatic cells, such as fibroblasts, to a pluripotent state using exogenous expression of the pluripotency-associated transcription factors OCT4, Krüppel-like factor 4 (KLF4), sex determining region Y-box 2 (SOX2) and c-MYC (Takahashi and Yamanaka, 2006). As the resulting induced pluripotent stem cells (iPSC) can be differentiated into any cell type, the ability to generate stem cells directly from patient cells has become a powerful tool for neurological disease research, permitting an essentially limitless supply of human neurons to be generated (Takahashi et al., 2007; Yu et al., 2007; Xie and Zhang, 2014).
**Figure 10. Simplified overview of fibroblast to iPSC to neuron protocol.**

A simplified diagram showing fibroblasts reprogrammed using OCT4, KLF4, SOX2, and c-MYC episomes to create stable iPSC lines. iPSC lines can be differentiated into 2D neuronal cultures using dual SMAD inhibition via SB431542 and dorsomorphin. Differentiating from iPSC to Neurons through an NPC stage. Original figure created using SMART Servier Medical Art (LesLaboratories Servier SAS, 2020).

iPSC derived from adult somatic cells contain the patients precise genetic background, including any pathogenic mutations, but also other potentially disease-modifying genes, which may be contributing towards the pathology. This means that iPSCs are a more physiologically relevant and translational model containing the patients precise genetic code in a physiologically-relevant cell type (Park *et al.*, 2008). Given the difficulties in generating models of tau pathology outlined in section 1.8, iPSC derived cell types represent an attractive method for the creation of human, neuronal models of tauopathy.

**1.10.2 Differentiation of iPSC into 2D cortical neurons**

In AD research, the most heavily studied cell type generated from iPSC is cortical glutamatergic neurons, as these are the main tau tangle bearing cells in disease. Differentiation of iPSC into glutamatergic neurons is accomplished through three stages: neural induction, patterning, and terminal differentiation, initiated via dual SMAD inhibition (Chambers *et al.*, 2009; Shi, Kirwan and Livesey, 2012). SMAD inhibitors used are dorsomorphin, a BMP inhibitor, and the TGF-β inhibitor SB431542. Once committed to a neural tissue lineage, neuroectoderm has a default differentiation capacity to become cortical tissue. Due to Wnt signalling, anterior and dorsal forebrain neuronal cells are the main cellular product from this differentiation (Li *et al.*, 2009). These take the form of ‘cortical rosettes’ in culture, which mimic the formation of the neural tube and are formed while adhered to the plate surface, after which their architecture is passaged repeatedly to acquire neuronal cultures of the correct density (Shi, Kirwan, Smith, Robinson, *et al.*, 2012). The neuronal rosettes express...
early forebrain markers such as FOXG1 and TBR1. Radial glia and neural progenitors migrate across the rosette and can undergo either asymmetric or symmetric division at the apical edge, successively generating neurons of the six cortical layers over a period of 100 days (Gaspard et al., 2008; Dolmetsch and Geschwind, 2011). Markers in tandem are used to identify neurons and culture progression, such as TBR1 for early born layer VI, CTIP2 for layers VI and V, BRN2 for layers IV, III, and II, and SATB2 expressed in layers II through IV (Ueta et al., 2013; Cortical Layer Markers | Atlas Antibodies, 2020). This also exhibits the temporal cortical development of neuronal layer VI, followed by V, IV, III, II, and I, in a manner following human development (Gilmore and Herrup, 1997).

Studies have shown that during the 100 days of differentiation, neurons undergo terminal differentiation to form mature neurons and astrocytes. Following the in vitro corticogenesis process, the culture is mostly comprised of excitatory glutamatergic neurons (Gaspard et al., 2008; Shi, Kirwan and Livesey, 2012).

It can be argued, however, that these cultures models are reductionist, with a limited number of cell types and the 2D architecture of the model being seen as a major drawback (Arber, Lovejoy and Wray, 2017b; Duval et al., 2017). These cultures potentially have reduced cell to cell interactions, as well as a lack of spatial, temporal and mechanical inputs that in-vivo neurons normally experience (Mueller et al., 2020).

Multiple groups have investigated whether 2D iPSC derived neurons form functional synapses. iPSC-neurons express pre and post synaptic markers including VGLUT1, PSD95, and synaptophysin (Shi, Kirwan and Livesey, 2012). iPSC-derived neuronal cultures are mostly homogeneous and have been shown to contain electrophysiologically active neurons and functional synapses (Lam et al., 2017). Single cell analysis of iPSC derived neurons show they largely reflect those found in the developing cerebral cortex (Kirwan et al., 2015). Synapses release neurotransmitters and can be used to investigate synaptic transmission (Meijer et al., 2019). Electrical activity was similar throughout developmental stages when compared to rodent primary cultures. Similarly, glutamatergic and GABAergic inputs recapitulated cortical activity, although unique patterns of synchronous bursting activity were reported (Hyvärinen et al., 2019). Further studies have shown networks of iPSC derived neurons respond to electrical stimulation with a physiological relevant collection of spike waveforms. These readings were after 3 months of culture, however waveforms suggested that this might not be enough for full function maturation (Amin et al., 2016).

It also important to consider the role of non-neuronal cell types including astrocytes, an important cell type that exhibits tau pathology in PSP and CBD (Matyash and Kettenmann, 2010; Yoshida, 2014). Astrocytes are an important cellular component of the human brain and outnumber neurons,
although the neuron to glia ratio in human brain is disputed, estimated to be approximately between 1:2 - 1:7, depending on the region (von Bartheld, Bahney and Herculano-Houzel, 2016; Verkhratsky, Zorec and Parpura, 2017). Following completion of corticogenesis in iPSC-neurons generated by dual SMAD inhibition, the remaining precursor cells undergo a gliogenic switch and astrocytes are produced from 80 days *in vitro* (DIV) onwards (Shi, Kirwan and Livesey, 2012). DIV is usually calculated from the day of neuronal induction, therefore the proliferative and NPC stages of differentiation are also included within this time frame.

Some groups have developed co-cultures to study the interaction between different cells types. For example, co-culturing hPSC-derived neuronal cells with rat primary astrocytes increases long-term culture survival as well as the functional maturation of these cultures (Paavilainen et al., 2018). Similar studies utilising human astrocytes have also shown to be useful in studying neurotoxic compounds and the relationship between neurons and astrocytes (De Simone et al., 2017). The addition of immune cells from a different lineage like microglia to neuronal cultures allow the study of microglia and neuronal interactions in a 2D plane (Haenseler et al., 2017).

An interesting development in the last ten years was the development of induced neurons (iN), or direct conversion of fibroblasts into neurons, a process known as transdifferentiation (Vierbuchen et al., 2010). Expression of three transcription factors via viral vector, Ascl1, Brn2 (also called Pou3f2), and Myt1l, was sufficient to convert adult post-mitotic differentiated cells (fibroblasts) in to function neurons with synapses (Carter, Halmai and Fink, 2020). This bypasses the embryonic stage (i.e. no iPSC or NPC stage) resulting in the retention of aged based phenotypes may remain, for example telomere length and certain gene expression via DNA methylation (Carter, Halmai and Fink, 2020). Although an exciting prospect, due to direct conversion skipping the proliferative NPC stage of differentiation, neuronal yields are low. Additionally, there is potential starting cell contamination, although protocols are currently improving with the use of blood lymphocytes, episomal vectors, and the addition of small molecules forskolin, dorsomorphin, and SB431542 (Tanabe et al., 2018). With these improvements over 50,000 iN cells from 1 mL of peripheral blood are possible, making them viable for large scale experiments.

Exogenous expression of the pro-neural transcription factor neurogenin 2 (NGN2) can accelerate the conversion of iPSC into neurons, appearing to bypass the NPC-like stage, when expressing NGN2 (Zhang et al., 2013). With the expression of NGN2 via lentiviral vector, plus puromycin selection of successfully transduced cells, homogenous neuronal cultures were obtained within two weeks (Zhang et al., 2013; Ho et al., 2016). Although a very efficient and fast method, this protocol does go through the embryonic cell stage, unlike direct conversion, meaning any age related factors are probably lost,
as with iPSC reprogramming (Carter, Halmai and Fink, 2020). Recent work has also questioned the identity of these cells, as single cell RNAseq showed the cultures were more heterogeneous than previously thought, including the expression of several PNS specific genes, and also did not appear to have a specific neuronal identity. Thus although the rapid and potentially homogenous nature of the cells is advantageous, the developmental relevance and utility of these cells to mimic a specific neuronal population must be treated with caution (Lin et al., 2020).

1.11 iPSC models of tauopathy

1.11.1 Tau splicing

Several groups have used iPSC-neurons to investigate the effects of tau mutations in a iPSC derived human neuronal system (Wray, 2017). An important question, given the developmental regulation of tau splicing described in section 1.1, is whether iPSC-neurons express and splice tau in the same way as observed in the adult CNS. Genome-wide, exon-specific expression analysis has shown that stem cell derived neurons cluster more closely to fetal neurons than adult neurons (Patani et al., 2012). Studies have specifically investigated tau expression and splicing in iPSC-neurons, showing that the fetal tau isoform (0N3R) is the most abundant isoform present in these neurons. However, expression of mature tau splice variants becomes apparent with extended culture times (Patani et al., 2012; Iovino et al., 2015; Sposito et al., 2015; Sato et al., 2018).

Sposito et al, 2015, verified that 0N3R tau is primarily expressed up to 100 DIV in control iPSC derived cortical neurons at the RNA and protein levels. To determine whether the splicing of tau changes developmentally in vitro, this study also cultured control iPSC neurons to 365 DIV, demonstrating a switch in tau expression to multiple tau isoforms with 0N3R, 0N4R, 1N3R, and 1N4R detectable (Sposito et al., 2015). However, 2N3R and 2N4R were still below the limits of detection, despite a year in culture. Using mass spectrometry and stable isotope labelling kinetics (SILK), Sato et al, (2018), compared levels of tau isoforms within human adult brain, CSF, iPSC derived neuronal cultures, and iPSC derived neuronal culture media (Sato et al., 2018). Results show expected levels of tau isoforms within human brain, with CSF exhibiting far less C-terminal repeats and reduced N-terminal tau isoforms compared to the brain sample. Cell lysates from iPSC derived neurons, cultured to 63 DIV, show a tau isoform profile similar to human brain, with the human brain samples being a mixture of ten adult brains ranging from 70 to 108 years old. However, other isoforms, especially 4R tau, are present in levels two orders of magnitude lower than in human brain samples. Confirming iPSC neurons predominately express 0N3R tau, but suggesting other isoforms are present, just at
magnitudes lower than in human adult brain. Conditioned cell media displayed lower levels of MTBR and C-terminus extracellular tau than detected within the cell lysates, a pattern similar to human brain compared to the CSF samples.

As discussed in section 1.1, specific mutations alter tau splicing, and it has been difficult to generate models of this due to the species-specific isoform patterns observed. Several groups have therefore generated iPSC models to determine whether altered splicing can be mimicked in this system.

Sposito et al were the first to show that the 10+16 MAPT mutation is able to override the developmental regulation of MAPT splicing, leading to early 4R tau expression (specifically 0N4R) (Sposito et al., 2015). This showed that iPSC-neurons can provide suitable models for the investigation of 4R tau within neurons and how tau isoforms ratio imbalances contribute towards FTD. However, it should be noted that in this study 0N3R and 0N4R are the predominant isoforms expressed as the N-terminal splicing is unaffected by this mutation and therefore still represent a fetal stage in development.

To further investigate MAPT splicing mutation effects on tau isoform expression, Verheyen et al, 2018, generated isogenic iPSC lines with a 10+16 MAPT mutation, both mono and bi-allelic variants, with additional lines also harbouring the P301S MAPT mutation (Verheyen et al., 2018). Following differentiation and culture for 65 days, WT iPSC expressed only 0N3R tau expression. However neurons harbouring the 10+16 mutation in both mono and bi-allelic forms, showed early expression of 4R tau, although fetal 0N3R tau was still predominant at this 65 DIV time point. In the presence of the P301S mutation alongside the 10+16 biallelic mutation potentially enhances 4R tau expression.

Paonessa et al, (2019), demonstrated the same splicing of 10+16 MAPT mutant iPSC neurons, with expression of 0N3R and 0N4R (Paonessa et al., 2019). However, this study also suggested P301L and WT control iPSC neurons also produce 0N4R tau, along with fetal 0N3R tau, at 120 DIV. No other tau isoforms are clearly detectable within any culture at this time point within this work.

Interestingly, lovino et al, 2015, not only showed disruption of the developmental regulation of MAPT due to another splicing mutation (N279K), but also suggested it accelerated the maturation of iPSC derived neurons (lovino et al., 2015). WT and P301L mutation iPSC used in the study, up to 150 DIV, show predominately 0N3R fetal tau, with possibly expression of 0N4R tau also. However, iPSC-neurons with the N279K MAPT mutation showed expression of multiple tau isoforms at 150 DIV, specifically 0N3R and 0N4R, together with 1N4R and 2N3R are also present. For unknown reasons, it appears that 1N3R and 2N4R are absent from these cultures, although this may be due to low levels of expression.
The predominant expression of fetal tau in iPSC-neurons complicates modelling adult diseases, as not all isoforms found in disease are present within the model system and, as discussed in section 1.6, isoform composition directly influences filament structures observed using cryoEM (Scheres et al., 2020). Furthermore, certain mutations, such as P301S, are located in exon 10 which is not present in the fetal tau isoform (Lossos et al., 2003). Notably, FTD mutations that alter tau splicing such as 10+14, 10+16, and N279K have been shown to override this developmental regulation and lead to increased 4R tau expression in iPSC-neurons (Iovino et al., 2015; Sposito et al., 2015; Wren et al., 2015). While these mutations allow both ON3R and ON4R tau to be present within these iPSC derived neurons, other isoforms are still missing (Sposito et al., 2015).

1.11.2 Tau pathology within iPSC models

The formation of NFTs within iPSC-neurons has not been reported, however precursors to NFTs including tau aggregates as well as hyperphosphorylated tau pathology has been seen within these models. Increases in phosphorylated tau levels have been reported within MAPT mutation iPSC derived neurons including 10 + 16, P301L, N279K, and V337M, individual studies discussed further below.

Ehrlich et al, (2015), not only confirmed abundant ON3R tau within iPSC neurons harbouring N279K and V337M MAPT mutations, but also an increase in S202 phosphorylation via AT8 antibody (Ehrlich, A. L. Hallmann, et al., 2015). This was corroborated by Iovino et al, 2015, who revealed increased S202 tau phosphorylation in iPSC neurons harbouring P301L and N279K cultures via AT8 antibody staining (Iovino et al., 2015). However, increased T212 and S214 phosphorylation was also shown via AT100 antibody staining, however mainly within the N279K neurons.

In addition to increased S202 phosphotau via AT8 antibody staining within 2D 10+16 MAPT neurons, Paonessa et al, (2019), also showed tau within these cultures caused nuclear incursions. Phosphotau was shown to invade the nucleus, creating nuclear invaginations within the nuclear membrane (Paonessa et al., 2019). The study conducted on the triple MAPT mutation neurons also showed increases in tau phosphorylation (García-León et al., 2018). iPSC derived neurons harbouring N279K, P301L, and 10+16 MAPT mutations were shown to have increased S202 phosphorylation via the AT8 antibody as well as increased T181 via the AT270 antibody.

However, increased tau phosphorylation needs to be carefully interpreted in iPSC models, as this phenotype is also displayed during neuronal development. Studies have argued that fetal tau during development is phosphorylated to a similar degree to PHF tau in AD brain lysate (Brion et al., 1993;
Kenessey and Yen, 1993): 7 moles of phosphate per mole of protein, which is 2-4 times the level of normal adult tau. Thus, it is important to consider whether tau phosphorylated is truly pathological or a reflection of the developmental status of these cells.

A152T patient iPSC derived neurons exhibit accumulation, redistribution, and decreased solubility of tau (Silva et al., 2016). Upregulation / accumulation of tau was linked increased susceptibility to proteotoxic, excitotoxic, and mitochondrial stressors, which resulted in increased cellular stress in mutant neurons compared with WT neurons. Cell stress was measured via carbonylated protein levels that accumulate in the cell due to excessive protein oxidation. The A152T mutant phenotype was rescued upon CRISPR/Cas9 mediated down regulation of tau or drug inducible activation of autophagy (Silva et al., 2016). This same mutation (A152T) in neurons was show by Fong et al, 2013, to exhibit distinct degenerative features, characterized by breaks, bends and bulges along neuronal processes and reduced neuronal survival. They also reported restoration of normal function when correcting the mutation, and exacerbating disease phenotypes by gene editing the line to be homozygous for the A152T mutation (Fong et al., 2013).

Imamura et al, 2016, demonstrated that 10+14 and R406W MAPT mutation iPSC neurons accumulated intracellular misfolded tau when labelled using an anti-oligomeric tau antibody, TOC1 (Imamura et al., 2016). It is important to note that this labels small aggregates/oligomers rather than NFT. AT100 positive tau inclusions have also been shown in N279K neurons, suggesting aggregation of tau phosphorylated at S212 and S214 (Iovino et al., 2015). However, these were low in number and were not present in P301L mutation neurons analysed in the same study.

Verheyen et al, 2015, used WT iPSC derived neurons and transduced them with a P301L tau mutation via AAV6-syn1-TAU-P301L adeno associated virus (Verheyen et al., 2015). No spontaneous tau aggregation was reported, however seeding with exogenous K18 P301L tau fibrils, tau aggregation within the cultures was seen after 2 weeks. K18 P301L tau is the MTBRs of the tau protein, harbouring the P301L mutation and aggregated into fibrils using heparin. WT neurons do not show aggregation of tau when treated with K18 fibrils.

In order to accelerate tau pathology, the García-León et al, 2018, paper engineered iPSC-neurons harbouring N279K, P301L, and 10+16 MAPT mutations, finding increased phosphotau. However despite harbouring three MAPT mutations and increased phosphotau, these cultures failed to develop tau aggregates (García-León et al., 2018). This demonstrates the resistance of iPSC-neurons to the formation of aggregates, although the mechanisms underlying this are currently unknown.
1.11.3  Tau Seeding

The observed spread of tau pathology observed in AD pressed researchers to ask whether pathological tau could be transmitted between neurons and other cell types, and template the mis-folding of normal tau in recipient cells, in a prion-like mechanism. The prion like spread of proteins in neurodegenerative disease is reviewed in Meisl et al, (2020) (Meisl, Knowles and Klenerman, 2020).

One of the first investigations of pathological tau spread and seeding was carried out by Clavaguera et al, 2009. This study utilised brain lysates from 6 month old P301S mice (a model that displays abundant tau filamentous inclusions) and injected it in to 3 month old ALZ17 mice (Clavaguera et al., 2009). The ALZ17 mouse model expressed wild type human 2N4R tau, the longest and does not exhibit any filamentous tau aggregates (Probst et al., 2000). On examination of injected mice tau pathology was discovered as well as significant distribution of pathology originating from the injection locus to other neighbouring brain regions. The same group in 2013 repeated this work but using human brain extracts from patients with AD, tangle-only dementia, PiD, AGD, PSP, and CBD (Clavaguera et al., 2013). The ALZ17 mice displayed similar spreading patterns to their previous work, as well as showing similar aggregations to the tau used for inoculation, suggesting tau spreads in a replicative prion like manner.

An important study from Hu et al, 2016 utilised the hTau mouse model (mouse tau KO expressing all 6 human tau isoforms (Andorfer et al., 2003)), showing injection of phosphorylated AD derived tau gave rise to significant highly phosphorylated tangles (Hu et al., 2016). This pathology was present at the injection site and spread to surrounding brain regions. Interestingly, dephosphorylation of AD tau drastically reduced the hyper phosphorylated tau pathology. Furthermore, tau pathology was present, but resembled argyrophilic grain-like morphology. Suggesting various phosphorylated states of tau play an important role in tau species aggregation.

Wegmann et al, (2015), used ECrTgTau mice, which limit expression of a human mutant P301L tau to the EC, where tau pathology initiates in AD. This model demonstrated trans-synaptic transmission of tau pathology to recipient neurons (De Calignon et al., 2012). On crossing the ECrTgTau mouse model with a tau knockout mouse model, creating a mouse that only expresses P301L human tau within the EC, brain sections from 18 month old mice revealed robust propagation of transgenic tau (Wegmann et al., 2015). This is despite no endogenous tau being expressed within other brain regions. Results such as these suggest tau seeds can propagate to neurons even without tau. Interestingly, the tau pathology toxicity was reduced when endogenous tau was removed, despite tau spread from the EC region. The same result was seen when tau knock out mice were injected with a viral vector (AVV expressing P301L human tau) within the EC. Surprisingly, immunostaining for human tau (using
human tau N-terminus-specific antibodies Tau13 or TauY9) in horizontal brain sections from 18-month-old mice revealed robust propagation of transgenic tau to DG neurons in mice that expressed human P301Ltau in the EC in the absence (ECrTgTau-Mapt0/0) and presence (ECrTgTau) of endogenous

Several in vitro studies have shown that tau uptake occurs in cells, moving it from extracellular space to the cell body itself (Frost, Jacks and Diamond, 2009). With this in mind, a Förster resonance energy transfer–based biosensor assay has been developed (Furman and Diamond, 2017). This biosensor assay consists of HEK cells overexpressing the human tau microtubule binding domain (MBD), which also contains the P301S mutation, with this peptide is tagged to either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) domains. Addition of seed-competent tau species will sequester tagged tau, leading to the aggregation of tau, with the fluorescent tags allowing for visualisation of these aggregates. Either by excitation and detection of CFY directly or by Förster resonance energy transfer (FRET), exciting CFP and detecting emission from the YFP tag due to close proximity energy transfer from one to the other. This assay has been previously used for the detection of early tau pathology (Takeda et al., 2016).

Holmes et al, 2014, confirmed previous work by utilising the biosensor assay and P301S mice (Holmes et al., 2014). With a time course of brain lysates applied to biosensor cells, robust seeding activity at one and a half month before the earliest histopathological stain was detected. In addition to this the Dujardin et al, 2020, work showed seeding capacity correlated with clinical severity (Dujardin et al., 2020).

This has system has been successfully utilised to show tissue from mice expressing pro-aggregating 1N4R tau harbouring a P301S mutation can seed tau aggregation (Kaufman et al., 2016). Following this, fixed tissue from the same mice, without tau pathology, was shown to also seed tau aggregation within the tau biosensor assay (Kaufman et al., 2017). This study also showed seeding from AD patient brains with comparative IHC. Interestingly, lysates isolated from white matter tracts from AD patients was also able to create tau aggregations, suggesting aggregate competent tau seeds are present in, or can be transported along neuronal connections (DeVos et al., 2018). Although it is important to realise biosensor assays with signal molecules conjugated to protein aggregation prone species do not aggregate in the same way as they do in the human brain (Kaniyappan et al., 2020).

Human iPSC neurons have been show to uptake tau. Evans et al, 2018, showed monomeric tau could be taken up via dynamin dependent endocytosis as well as actin dependent micropinocytosis (Evans et al., 2018). With work in neuroblastoma cells and again iPSC neurons showing the knockdown of
lipoprotein receptor related protein 1 (LRP1) significantly reduces the cells ability to uptake tau (Rauch et al., 2020). Furthermore, this mechanism is mediated by lysine resides within the MTBD.

Inducing pathology via iPSC neurons harbouring mutations has proven difficult, with most studies utilising exogenous tau seeding. For example Verheyen et al, 2015, demonstrated P301L neurons failing to demonstrate any pathology, however tau K18 seeding revealed AT8 positive tau aggregation. The same group also utilised their 10+16 biallalic with P301S biallelic mutation neurons in 2018, again unable to see any spontaneous tau pathology (Verheyen et al., 2018). Interestingly total tau levels were unchanged after seeding with K18 tau, measured by ELISA, and no AT8 positive inclusions were seen. Another interesting study looked at the relationship between neuronal activity and tau (Wu et al., 2016). Results not only confirmed the release of tau from iPSC neurons, but also that the release of tau can be stimulated via neuronal activity.

1.11.4 Disease mechanisms

Multiple studies have used iPSC-neurons is the ability to mechanisms of neuronal dysfunction linked to MAPT mutations. Mitochondrial function was examined within 10+16 neurons showing mitochondrial hyperpolarisation reduced ATP production and increased reactive oxygen species (Esteras et al., 2017). As tau is a microtubule binding protein, this could be related to disrupted mitochondrial transport (Esteras et al., 2017). Recent research also suggests MAPT mutations can cause microtubule facilitated deformation of the nucleus, specifically in 10+16 and P301S neurons. Defective nucleocytoplasmic transport is a consequence of these nuclear distortions, and models the deformed neuronal nuclei phenotype seen in post-mortem frontotemporal dementia patients (Paonessa et al., 2019).

In addition to the 10+16 and P301S FTD MAPT mutations discussed earlier in this thesis, other FTD MAPT mutations have been investigated using iPSC culture to investigate the effects of mutant tau. For example the N279K mutation is a coding mutation, which resides within an exonic splicing enhancer site, and increases the ratio of 4R to 3R tau (Iovino et al., 2015). iPSC derived N279K neural stem cells exhibit accumulation of stress granules as well as impaired endocytic trafficking, and a reduction of lysosomes. In contrast, N279K fibroblasts did not show any difference compared to controls, indicating that this phenotype is neuronal specific (Iovino et al., 2015; Wren et al., 2015).

Multiple studies have examined the impact of the A152T variant on neuronal health using iPSC models (Fong et al., 2013; Silva et al., 2016). Unlike most pathogenic mutations in MAPT, A152T does not
cause autosomal-dominant FTD, although it is thought that it may act as a risk modifier for tauopathies such as PSP (Kovacs et al., 2011).

Finally, chimeric models have been utilised to investigate the selective vulnerability of human neurons to tauopathy. Chimeric models were generated by transplanting human iPSC derived NPCs into WT mice, NPC cells differentiated and the subsequent human neurons integrated into the mouse circuitry (Espuny-Camacho et al., 2013). In a model by Espuny-Camacho et al, 2017, human WT neurons were transplanted into a KM670/671NL mutated APP and L166P mutated PS1 mouse model (Espuny-Camacho et al., 2017). Remarkably the human neurons express 3R/4R human tau isoforms after 4 months post transplantation, however the expression level of 4R tau was only detectable in 0.7% of human neurons. However, 6 months post transplantation this has risen to 89%, with 8 months post transplantation animals showing a 1:1 ratio of 3R/4R tau, as expressed in human adult brain. Human neurons did show abnormal phosphorylation at S202 using AT8. Tau was also shown to undergo conformation change within the transplanted human neurons via MC1 positivity, an antibody that detects the early structural changes of tau, potentially leading to NFT formation (Jicha et al., 1997). Further to this, human neurons within the chimeric model undergo cell death and neurodegeneration, but the mouse neurons do not. This suggests that human neurons are selectively vulnerable to the Aβ generated in this model.

However, NFTs were not detected in human neurons or in human tau expressing murine derived neurons, despite 8 months of maturation within the animal (Espuny-Camacho et al., 2017). This adds further support to the idea that pre-tangle, oligomeric tau species are more neurotoxic than NFTs.

1.12 Cerebral organoids and other 3D culture systems

1.12.1 Early 3D culture

2D neuronal cultures from human iPSC offers many benefits for disease modelling as discussed previously, however they lack the cellular diversity, structural complexity and physical architecture seen in vivo. To circumvent these drawbacks, there has been a significant effort to develop 3D alternatives in order to better model the human brain in vitro. It must be highlighted that 3D culture systems have increased cellular heterogeneity and complexity compared to 2D cultures, this makes their culture more demanding and subsequently more challenging to analyse (Lancaster et al., 2013; M. A. Lancaster and Knoblich, 2014).
3D culture models of the brain were initially attempted in the 1970s. Utilising both serum and serum free media to reduce exogenous protein influences, cell aggregates created from mechanically dissociated rat fetal brain were grown in suspension. This model was originally touted as being valuable for the study of nutritional and hormonal influences on brain development (Honegger, Lenoir and Favrod, 1979). Through the 1980s other models utilised purified astrocyte cultures and attempted to grow the cells in 0.5mm cellulose tubes looking at co-culture dorsal root ganglion (DRG) axonal growth (Fawcett et al., 1989). 3D bio-printing was also trialled, with limited success, as a tissue culture method in the 1980s (Klebe, 1988). Bioprinting involves the extrusion of cells in a substrate, usually on to a scaffold, to create a three-dimensional culture / tissue (Murphy and Atala, 2014). Bioprinting has subsequently improved a great deal, however this technology is still not utilised as frequently today as free floating or scaffolded cultures, mainly due to the mechanistic costs involved (Thomas and Willerth, 2017). Costly sterile bioprinting machines are required, usually with high running costs, although cheaper microextrusion machines have proved popular recently, even if this does sacrifice resolution (Jones, 2012; Murphy and Atala, 2014).

Early precursors to the currently used cerebral organoid 3D neuronal models started in the 1990s. Neurospheres were grown by Reynolds et al, 1992, and tried to recapitulate some developmental aspects of the brain. Cells from the striatum of an adult mouse brain were isolated and pushed to proliferate using epidermal growth factor (Tropepe et al., 1999). These cells were cultured without any tissue culture adherent compatible surface (Reynolds and Weiss, 1992). These cells proliferate further, forming small clusters of cells, coined neurospheres, which continue to grow in suspension. The multipotent cells that make up these neurospheres harbour the ability to differentiate into both neurons and astrocytes (Reynolds and Weiss, 1992).

With the isolation of embryonic stem cells (ESC), and later the generation of induced pluripotent stem cells (iPSC), a merging of early 3D and 2D protocols gave rise to neuronal aggregation cultures. These utilised a 3D embryoid body stage, comprised of embryonic stem cells, with treatment of fibroblast growth factor 2 (FGF-2) to generate neuronal precursors. These precursors can then be either transplanted into animal models for further differentiation, or plated on to adherent tissue culture surfaces for further differentiation into neurons and astrocytes after FGF2 withdrawal (Zhang et al., 2001). Later on these protocols were improved upon by using iPSC cultures, and furthermore efficiency was improved with dual SMAD inhibition patterning as mentioned above in 1.10 (Chambers et al., 2009; Shi, Kirwan and Livesey, 2012).
1.12.2 Free floating cultures and extra cellular matrices

Moving forward from aggregate cultures, cortical spheroids were developed, taking tissue culture another step towards mimicking in vivo structures. The serum-free suspension culture (SFEB) method was developed by Watanabe et al., 2005, who discovered that early treatment of suspended ESCs with Wnt and Nodal antagonists (Dkk1 and LeftyA) causes selective neural differentiation (Watanabe et al., 2005a). Three years on from that work, Eiraku et al (2008) developed serum-free floating cultures of embryoid body-like aggregates with quick reaggregation (SFEBq) (Eiraku et al., 2008). Similar to the 3D protocols before it, SFEBq requires aggregated ESCs, however these stem cells form neuroectoderm-like epithelium tissue. This tissue then generates NPCs and cortical neurons while self-organising, all similar to the process in vivo (Eiraku and Sasai, 2012). This step towards self-organisation lead to the development of human cortical spheroids which are 3D cultures that utilise iPSCs but without any extra cellular matrix (ECM) and uses minimal patterning factors. These cultures were shown to develop deep and upper layer neurons as well as astrocytes throughout the tissue, which are important for synapse formation (Pasca et al., 2015).

As an alternative, some groups investigated the utility of ECMs and hydrogels as a medium harbouring cells. Utilising cells suspended within these materials to allow 3D cell morphologies and cell to cell interactions. Hydrogels are polymeric materials that create 3D crosslinked hydrophilic polymer networks. When mixed with cell media they are capable of swelling or shrinking, retaining large amount of water when in the swollen status. Neuronal cultures, including cellular axonal growth cones, have been shown to adhere to this matrix, providing a medium for growth and support (Ahmed, 2015; Madhusudanan, Raju and Shankarappa, 2020).

These groups utilised hydrogels to suspend their cell type of interest, allowing for more physiologically relevant individual cell morphologies, for example longer axons and cellular domains, when compared to 2D cultures. Early suspension cultures of primary human schwann cells and neonatal astrocytes in the 1990s were maintained for short periods of time and showed low cell survivability, only seeing 10-20% cell survival rates after 6 days in culture (Woerly, Plant and Harvey, 1996). Many variables for scaffolds were investigated (Woerly et al., 1993). Studies in 1993 for example demonstrated that N-(2-hydroxypropyl) methacrylamide (pHPMA) hydrogels with added carbohydrate and ECM proteins promoted adhesion of neuronal cultures (Woerly et al., 1993). The addition of collagen to these cultures further stimulating neurite formation.

Xu et al, (2009), showed improved morphology of embryonic rat hippocampal neurons, as well as improved survivability post 21 days in culture, within hydrogel cultures vs 2D (Xu et al., 2009). However similar electrophysiological characterization within 3D were observed when compared to
monolayer cultures. 2D and 3D cultures, at 14 and 21 days old, both showed spontaneous postsynaptic currents, demonstrating functional synapses. Spontaneous postsynaptic currents were recorded in 14- and 21-day-old 3D cultures evidencing functional synapse formation.

Collagen and early versions of Matrigel™ were also tested for their abilities to support 3D suspension cultures, however it was found that collagen stunted the growth of neurons encapsulated within (Koutsopoulos and Zhang, 2013). Matrigel is a murine tumour matrix extract, which has been shown to be abundant in basement membrane proteins such as laminin, collagen IV, and heparan sulphate (Kleinman et al., 1982). At room temperature matrigel solidifies in to a gel, creating a structure similar to an authentic cellular matrix. (Benton et al., 2014; Kleinman, Kim and Kang, 2018). Matrigel allowed increased NPC proliferation and differentiation compared to collagen, however tissue survival rates decreased when taken to long culture time points (Koutsopoulos and Zhang, 2013).

Several groups have also used alginate to encapsulate small numbers of human embryonic stem cells to improve differentiation efficiency and decrease the labour needed per culture (Kim, Sachdev and Sidhu, 2013).

Kevin Alessandri in Switzerland optimised alginate encapsulation to examine neuronal morphology within these cultures (Alessandri et al., 2016). Control iPSC were induced via dual SMAD inhibition until rosettes and neural stem cells were visible, NSCs were used due to post mitotic neurons not being able to survive disassociation. NSCs were disassociated and encased in spherical matrigel and alginate capsules where differentiation continued and post mitotic neurons filled the structure. This allowed for more uniform and homogenous 200 µm neuronal cultures and permitted neurons to adopt a more in vivo like morphology with imaging of tubulin subunit beta3 positive mature neurites via confocal microscopy. This model also benefits from supporting neurons with an ECM, and allowing nutrient diffusion due to the 200 µm culture limit (Alessandri et al., 2016).

1.12.3 Organoid technology

In what could be said to be a culmination of these models, iPSC derived cerebral organoids (COs) utilised embryoid bodies along with ECM embedding and minimal patterning. This allows the cells to self-organise and respond to intrinsic signalling to recapitulate in vivo architecture and development (M. A. Lancaster and Knoblich, 2014; Kelava and Lancaster, 2016).

iPSC derived neuronal organoid models have been popular since their advent by Madeline Lancaster in 2014 (M. A. Lancaster and Knoblich, 2014). Starting from iPSC, embryonic bodies (EBs) can be
induced to neural ectoderm followed by self-guided differentiation. Self-organisation allows cell autonomous signals guiding events such as migration, polarisation of neuroepithelium and generation of distinct cell subtypes, concluding in self-organised heterogeneous neuronal tissue. This self-guided architecture resembles the developing brain much more closely than the previously mentioned suspension cultures in ECM (Nascimento et al., 2019). 2D dual SMAD patterned cultures create flat neural tube-like structures called rosettes, however these neural tube like structures are recapitulated in 3D within organoids. Retention of these neural tube-like structures, and hence their architecture, allow for more in vivo-like development of the subventricular zone (SVZ). The basal area of the neural tube harbours radial glia, which divide and begin neuronal migration to the basal surface and subsequent differentiation (Lancaster et al., 2013, 2017; M. A. Lancaster and Knoblich, 2014). Cellular migration from the SVZ, through NPC layers allows the creation of the cortical plate and marginal zone. Human cortical formation and the subsequent layers it creates is done so in a manner more complex than other animal models like rodents (Semple et al., 2013). Multiple distinct layers of specialised cells and neurons are present in human neuronal development, with morphological differences that would be more viable within 3D culture (Tkachenko et al., 2016). Cell to cell interaction have been shown to be very important in multicellular models. Impressive work from Mueller et al, (2020), demonstrated this via photoswitchable connections between cells. Photoswitchable proteins are peptides that alter their interaction depending on the wavelength of light applied to them (Kawano et al., 2015). Cell surface protein pairs that specifically heterodimerize under blue light were utilised to examine cell to cell interaction and its effects. This revealed different cell structures develop depending on cell to cell interaction dynamics (Mueller et al., 2020).

COs allow for cell to cell interactions to be studied as development progresses. COs contain a variety of cell types identified by cell-type specific markers, including neurons (βIII-tubulin), neural progenitors (SOX2), forebrain region (FOXG1), choroid plexus (TTR), hippocampal regions (PROX1 & FZD9), mitotic radial glia (P-Vimentin), later-born superficial layer identity neurons (SATB2), and early-born deep layer identity neurons (TBR1 & CTIP2) (M. A. Lancaster and Knoblich, 2014; Pasca et al., 2015). Tightly regulated signalling events, protein cascades, and spatially controlled responses strictly govern these complex cell behaviours. Expression of these signals all strongly recapitulate gene expression programs of fetal neocortex development (Camp et al., 2015; Grenier, Kao and Diamandis, 2019; Fiock et al., 2020).

This relatively new system has already been widely used to model neurodevelopmental disorders. For example, organoids gave powerful insights into the effects of Zika virus on cortical development. Elegant studies have shown that the virus attacks proliferate cells in developing cortex, leading to the microcephaly phenotype associated with ZIKA exposure within the womb (Clevers, 2016; Qian et al.,
2016). Specifically, organoids infected with Zika virus showed a significant decrease in 5-ethynyl-2'-deoxyuridine (EdU) and SOX2 stained progenitor cells, showing a reduction in NPCs and proliferation. Infected organoids also displayed a significant reduction in CTIP2 positive cells in layers V and VI as well as a large reduction in total neurons via βIII-tubulin staining.

COs have also successfully been used to examine the developmental basis of microcephaly. Patient fibroblasts from an individual with severe microcephaly caused by a truncation mutation in CDK5RAP2. This mutation resulted in no CDK5RAP2 protein being produced. iPSC from this patient were used to generate COs, which revealed decreased numbers of radial glia cells as well as increased differentiated neurons when compared to control at 22 DIV, suggesting premature terminal differentiation resulting in microcephaly (Lancaster et al., 2013).

This self-organisation, related architecture and expression profiles give 3D organoids more in vivo relevance than 2D neurons or suspension 3D cultures. Single cell RNA sequencing data has shown gene expression to be remarkably similar to the developing cerebral cortex of fetal tissue (Camp et al., 2015). Similar to 2D cultures, dual SMAD inhibition may also be applied to EBs to generate region specific, forebrain organoids (Qian et al., 2016). This approach enhances the content of cortical glutamatergic neurons within the organoid, generating region-specific forebrain organoids.

This approach of combining patterning factors with 3D culture has also been used to generate midbrain, cerebellar and hippocampal organoid cultures (Muguruma et al., 2015; Sakaguchi et al., 2015; Jo et al., 2016; Qian et al., 2016). This has been further extended to model non-neuronal components of the brain, for example work from Pellegrini et al. (2020), described a method for the development of choroid plexus organoids, which showed CSF-like fluid production, and SARS-CoV-2 disruption of the blood brain barrier (Pellegrini, Albecka, et al., 2020; Pellegrini, Bonfio, et al., 2020).

These ‘patterned organoids’ reduce the heterogeneity and variability seen in COs, however this also means the ability to look at multiple neuronal subtypes within a single system is lost (Watanabe et al., 2005b; Smith et al., 2007; Qian et al., 2016). These region-specific organoids have been used as the basis to generate multi organoid cultures known as ‘assembloids’, which allow the interactions between distinct brain regions to be modelled. These 3D models merge brain-region specific organoids to examine cell-to-cell interactions and migration (Sloan et al., 2018).

For example, the ventral forebrain area deemed the subpallium comprises of divisions called the ganglionic eminences (GEs). Interneurons are generated from these regions in development and migrate towards cortical and other regions (Anderson et al., 2001). GEs and interneurons are rarely seen in cortical organoids due to the forebrain and dorsal cortical identity within them. Cortical
glutamatergic organoids were fused with ventral interneuron organoids expressing GFP. Sloan et al., 2018, was able to show interneuron migration in to the cortical organoid, which recapitulates the interactions of interneurons and glutamatergic neurons seen in vivo (Sloan et al., 2018).

This work was extended in a recent paper that generated cortical-striatal assembloids while utilising viral tracing, a technique involving replicating neurotropic viruses to travel along neuronal pathways as a self-replicating neuronal and axonal marker (Ugolini, 2010). Numerous projections from cortical neurons in to the striatum organoid were observed, forming synaptic connections and in vivo like dendritic spine morphology. Medium spiny neurons (MSN) of the striatum organoid were also shown to be mature and electrophysiologically active via analysis of calcium signalling (Miura et al., 2020).

Chimeric models with rodent explants and human iPSC derived 3D models is another avenue of research currently being explored. For example, human neuronal organoids has been grafted on to mouse spinal cord–muscle explants. Organoids were able to elicit coordinated muscle contractions when co-cultured, showing that the organoid can integrate and influence the mouse muscle independently of the murine CNS (Giandomenico et al., 2019). Chimeric models have been further used to enhance vascularisation, something discussed later on in this introduction.

Similar to 2D neuronal cultures, the utility of 3D models to study age-related NDD has been debated, particularly the maturity of the neurons. For example fetal brain under 22 weeks is shown to express mRNA only for N-methyl-D-aspartic acid (NMDA) receptors, while mature neurons are shown to express this receptor at the protein level (Eugenin et al., 2011). Cerebral organoids have demonstrated NMDA protein expression in 10 week old organoid cultures, suggesting mature NMDA receptors have developed (Yakoub and Sadek, 2018).

Evidence suggests 3D organoids are electrically active and functionally mature at earlier stages than 2D cultures, offering hope of mature neuronal models for tauopathies without exceedingly long culture times (Jo et al., 2016; Yakoub and Sadek, 2018). Further to this, functional dopaminergic and neuromelanin producing neurons have been reported within midbrain-like organoids (hMLOs) derived from iPSC (Jo et al., 2016). hMLO slices underwent patch clamp recording, showing both inward and outward currents, which likely correspond to the opening of sodium and potassium dependent channels.

As mentioned earlier, organoid gene expression is similar to fetal tissue, an advantage when recapitulating development in vitro (Camp et al., 2015). Further studies in 2019 by Nascimento et al, confirmed this as well as showing proteomic similarities to developing fetal tissue (Nascimento et al.,
It is important to note the developmental aspect of these 3D iPSC derived models and carefully address the questions of maturation.

A limitation associated with 3D models includes increased heterogeneity between batches, which arises due to their autonomous intrinsic signalling nature. This makes the analysis of sample materials from COs challenging (Qian et al., 2016). One challenge is the limiting factor of whole organoid analysis. Each organoid as a sample represents the whole cell population protein content and mRNA expression, which needs careful analysis and interpretation. For more cell specific analysis single cell analysis is a method that is becoming more routine in the 3D tissue culture field, however it can increase experimental costs significantly (Shapiro, 2018). Being able, via FACs for example, to only remove and analyse the mRNA of that population of cells is a powerful tool, allowing 3D maturation of neurons, with cell specific sample collection. Work by Camp et al, (2015) utilised single cell RNA sequencing, showing gene expression profiles in neuronal populations within organoids recapitulated expression in the fetal neocortex (Camp et al., 2015; Hodzic, 2016).

Another limitation of COs is the absence of overlying meninges tissue and vasculature as this severely limits the growth potential of the organoids and increases the chances of the formation of necrotic centres in larger COs (Lancaster and Knoblich, 2014). In an attempt to counteract heterogeneity, and improve the self-organisation of COs, Lancaster et al (2017). developed engineered cerebral organoids (enCORs) (Lancaster et al., 2017). As previously mentioned, the CO protocol begins with spherical embryoid bodies, however spheres possess the lowest surface area to volume ratio of any shape (Sekine and Haraguchi, 2011). This means that the induction media comes into contact with a limited number of cells and at a limited depth around the surface of the EB (up to its diffusion limit of potentially 0.4-2mm (Rouwkema et al., 2009; McMurtrey, 2016). This allows the centre of the EB to differentiate along other lineages such as mesoderm. The presence of these cell types can influence the differentiation of neighbouring cells into unwanted identities, despite the presence of pro-neural factors in the induction media (M. A. Lancaster and Knoblich, 2014).

enCORs incorporate internal scaffolds to enhance neuronal patterning using intrinsic patterning principles employed in the generation of COs. Scaffolds constructed from sea sponge and cellulose have been trialled, however these showed inconsistent cellular clumping or did not elongate the EB for increased surface area. Polylactic-co-glycolic acid (PLGA) fibres out performed other materials in these areas, showing consistent elongated EB creation within 96 well plates (Lancaster et al., 2017).

PLGA is a biodegradable copolymer used as sutures, allowing the stiches to hydrolyse within 70 days of introduction to a wound (Lim, Poh and Wang, 2009). These characteristics make it perfect as a tissue engineering substrate, lending structure to the enCOR initially, but being degraded by 50-70 DIV. As a
result, enCORs are less variable within and between batches, as well as fewer unwanted tissue linages and more cortical plate formation than COs (Lancaster et al., 2017).

Park et al, 2018, investigated a microfluidic tri-culture system as an alternate means to reduce heterogeneity in 3D cultures and investigate cellular migration (Figure 11). Neurons and astrocytes were separately differentiated from immortalised human NPCs (ReN cells), before being seeded into co-culture with matrigel, within the 3D microfluidic main chamber. This was followed by the subsequent addition of differentiated immortalized human microglia SV40 cell line at different time points via microfluidic chamber channels, allowing microglial migration within the culture (Park et al., 2018). This system reduces variability and allows addition of cells at predetermined time points. If using iPSC it would also allow only cell types of interest from efficient separate differentiations to be added to the culture with more control. However, it also increases the labour required for each 3D culture as well as arguably being a slightly reductionist model since cells are initially monocultures, with the final step combining them all, rather than early development of neurons and related cells within that 3D environment (Krencik et al., 2017).

Figure 11. Park et al, 2018, tri-culture system.

Diagram showing the Park et al, 2018, tri-culture system. NPCs added to the Matrigel™ 3D culture central chamber and differentiated in to neurons and astrocytes. After which immortalized human microglia are added at different time points through microfluidic channels running in to the 3D ECM culture. Stimulus can be added from above, for example Aβ peptides. Original figure created via SMART Servier Medical Art, (LesLaboratories Servier SAS, 2020), adapted from Park et al, 2018.

Organoid models currently do not include microglia due to this cell type arising from the yolk sac, rather than neuralectoderm that organoid models generate (Alliot, Godin and Pessac, 1999). This
makes this tri-culture system a unique way to investigate human neuronal and astrocyte interactions with microglial cells. Recent publications have described methods to generate microglial-like cells from iPSC (Abud et al., 2017; Speicher et al., 2019) and efforts to introduce microglia in to both organoid and spheroid cultures are being undertaken (Song et al., 2019). Microglia-like cells implanted on to human cortical organoids cultures showed they secrete cytokines in response to inflammatory stimuli such as lipopolysaccharides (LPS). In addition anti-inflammatory cytokines such as VEGF, TGF-β1, and PGE2 were detected when organoids were stimulated with amyloid β42 oligomers. Microglia-like cells also displayed ramified morphology, with cells taking on a globular morphology when the organoids were injured.

Finally, one report described the innate formation of microglia-like cells within organoid cultures (Ormel et al., 2018). However, it is difficult to understand how this could be possible from a developmental perspective given the unique origin of microglia, and raises questions about whether this observation could be a result of non-specific patterning/differentiation.

A major limitation of organoid systems is the lack of vasculature, which leads to an oxygen and nutrient deficient centre of the organoid, resulting in necrosis (Lancaster and Knoblich, 2014). Multiple groups have tried to address this issue. Chimeric models generated by the implantation of organoids within immune deficient mice was one of the first approaches investigated. Many groups have opted for this method, showing that following implantation, blood vessels were detected within the grafted organoids vivo two-photon imaging (Mansour et al., 2018). A cranial glass window allows visual access to the grafted organoid, which indicated blood flow through these newly created vessels. Using murine and human CD31 staining it was shown the blood vessels were of murine origin, not human epithelia cells. This is not regarded as a problem, however it is important to bear in mind that the organoids will contain murine tissue and be a chimeric model. Another interesting finding was increased NeuN+ neurons within implanted organoids when compared to non-implanted organoids, indicating potential earlier maturation of cultures when grafted (Takebe et al., 2013; Lancaster, 2018; Mansour et al., 2018).

An alternative approach to vascularisation method involves sacrificial networks of scaffolds. Scaffolds/3D filament networks can be 3D printed utilizing carbohydrate glass, on which cells can be grown. After harmlessly dissolving the scaffolds, the voids left within the culture could be seeded with separately differentiated epithelial cells, creating vasculature that is entirely human in origin (Miller et al., 2012). Utilising scaffold ideas and bio-printing, Kolesky et al, 2016, used a ‘cellular ink’ to print 3D filament networks. These networks are impregnated with human umbilical vein endothelial cells (HUVECs), meaning as scaffolds dissolve, HUVECs are left in their place, differentiating and organising
to create vasculature (Kolesky et al., 2016). Confocal microscopy demonstrated vasculature with open lumens which is even perfused via an external pump. This method would therefore utilise scaffolds in a similar way as described previously to generate engineered organoids, but with the potential of incorporating blood vessels into the culture.

Other methods have tried combining both human endothelial cells and animal transplantation in order to take advantage of angiogenesis signals from the host. Pham et al., (2018), produced organoids from control patient iPSC as well as endothelial cells (Pham et al., 2018). After 34 DIV, organoids were re-embedded with endothelial cells. At 54 DIV organoids were transplanted in to immune-deficient mice, leading to vascularisation of the organoids. Further still, CD31 staining conformed vessels were comprised of human endothelial tissue. This method allows human derived vasculature to grow, however murine cell encroachment cannot be ruled out (Pham et al., 2018; Wörsdörfer et al., 2020).

Similar methods involve engineering a proportion of organoid cell populations to overexpress ETV2, a master regulator of hematoendothelial lineages (Cakir et al., 2019). It was discovered that 20% of the organoid cell population expressing ETV2 was the most efficient if induced to express ETV2 at day 18, with these organoids forming vascular-like structures in vitro. After implantation within immune-deficient mice, significant human CD31 positive vasculature was reported. Interestingly tight junctions, important structures between endothelial cells that prevent leakage between cells, were also reported via tight junction marker α-ZO1 (Cakir et al., 2019; Tervonen et al., 2019).

In summary, multiple novel approaches to generate 3D culture systems are now available, with recent (not peer reviewed) research even utilising low cost baked bread scaffolds for 3D tissue engineering (Holmes et al., 2020). These techniques outlined above provide new opportunities to investigate neuronal development, neuronal development disorders, and neurodegenerative disease (Lancaster et al., 2017).

Below is a table summarising the main advantages and disadvantages of 2D and 3D neuronal cultures which have been discussed in this thesis.

<table>
<thead>
<tr>
<th></th>
<th>2D</th>
<th>3D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Labour</strong></td>
<td>Many honed protocols available with high but manageable labour required</td>
<td>Less protocols available with increased learning curves and labour required (Very dependent on protocol)</td>
</tr>
<tr>
<td><strong>Cost</strong></td>
<td>Relatively cheap, with many commercially available protocols and kits (Although expensive when compared to classic 2D cell line culture)</td>
<td>More expensive, with less commercial kits and protocols available. Extra equipment also required over 2D culture</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>in vivo imitation</strong></td>
<td>Mimics neuralectoderm, however techniques do not mimic <em>in vivo</em> structure. Extended steps break apart any early neuronal structures</td>
<td>Mims <em>in vivo</em> early developmental structures that are allowed to retain <em>in vivo</em> architecture and develop</td>
</tr>
<tr>
<td><strong>Cellular Interactions</strong></td>
<td>Reduced cell to cell interactions</td>
<td>Increased cell to cell interactions</td>
</tr>
<tr>
<td><strong>Culture time</strong></td>
<td>Indefinite. However, prone to lifting and detaching over extreme culture times</td>
<td>Indefinite. Larger 3D cultures potential exhibiting necrosis within their cores at after extended time points</td>
</tr>
<tr>
<td><strong>Culture variability</strong></td>
<td>High reproducibility, highly optimised with low variability</td>
<td>Lower reproducibility, early stages of optimisation, with increased variability between batches</td>
</tr>
<tr>
<td><strong>Nutrient and oxygen access</strong></td>
<td>Unlimited access to oxygen and nutrients from the media above the monolayer</td>
<td>Access reduced as the thickness of the culture increases, leading to variable access as well as necrotic cores in larger cultures</td>
</tr>
<tr>
<td><strong>Microenvironment</strong></td>
<td>Media changes remove most cellular secreted proteins, replacing it with fresh media</td>
<td>Secreted proteins removed in the media, however within the 3D tissue secreted proteins are retained</td>
</tr>
</tbody>
</table>

*Table 1. Comparison of the advantages and disadvantages of 2D and 3D neuronal cultures*

Comparison of the main advantages and disadvantage of 2D and 3D culture discusses within this thesis.
1.13 Tau pathology using 3D models

Although the field is in its infancy in comparison to studies in 2D cultures, multiple reports have used 3D culture systems to investigate tau biology and pathology.

One of the first reports of tau pathology within 3D cultures was Choi et al, 2014, utilising ReN cells integrated within an ECM of Matrigel (Choi et al., 2014; Kim et al., 2015). These cells exogenously overexpressed fAD mutated APP or APP and PSEN1 genes. ReN cells are human neuronal progenitors that have been immortalised, but still have the ability to differentiate in to neurons (Donato et al., 2007; Choi et al., 2014).

Aβ immunoblot via 6E10-antibody revealed Aβ monomers, dimers, trimers and tetramers in SDS-soluble fractions, as well as dimers and trimers within the folic acid soluble fractions in cultures after 6 weeks *in vivo*, demonstrating aggregation of insoluble Aβ within this 3D model. ReN cell cultures only expressing m-cherry showed no Aβ immuno-reactivity, and these aggregates were not observed in 2D cultures of the same cells. ICC on these cultures also highlighted 3D6 positive aggregates of Aβ. Remarkably treatment of the cultures with secretase inhibitors (β-secretase inhibitor IV, DAPT, or SGSM41) removed Aβ plaque staining when utilising ICH teqniques and 3,39-diaminobenzidine (DAB, BA27).

Tau pathology was analysed, with significantly increased levels of tau phosphorylated at the AT8 epitope (S202) detected within both SDS-soluble fractions, as well as folic acid soluble fractions. Sarkosyl insoluble fractions revealed insoluble, highly phosphorylated tau protein positive to both AT8 (S202) and PHF1 (S396 and S404). Tau aggregations could also been see using ICC with the PHF1 antibody. Similarly to Aβ treatment of cultures with β and γ secretase inhibitors reduced the levels of phosphorylated tau when anyalsed using IHC and DAB staining. A remarkable finding from this model was that of tau PHF within these cultures, positively identified utilising a modified Gallyas silver staining, the “gold standard” for detection of NFTs (Nadler and Evenson, 1983). Further to this, electron microscopy was employed, allowing visualisation of the PHF within this model, verified by tau-46 antibody staining (Choi et al., 2014). Although a step forward in the modelling of tauopathy *in vitro*, it should be noted that these are immortalised progenitor cells where the phenotypes are driven by overexpression.

A recent paper by Hernández-Sapiéns et al, 2020, used a similar culture protocol, but this time with patient derived iPSC cells with a single PSEN1 mutation, A246E. They also report extracellular Aβ deposits using ICC, showing Aβ deposits can be generated in the absence of overexpression. However, they do not comment on tau aggregation (Hernández-Sapiéns et al., 2020).
Raja et al., 2016, generated COs according to the Lancaster protocol from iPSCs with APP duplication as well as PSEN1 M146I, and PSEN1 A264E mutations (Raja et al., 2016). These organoids were shown to recapitulate AD pathology with Aβ aggregation present when sections were analysed for Aβ aggregates. ICC revealed both intra and inter cellular aggregates for Aβ when probed using two anti-Aβ antibodies, D54D2 and 4G8.

No NFTs were observed, however higher levels of tau phosphorylated at S396 were detected by ICC. On immunoblot analysis, 60 DIV organoids showed no difference in S396 phosphorylation, however 90 DIV organoids showed a significant increase, suggesting temporal changes to tau can be modelled in this system. Tau phosphorylated at T181 was also significantly increased at 90 DIV. Interestingly application of β and γ secretase inhibitors to the organoids reduced Aβ staining via D54D2, as well as reducing S396 phosphorylated tau (Raja et al., 2016).

A separate study by, Gonzalez et al., 2018, showed the accumulation of proteins that were described as ‘highly reminiscent to amyloid plaques and neurofibrillary tangles’ (Gonzalez et al., 2018). Organoids were generated by the Lancaster et al., 2014, protocol, utilising iPSCs from a patient with fAD harbouring an A246E mutation in PSEN1 as well as a patient with Downs syndrome, and therefore an extra APP allele (Antonarakis et al., 2004). ICC utilising both 4G8 and 6E10 anti-Aβ antibodies revealed extracellular Aβ deposits measuring 25–30 μm in both fAD and DS COs. Sections of organoids and patient brain samples were also treated with amyloid binding dye BTA-1, showing similar staining for amyloid plaques and aggregations. This was followed by ELISA analysis of organoid formic acid fraction lysates, revealing Aβ42:40 ratios were significantly higher in fAD organoids compared to controls, and higher still in DS organoid lysate.

Tau was also examined with ICC, using PHF1 and AT8, showing increased staining of tau phosphorylated at these epitopes within PSEN1 mutant and DS organoids compared to control. IHC was also conducted with positive Gallyas silver staining for aggregated tau within APP and DS organoids, revealing tau aggregates similar to those observed in AD brain (Gonzalez et al., 2018).

Organoid models have also been used to look at sAD, a more challenging task than fAD due to the absence of causal mutations. The strongest genetic risk factor for sAD is the apolipoprotein E4 (APOE4) variant. Lin et al., 2018, utilised organoid techniques with iPSC derived microglia-like cells, both harbouring the risk variant of APOE as well as the more common, neutral variant, APOE3 (Lin et al., 2018). APOE4 organoids exhibited increased Aβ aggregates and hyperphosphorylation of tau, however Aβ staining was present within the APOE3 organoid / microglial co-cultures, just to a lesser extent. More work would be needed to investigate the underlying mechanisms for these differences.
The 3D human tri-culture system employed by Park et al, 2018, (Figure 11), has also been used to investigate AD pathology. Neurons and astrocytes were differentiated from ReN cells, in a similar manner and model to Choi et al, 2014 (Choi et al., 2014; Park et al., 2018). Overexpression of APP harbouring two mutations (K670N/M671L (Swedish) and V717I (London)) were induced via cytomegalovirus transduction. Insoluble SDS resistant Aβ species were confirmed with immunoblot and 6E10 antibody, as well as highly phosphorylated tau aggregates using ICC and PHF1 staining.

WT microglia were added to this 3D AD model via microfluidic chambers, allowing time lapse videography to track migration. Microglial migration was significantly higher in the AD model than control. Measurement of chemokine and pro-inflammatory factors associated with AD neuro-inflammation were measured via a human cytokine array kit, with AD cultures showing increases in CCL2, IL8, TNF-α, and IFN-γ.

Interestingly, addition of microglia appeared to be toxic to neurons and astrocytes to the AD cultures. Cellular density around microglia was reduced by 21% after only 6 days of triculture. A reduction of 37% when compared to older (9 week) AD cultures without microglia. This suggests microglia are a causal factor in neuronal and astrocytic loss (Park et al., 2018). In summary, these preliminary reports show the potential of 3D culture systems to model disease-relevant pathologies.

### 1.14 Tau splicing in 3D culture models

As described previously, the developmental regulation of tau splicing is conserved in 2D iPSC-neurons, and extended cultures times of up to a year are required for mature tau isoform expression (Sposito et al., 2015; Wray and Fox, 2016; Lines et al., 2020).

Interestingly, faster maturation of 3D neuronal cultures has been suggested, with previous work displaying increased PAX6 and NESTIN double positive NPC populations as well as significantly longer neurites than in 2D cultures (Chandrasekaran et al., 2017). Further, electrophysiology showed postsynaptic currents as well as spontaneous firing of action potentials within 3D culture neurons as early as 3-4 weeks in vitro (Zhang et al., 2016; Sood et al., 2019). Organoids have also been shown to recapitulate the developing cortex, pointing to a more in vivo-like developmental time line within these cultures when compared to in vivo fetal tissue (Camp et al., 2015). Increased tau expression has also been show to correlate with neuronal maturation within the developing human cortex (Fiock et al., 2020).
This prompted several groups to ask whether tau splicing would be more mature in 3D cultures, and the first reports investigating MAPT splicing within 3D neuronal models have now been published.

As discussed earlier, Choi et al, 2014, showed Aβ and tau pathology with their ReN cell derived ECM suspension cultures, however they also investigated tau expression (Choi et al., 2014). Via qPCR they were able to show increased expression of 4R tau isoforms when compared to 2D differentiated cultures at 7 weeks in vitro. The same technique was used to look at a variety of neuronal markers, showing significant increases in expression of VGULT1, NCAM1, SYT5, and MAPT when compared to 2D at the same time point and normalised to β-actin. This suggests earlier neuronal maturation of 3D cultures, at least with respect to neurons and MAPT splicing.

Immunoblots were carried out revealing multiple tau isoform bands when probed with total tau antibodies. Multiple bands can also be seen when probing with phosphotau antibody AT8. However without dephosphorylation, it is difficult to quantify what protein isoforms are present (Choi et al., 2014).

Kim et al, 2015, repeated the suspension differentiated ReN cell culture and showed a clearer tau immunoblot which exhibits multiple bands, representing multiple tau isoforms present after 8 weeks differentiation in 3D conditions (Kim et al., 2015). However, as mentioned, it is difficult to assign specific isoform identities by western blot without prior dephosphorylation of samples, which would provide confirmation of different splice variants rather than phosphotau species (Hanger et al., 2002; Kim et al., 2015).

The most promising paper showing multiple tau isoforms within 3D cultures of iPSC-neurons is from Miguel et al, 2019. Cortical neurons were generated using dual SMAD inhibition before being differentiated and cultured within matrigel coated alginate capsules (Shi, Kirwan and Livesey, 2012).

Analysis of the MAPT transcripts was carried out with long read PCRs utilising fluorescent primers spanning exon 1 to 11, allowing amplification and analysis of all 6 isoforms within a single sample.

On analysis of MAPT mRNA isoforms, cultures at 25 weeks showed 5 isoforms of MAPT mRNA, although over 90% of the tau was still the fetal ON3R isoform. They subsequently cultured cells in Brainphys media, which has been suggested to increase the maturity of iPSC-neuronal cultures (Bardy et al., 2015). Interestingly, at the same time point, but cultured in Brainphys media, all 6 isoforms were detectable, however almost 80% of mRNA transcripts were also fetal in nature (ON3R).

Due to this PCR and primer technique, quantification of each isoform is possible (Table 2). This result shows that BrainPhys not only improves neuronal cell culture maturation from a synaptic stance, but also from a tau isoform standpoint (Bardy et al., 2015; Satir et al., 2020).
Table 2. Expression of MAPT isoforms in Miguel et al, 2019

Percentages of MAPT isoform transcripts within 3D alginate cultures at 25 weeks. Cells cultured in neurobasal and BrainPhys media shown, revealing increased MAPT transcripts within cultures grown in BrainPhys. Table adapted from Miguel et al, 2019.

The authors subsequently examined tau isoforms at the protein level, observing that 0N3R was again the most prevalent isoform, with 0N4R, 1N3R, 1N4R also being present but at low levels (Miguel et al., 2019). The 2N isoforms, 2N3R and 2N4R, are absent from these 3D culture samples, however they could be present at extremely low levels that could not be detected by western blot.

With increasing evidence for neuronal maturation within 3D cultures as well as other advantages, examining MAPT splicing within cerebral organoid models was determined to be an interesting path of investigation.

1.15 Project aims

Tau splicing is intrinsically linked to neuronal health and disruption of tau splicing is sufficient to cause neurodegeneration. In vitro models which capture the complexity of tau splicing in a human neuronal system do not exist. To date, a detailed analysis of tau expression and splicing in cerebral organoids has not been performed. We hypothesised that cerebral organoids would express and splice tau in a similar way to what is seen in the adult human brain, and that this would be disrupted by splicing mutations in tau, this providing a physiologically relevant model to investigate disease mechanisms. The aim of this project was to establish cerebral organoids from iPSC harbouring MAPT mutations linked to FTD. Specifically, using isogenic iPSC lines with the 10+16 monoallelic, 10+16 biallelic, and 10+16 biallelic plus P301S biallelic mutations (Verheyen et al., 2018).
The specific aims of the project were to:

- Reprogram somatic patient cells into iPSC via episomal reprogramming.
- Establish the differentiation of iPSC into cerebral organoids utilizing different protocols.
- Determine whether iPSC carrying mutations in MAPT are competent to differentiate into cerebral organoids using molecular biology techniques.
- Investigate tau expression, splicing, and post-translational modifications within cerebral organoids and compare this to 2D iPSC-neurons via molecular biology techniques.
- Determine whether markers of tau pathology, such as hyperphosphorylation, are present within organoids using phosphotau specific antibodies.
- Investigate if cerebral organoids harbouring MAPT mutations produce seed-competent tau species via a cell based tau seeding bioassay.
2 Materials and Methods

2.1 Fibroblast culture

Dermal fibroblasts were obtained from skin biopsies taken from individuals carrying FAD mutations. The study was approved by the joint research ethics committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology (Study Title: Induced pluripotent stem cells derived from patients with familial Alzheimer’s disease and other dementias as novel cell models for neurodegeneration, Reference: 09/H0716/64). The protocol for generation and characterization of fibroblast lines was followed according to Wray et al, 2012 (Wray et al., 2012). Briefly, biopsies were sliced thinly using sterile disposable scalpels and placed in 5cm biopsy dishes (Corning, #430589). Fibroblast media, DMEM + GlutaMAX (Gibco, Thermofisher scientific, Paisley UK, #31966-021), 10% FBS (Gibco, Thermofisher scientific, Paisley UK, #11573397) and 0.5% Penicillin Streptomycin (Gibco, Thermofisher scientific, Paisley UK, #H4034-100G), was added to cover the biopsy pieces and carefully replaced every few days until explants adhered to the culture plate. When confluent, fibroblasts were harvested using 100µl trypsin (Gibco, Thermofisher scientific, Paisley UK, #25200056) and replated into T75 culture flasks (Nunc, Thermo Scientific, 156499). Once confluent and these cultures were passaged every 4-7 days using 1mL trypsin in to new T75 flasks and maintained in fibroblast media. Cell cultures were carried out in class 2 hoods in sterile conditions. All cells were incubated at 37°C and 5% CO₂.

2.2 Passaging of fibroblast cultures

Spent fibroblast media was removed and 5mL of Dulbecco’s phosphate-buffered saline (PBS) (ThermoFisher Scientific, 14190-326) added to the flask. After removal of the PBS, cells were detached by the addition of 1mL trypsin and incubated for 5 min at 37°C and 5% CO₂. Fibroblast media was added to neutralise the trypsin, and detached fibroblasts were split at a ratio of 1:4 into new T75 flasks (section 2.1). Cells were then placed back in the incubator at 37°C and 5% CO₂ and left to attach overnight.

2.3 Cryopreservation of fibroblast cultures

Cells were detached by the addition of trypsin for 5 min at 37°C and 5% CO₂ and suspended in excess fibroblast media. Cell suspensions were transferred to 15mL falcon tubes and centrifuged at 150g (rev)
for 3 min. Excess media was removed, and the pellet was re-suspended in fibroblast media + 10% DMSO (Sigma-Aldrich, D2650), before being placed into 2ml cryovials (Corning, CLS430488). A Nalgene® Mr. Frosty (Sigma-Aldrich, C1562) was prepared by filling with RT isopropanol and the fibroblast cryovials were added before being placed in the freezer at -80°C for 24h. For long term storage cells were transferred to liquid nitrogen.

### 2.4 Thawing Fibroblast Lines

Cyrovials were removed from the liquid nitrogen and thawed using either the incubator at 37°C or body heat. Once thawed, 500µL fibroblast media was added to the cryovial. The subsequent cell suspension was transferred to a 15mL falcon tube containing 4ml of fibroblast media and centrifuged at 150g(ave) for 3 min. Freezing media above the pellet was removed, cells re-suspended in fibroblast media and added to a T75 flask. Cells were incubated at 37°C and 5% CO2 overnight to allow cells to attach. The following day media is replaced to remove dead cells and any residual DMSO.

### 2.5 Reprogramming of fibroblasts into iPSC

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS15</td>
<td>PSEN1 - R278I</td>
<td>M</td>
<td>60</td>
<td>ION Biopsy</td>
</tr>
<tr>
<td>PS22</td>
<td>PSEN1 - Y115H</td>
<td>M</td>
<td>39</td>
<td>ION Biopsy</td>
</tr>
<tr>
<td>PS10</td>
<td>PSEN1 – E280G</td>
<td>M</td>
<td>46</td>
<td>ION Biopsy</td>
</tr>
<tr>
<td>APP15</td>
<td>APP – V717I</td>
<td>F</td>
<td>44</td>
<td>ION Biopsy</td>
</tr>
</tbody>
</table>

Table 3. Patient fibroblast cell lines used for episomal reprogramming to iPSC lines.

Fibroblast lines harboured mutations in PSEN1 and APP. Fibroblast lines were from three males and one female and were all collected in the institute of neurology.

Patient fibroblasts (Table 3) were used at low passage numbers (P2 – P3) for the generation of iPSC. When confluent, fibroblasts were detached from T75 flasks by the addition of trypsin and incubated at 37°C, 5% CO2 for 5 minutes. Fibroblast media was added to inhibit further enzymatic action and the flask gently tapped to enhance lifting of cells from the flask surface.
Plates were pre-coated with Geltrex (Gibco, A14133-02) (diluted 1:100 with DMEM/F12 (Gibco, #10565-018)) for 1h at 37°C. 10µL of the fibroblast suspension was removed for cell counting. The cell suspension sample was mixed with equal parts trypan blue and counted using a disposable haemocytometer. The remaining cells were collected by centrifugation at 150g (av) for 5 min at RT. The cell pellet was re-suspended at a concentration of two million cells per 100µl in nucleofection solution (Lonza, PBP2-00675) containing episomal DNA coding for Oct4, Klf4, Sox2 and L-myc, together with Lin28A and shRNA to p53 (AddGene, #27077, #27078, #27080) (Okita et al., 2011). The cell suspension was transferred to an Amaxa Nucleofector 2b cuvette (Lonza, AAB 1001) and electroporated using the program setup: NHDF, human, neonate. Electroporated cells were removed from the cuvette with an Amaxa pipette and plated equally between two geltrex coated (A1413302, Life Technologies) wells of a 6-well plate (10119831, Fisher Scientific). Electroporated cultures were initially maintained in fibroblast media which was replaced every three days.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Genes</th>
<th>Amount used for electroporation</th>
<th>Company</th>
<th>Ref #</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCXLE-hOCT3/4-shp53-F</td>
<td>OCT3/4 and shRNA against p53</td>
<td>1.7µg</td>
<td>AddGene</td>
<td>#27077</td>
</tr>
<tr>
<td>pCXLE-hSK</td>
<td>SOX2, KLF4</td>
<td>1.6µg</td>
<td>AddGene</td>
<td>#27078</td>
</tr>
<tr>
<td>pCXLE-hUL</td>
<td>L-MYC</td>
<td>1.7µg</td>
<td>AddGene</td>
<td>#27080</td>
</tr>
</tbody>
</table>

Table 4. Episomes from AddGene used for reprogramming of fibroblasts to iPSC.

Six days after electroporation, the reprogrammed fibroblasts were incubated for 5 mins at 37°C, 5% CO₂ with trypsin (15090-046, Gibco) before they were detached using fibroblast media and placed equally into two 15ml falcon tubes. Cells were centrifuged at 150g (av) for 5 min and the pellets re-suspended in 10ml of required media. Suspended cells were plated on either geltrex coated 10cm dishes (feeder free) and cultured in fibroblast media, or geltrex coated 10cm dish with a mouse embryonic fibroblast feeder layer. Mouse embryonic fibroblast CF-1 feeder cells (ATCC® SCRC-1040TM), MEFs, were plated on a geltrex coated 10cm dish (172958, Nunc) at approx. 0.8 X 10⁴ cells/cm² in fibroblast media. The next day, MEF cultures were transferred to human embryonic stem cell media (HES), consisting of: DMEM (Gibco #10565-018), 10% knock out serum replacement (KOSR) (Thermo #10828028), 1% GlutaMAX (Invitrogen, #35050-038) , 1% non-essential amino acids (NEAA) (Sigma-Aldrich, #M7145) and 0.1 mM 2-mercaptoethanol (Gibco, #31350-010). The media was then
supplemented with 10ng of FGF2 (Peprotech, #100-18). Eight days after electroporation, fibroblast feeder-free cultures were switched to Essential-8 (E8) stem cell media (Gibco, #A15169-01, + Supplement, Gibco, #A15171-01) and the media was changed daily. By day 21-35 iPSC colonies began to emerge on both feeder and feeder-free plate types.

2.6 iPSC colony picking and expansion

Colonies exhibiting an iPSC morphology were manually picked using a 1000ul pipette tip from both feeder and feeder-free cultures and each colony was plated in to a single well of a geltrex coated 24-well plate containing E8 media. Nascent iPSCs were fed daily and passaged every 5-7 days by removing the media, adding 0.5mL of 0.5mM Ethylenediaminetetraacetic acid (EDTA) for 5 min (15575-038, Invitrogen), and detaching the cells with E8 media. Once detached the clone was manually titrated and maintained into multiple wells, of a 24 well plate at 1:2 ratio and fed daily. Once clones reached 80% confluency, each well was plated in to a single well of a 6 well plate for further expansion. iPSC morphology was defined as a colony of tightly packed cells with a high nucleus to soma density ratio, smooth colony edges and no evidence of cellular migration or differentiation. Morphology was analysed visually on an Olympus CKX41 phase contrast microscope.

2.7 iPSC cell line maintenance

iPSC lines were maintained on 6-well plates (Fisher, #10119831) at 37°C and 5% CO₂. Plates were pre-coated with Geltrex. iPSC were fed daily using E8 media (Gibco, #A15169-01, + Supplement, Gibco, #A15171-01). iPSC were passaged every 4-5 days using EDTA. Briefly, media was aspirated and 1ml of 0.5mM EDTA (Invitrogen #15575-038) was added to each well, followed by incubation for 5 min at 37°C. EDTA was aspirated and colonies were manually detached using E8 media. Cells were seeded into new geltrex coated plates at a split ratio of 1:6. The iPSC lines used in this study are detailed in Table 5.

PSEN1 – R278I, PSEN1 – Y115H, and APP – V717I 2 were generated as part of this thesis.

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Table 5. iPSC lines used in this thesis.

### 2.8 Cryopreservation of iPSC lines

Cells were detached by the addition of EDTA for 5 min at 37°C and 5% CO₂. EDTA was aspirated and cells were re-suspended in E8 media + 10% DMSO (D2650, Sigma-Aldrich), before being placed into 2ml cryovials (CLS430488, Corning) and placed at -80°C in a Nalgene® Mr. Frosty (C1562, Sigma-
Aldrich) which was filled with RT isopropanol. For long term storage cells were transferred to liquid nitrogen after 24 hours at -80°C.

### 2.9 Thawing iPSC lines

Cyrovials were removed from the liquid nitrogen and warmed using either the incubator or body heat. Once thawed, 500μL E8 media was added to the cryovial. The subsequent cell suspension was transferred to a 15mL falcon tube containingXml of E8 media and centrifuged at 150g(ω) for 3 min. Freezing media above the pellet was removed, cells re-suspended in fresh E8 media and added to single well in a 6-well plate. Cells were incubated at 37°C and 5% CO2 overnight to allow cells to attach. The following day media is replaced to remove dead cells and any residual DMSO.

### 2.10 Differentiation of iPSC into 2D cortical neurons

Differentiation of iPSC into cortical neurons was performed using dual SMAD inhibition as described in Shi et al, 2012 (Shi, Kirwan, Smith, Robinson, et al., 2012). Briefly, 4-5 wells of an iPSC line were grown to 80% confluence, passaged with EDTA and pooled into 1 geltrex coated well of a 6-well plate. 24h later when the cells were 100% confluent, neural induction media was added: N2B27 containing 10 μM SB431542 (1614 - R&D Systems) and 1 μM dorsomorphin (R & D systems, #3039). N2B27 media consisted of a 1:1 mixture of Dulbecco’s modified eagle medium F12 and Neurobasal (Gibco, #12348-017) supplemented with 0.5% N2 (Gibco, #17502-048), 1% B27 (Gibco, #17504044), 0.5% non-essential amino acids (NEAA) (Sigma-Aldrich, #M7145), 1 mM L-glutamine (Gibco, #25030081), 25U pen/ strep (Gibco, #H4034-100G), 10 μM β-mercaptoethanol (Gibco, #31350-010) and 25U insulin (Sigma-Aldrich, #I9278-5ML).

Cells were fed daily with induction media. After 10 days the neuroepithelial layer was detached using dispase (50 U/ML, Invitrogen, #17105-041) and re-plated onto a laminin-coated 6-well plate (Sigma-Aldrich, #L2020, 1:100 diluted in PBS, Gibco, 10010023) in N2B27 media without SB43142 and Dorsomorphin.

Neuronal rosettes began to form from the plated neuroepithelial layer, and were fed every 48h with N2B27. Dispase was prepared by dissolving 0.5g (878 units) of dispase powder (#17105-041, Gibco) in 50ml PBS for 30 min at 37°C. The solution was then filtered using a syringe and 0.22μm filter and stored in aliquots at -20°C until needed. Laminin coated 12-well plates were also prepared via application of 1mL PBS solution containing 10μg/mL laminin (#L2020, Sigma-Aldrich) to 12-well plates.
followed by incubation overnight at 37°C, 5% CO₂. After incubation the laminin solution is removed and replaced with PBS. Plates can be sealed with Parafilm (#11772644, Fisher Scientific) and kept at 4°C until needed. Once cultures were confluent, each well of rosettes was passaged using dispase at 37°C and 5% CO₂ for 15 to 30 min. The clusters of cells were lifted, transferred to a 15ml falcon tube containing 10ml PBS and left to settle at the bottom of the tube. 95% of the DPBS was removed and the cell clumps were washed three more times with PBS to remove residual dispase. Cells were re-suspended in N2B27 media and were gently triturbated to break the larger clumps before plating into laminin coated 12-well pates (10098870, Fisher Scientific) at a split ratio of 1:2-1:5.

Cells were split at a ratio of a 1:2 and media was replaced with fresh media every 2-3 days. Cells were passaged again at around day 20, allowing further expansion of precursors. Once substantial neurogenesis could be observed (around day 27-31), cells were split a final time at a ratio of 1:2 – 1:5 using accutase, at 37°C, 5% CO₂ for 5-10 minutes (500µl per well on 12-well plate) (A11105-01, Gibco), to generate close to a single cell suspension. Once detached, cells were centrifuged at 180g (av) for 3 min, re-suspended in N2B27 and plated at a final density of 50,000 cells per cm² onto 12-well plates coated with poly-ornithine (Sigma-Aldrich, #P4957) and laminin. Media was replaced every 3-4 days and samples were taken at the required time points.

2.11 Differentiation of iPSC into Cerebral Organoids (COs)

Organoids were generated using the protocol described by Lancaster et al (Lancaster et al., 2013). Briefly, iPSC lines were detached from plates using EDTA as described in section 2.1, after which 500 µL of accutase (Gibco #A11105-01) was added to the well for 3 min at 37°C in order to generate a single cell suspension. The dissociated cells were collected with an additional 500uL of E8 media (total volume 1mL) and 10uL were taken for cell counting purposes. The 10uL sample was added to 10uL of
trypan blue, to label dead cells, and counted using a disposable haemocytometer. The remaining cells were centrifuged at 270g (av) for 5 min at RT, after which the cells were suspended in standard knockout serum media (KSM): DMEM F12 containing 10% knock out serum replacement (KOSR) (Thermo #10828028), 3% embryonic stem cell quality fetal bovine serum (ESC-Quality FBS) (Invitrogen, #10828-028), 1% GlutaMAX (Invitrogen, #35050-038), 1% non-essential amino acids (NEAA) (Sigma-Aldrich, #M7145) and 0.1 mM 2-mercaptoethanol (Gibco, #31350-010). Cells were diluted to a concentration of 1000 cells per 1µL and 9000 iPSC (9uL of the cell suspension) were seeded per well into a low attachment round bottomed 96 well plate (Thermo #145399), using 141µL of KSM with the addition of 4ng/mL of bFGF (Peprotech, #100-18B) to bring each well to 150µL in total. To ensure cell numbers were homogenous between wells, a master mix of cells and media was made depending on the number of wells being seeded. 150µl of this master mix cell suspension was then aliquoted out to each well.

24h post-seeding, embryoid bodies were fed with KSM. Embryoid bodies were then fed every 48h. For the first 4 days bFGF was added at 4ng/mL and 50mM Y-27632 ROCK inhibitor (VWR, #688000-5). After which both were removed from the KSM. At day 6 the embryoid bodies were manually transferred to low attachment 24 well plates (Corning, #3473) and induction media was added: DMEM F12 containing 1% N2 supplement (Gibco, #17502-048), 1% NEAA (Sigma-Aldrich, #M7145) and Heparin (Sigma-Aldrich, #H3149-25KU) to a final concentration of 1µg/mL.

At day 10-12 each neuroepithelial tissue / organoid was examined visually, any that had failed to form clear neuroectoderm layers, or were damaged, were discarded. Organoids that passed this assessment were transferred to a parafilm mould. Excess media was removed and 20-30µL of Matrigel (BD Biosciences, #356234) was placed on top of each organoid, before centring the tissue within the drop of matrigel. These were placed in the incubator for 10-12 min until the Matrigel polymerised. The moulds were gently pushed from behind to release the embedded organoids and then placed in a 5cm tissue culture dish (Corning, #430589) containing Cerebral Organoid Differentiation Media (CODM) without Vitamin A: 1:1 ratio of DMEM-F12 Medium and Neurobasal Medium (Gibco, #12348-017), 0.5% N2 supplement, 0.25% Insulin (Sigma-Aldrich, #19278-5ML), 1% GlutaMAX supplement, 0.5% NEAA, 0.5% Penicillin Streptomycin (Gibco, #H4034-100G), 50uM 2-mercaptoethanol, of B27 supplement without Vitamin A (Gibco, #12587010).

Following 48h static incubation, the dishes were moved onto an orbital shaker (IKA, #0009019200) at 55-85 rpm with a minimum orbital diameter of 10mm to achieve organoid and media movement.

The media was replaced with CODM at this stage with the addition of vitamin A within the B27 supplement (Gibco, #17504044). Samples were kept on an orbital shaker for as long as needed with
media replacement every 72 hours. Samples were taken for experimental analysis at the required time points. The protocol for the generation of cerebral organoids is summarised in Figure 12.

### 2.12 Generation of forebrain organoids

**Figure 13. Diagram of forebrain organoid culture.**

*Simplified diagram showing the stages of forebrain organoid culture. The top grey bar shows simplified main stages of organoid culture, with the bottom yellow to orange bar showing media changes through organoid culture.*

Cerebral organoids with a forebrain identity were generated using dual SMAD inhibition according to the protocol published in Qian et al 2016 (Qian et al., 2016). Briefly, EBs were generated as described in the CO protocol (section 2.X) by the seeding of iPSC, but maintained in Dual SMAD media: KSR media (described above) with the addition of 2 μM dorsomorphin (R & D systems, #3039) and 2 μM SB431542 (1614 - R&D Systems). 50mM Y-27632 ROCK inhibitor (VWR, #688000-5) was added for the first four days.

At day 5 the media was replaced with Dual SMAD induction media: DMEM F12 containing 1% N2 supplement (Gibco, #17502-048), 1% NEAA (Sigma-Aldrich, #M7145), Heparin (Sigma-Aldrich, #H3149-25KU) to a final concentration of 1μg/mL, 1 μM SB431542 (1614 - R&D Systems) and 1μM CHIR99021 (Tocris, #4423). On day 7 the forebrain organoids were embedded in clear matrigel as described in 2.11 and maintained in Dual Smad induction media. On day 14 the forebrain organoids were switched to Cerebral Organoid Differentiation Media (CODM) with added vitamin A, as described in 2.11. In addition, DS organoids were transferred to the orbital shaker (IKA, #0009019200) at 55-85 rpm on day 14 of differentiation.
2.13 Generation of Engineered Cerebral Organoids (enCORs)

EnCORs were generated according to the protocol described by Lancaster et al (Lancaster et al., 2017). PLGA Vicryl violet sutures (Ethicon, #W9567) were cut in a petri dish using a scalpel and tweezers into small pieces approximately under 1mm in length, all cutting was done in a tissue culture hood. 5-15 fibres were placed in each well of a 96 well round bottomed low attachment plate (CoStar, #7007).

18,000 iPSC (prepared as described in 2.11) were seeded per well on top of the PLGA fibres. The embryoid bodies were maintained as described in 2.11, embedded in Matrigel at day 11-12, but kept in induction media. Two days post-embedding, the media was changed on the enCORs to improved differentiation media minus vitamin A (IDM-A): 1:1 ratio of DMEM-F12 Medium and Neurobasal Medium, 0.5% N2 supplement, 0.025% Insulin, 1% GlutaMAX supplement, 0.5% NEAA, 0.5% Penicillin Streptomycin, 50µM 2-mercaptoethanol, 2% of B27 supplement without vitamin A. On days 13-16 3µM CHIR99021 (Tocris, #4423) was added to the media.

On day 18-20 the enCORs were moved to the orbital shaker at 85rpm, with a minimum orbital diameter of 10mm to achieve organoid and media movement. The media was also changed to Improved Differentiation Media plus Vitamin A & C+HEPES (IDM+vA): 125mL DMEM-F12 Medium, 125mL Neurobasal Medium, 0.5% N2 supplement, 0.025% Insulin, 1% GlutaMAX supplement, 0.5% NEAA, 0.5% Penicillin Streptomycin, 50mM 2-mercaptoethanol, 2% of B27 supplement with Vitamin A, 0.5µg/mL Vitamin C / Ascorbic acid (Sigma-Aldrich), 1.49g HEPES per 500mL (Sigma-Aldrich, #H4034-100G). The media was changed every 3-4 days and 1% Matrigel was dissolved in the media from day 40 onwards.

Figure 14. Diagram of engineered cerebral organoid culture.

Simplified diagram showing the stages of engineered cerebral organoid culture. The top grey bar shows simplified main stages of organoid culture, with the bottom yellow to orange bar showing media changes through organoid culture.
2.14 Collection of conditioned media

Conditioned media was collected from iPSC derived cortical neurons, cerebral organoids and engineered cerebral organoids. 72h before the desired time point, the media was completely changed on the cultures. At the desired time point, all the media was collected into a 15mL falcon tube and replaced with fresh media. Conditioned media was centrifuged at 2000g (av) for 5 min at RT to remove dead cells and cell debris. Media was aliquoted into 1ml volumes in low binding screw cap tubes (72.694.406, Sarstedt) and immediately stored at -80°C.

2.15 Fixation of organoids and preparation of frozen sections

Whole COs and enCORs were fixed in 4% paraformaldehyde for 30min at RT, washed 3 x 1min with PBS and immersed in 30% sucrose overnight at 4°C. Sucrose was removed and the tissue placed in premade foil moulds with optimal cutting temperature compound (OCT; VWR - 361603E). Moulds were frozen on dry ice and 10 µm sections were prepared using the cryostat. Sections were placed serially on frost plus slides (VWR 631-0108) and allowed to air dry for 10min before being frozen at -80 degrees.

2.16 Immunocytochemistry

Tissue sections were permeabilised using PBS 0.3% Triton X-100 (PBS-T), (3 x 5min washes) before blocking with 5% bovine serum albumin (BSA) in PBS-T for 1h at RT. Primary antibodies (Table 6) diluted in blocking solution were applied to the sections overnight at 4°C. Following 3 x 5 min washes with PBS-T, cells were incubated with secondary antibodies (Table 5) at 1:1000 concentration in blocking solution for 1h at RT. Following 3 x 5 min PBS-T washes, nuclei were stained with 0.1 µg/ml 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, D9542-1MG) in BSA solution for 5min and then washed with PBS-T. Fluorescent Mounting Medium (DAKO, #53023) was applied to the slide and a coverslip placed on top before being stored at 4°C.

Slides were imaged using a Leica CTR6000 microscope and Hamamatsu C10600 camera.
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Table 6. Antibodies used for immunocytochemistry in this study.

2.17 Preparation of protein lysates

Organoids or 2D neuronal cultures were lysed in RIPA buffer (10mM Tris-Cl, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140mM NaCl). Protease inhibitors (Roche, #11836170001) and phosphatase inhibitors (Roche, #04906845001) were added to the RIPA buffer immediately prior to use. Disposable cell scrappers (Fisher, #08-100-241) for 2D cultures or disposable pestles (Corning, #PES-15-B-51) for 3D cultures were used to help mechanically lyse samples, after
which they were incubated at 4°C with agitation for 20 min. Samples were centrifuged at 12,000g_{av} for 15 min at 4°C to remove debris, and lysates were then stored at -80°C until use.

2.18 SDS-PAGE and Western Blotting

Bicinchoninic acid (BCA) assay was used to determine total protein concentrations of cell/organoid lysates. Briefly a Bio-Rad detergent compatible (DC) protein assay kit was used (5000111, Bio-Rad). Standard protein solutions of 4, 2, 1, 0.5, 0.25 and 0.125mg/mL were made using bovine serum albumin (BSA) (A9647-100G, Sigma-Aldrich) solubilise in RIPA buffer. 20 µl of reagent S is added to each ml of reagent A that will be required, creating reagent SA. 5µL of each standard and each sample was loaded into an individual well of a 96-well plate (655061, Greiner bio-one), all in triplicate. 25µL of reagent SA was added to each well after which 200µL of reagent B was added to each well. After removing any bubbles from the wells and incubating at RT and away from light for 15 min, absorbance was read using a plate reader (Spark 10M, Tecan) at 750nm. A standard curve was prepared using the readings from the protein standards, which was then used to calculate sample protein concentrations.

Equal amounts of protein were mixed with 4x LDS loading buffer (Invitrogen, #NP0007) supplemented with 20mM DTT and heated at 98°C for 10 min before being centrifuged for 5 min at 12,000g_{av} at RT. Protein samples were separated on 4-12% SDS-PAGE gels (Invitrogen, #NP0321BOX) at 150V for 3h in running buffer (MES NuPAGE SDS, NOVEX, #NP0002), on ice. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (GE Healthcare, #10600003) for 1hr 15 min at 30V on ice in transfer buffer consisting of 25 mM Tris, 192 mM glycine, (Geneflow, #EC-880) and methanol was added to achieve 4.9095 molar concentration (VWR, #UN1230) (20% of final solution). Membranes were heated in an oven at 37°C until dry, rehydrated with transfer buffer and blocked in 5%-non-fat milk in PBS for 1h prior to overnight incubation at 4°C in primary antibody (Table 4) diluted in 5% milk in PBS-T. The next day, membranes were washed 3 X 5 min in PBS-T before incubation for 1h with secondary antibodies (Anti-mouse Alexa Fluor, #A21058 & anti-rabbit Rockland, #611-145-122) diluted (1:20,000) in 5% milk in PBS-T. After incubation, 3 x 5 min PBS-T washes were performed with a final PBS wash to remove detergent before imaging. Secondary antibodies were visualised at 700nm and 800nm using the Li-Cor Odyssey Fc system.
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<td>AT8</td>
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<td>MN1020</td>
<td>Thermo</td>
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<td>PHF1</td>
<td>Mouse</td>
<td>N/A</td>
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<td>Mouse</td>
<td>801202</td>
<td>BioLegend</td>
<td>1:1000</td>
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<tr>
<td>β-Actin</td>
<td>Mouse</td>
<td>A2228</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>AM4300</td>
<td>Invitrogen</td>
<td>1:1000</td>
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Table 7. Antibodies used for immunoblotting in this study.
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<tr>
<th>Name / Target</th>
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<th>Product Code</th>
<th>Company</th>
<th>Dilution</th>
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<td>Rockland</td>
<td>1:20,000</td>
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<tr>
<td>Secondary</td>
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<td></td>
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</tr>
<tr>
<td>Immunoblot</td>
<td>Goat anti Mouse</td>
<td>A21058</td>
<td>Invitrogen</td>
<td>1:20,000</td>
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<td>Secondary</td>
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</tr>
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</tr>
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<td>Donkey anti Rabbit</td>
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<td>Invitrogen</td>
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<td>568nm</td>
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<td>Invitrogen</td>
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<td></td>
</tr>
<tr>
<td>ICC Secondary</td>
<td>Donkey anti Rabbit</td>
<td>A21204</td>
<td>Invitrogen</td>
<td>1:1500</td>
</tr>
<tr>
<td>488nm</td>
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<td></td>
</tr>
</tbody>
</table>

Table 8. Secondary antibodies used in this study.

2.19 Protein dephosphorylation

Dephosphorylation was carried out as first described in Hanger et al 2002 (Hanger et al., 2002). Equal amounts of protein were diluted in RIPA buffer with the required amount of Protein MetalloPhosphatases (PMP) buffer (New England Biolabs, #B07615) and 10mM MnCl2 (New England Biolabs, #B17615) and 2000 units of Lambda protein phosphatase (New England Biolabs, #P0753L). Samples were vortexed gently for 10s before being incubated at 30°C for 3h. Dephosphorylation reactions were stopped by the addition of LDS loading buffer and heating at 100°C for 5 min.
2.20 RNA Extraction

Organoids were lysed in 1mL of Trizol using disposable pestles (Corning, #PES-15-B-51) and incubated at 4°C with agitation for 10 min. RNA was extracted from these lysates according to the manufacturers protocol.

Briefly, 0.2mL of molecular biology grade chloroform (Fisher, 11426461) was added to the samples per 1mL of Trizol used and incubated at RT for 2-3 min, before centrifugation at 12,000g for 15 min at 4°C. The aqueous phase was pipetted to another tube and 0.5mL of isopropanol was added, incubated for 10 min and centrifuged at 12,000g for 10 min at 4°C. The supernatant was discarded and 1mL of cold 75% ethanol was added to precipitate the RNA. Samples were briefly vortexed before centrifugation at 7,500g at 4°C. The supernatant was removed and the pellet was air dried for 10 min at RT. RNA pellets were diluted in 20µL of RNase free water (Ambion, #AM9937) and stored at -80°C.

RNA concentration in ng/µL was determined by Nanodrop (Nanodrop, ND-1000). The 260/280 ratio was used to determine protein contamination, pure RNA and DNA. 260/280 ratios should be somewhere around 2.1 and 1.8 for RNA and DNA, respectively. A lower ratio indicates the sample is contaminated with protein. The 260/230 ratio was used to determine the presence of organic contaminants, such as phenol, Trizol and other aromatic compounds. A pure sample 260/230 ratio should be close to 2.0. Samples with 260/230 ratios below 1.8 are considered to have a significant amount of these contaminants.

2.21 Reverse Transcription

1000ng of RNA was reverse transcribed using Super Script IV kit (SSIV, Invitrogen 18090010) according to manufacturer’s instructions. Along with samples, control brain RNA was reverse transcribed at the same time (Thermo, AM7962). Briefly, 1000ng of template RNA was added to RNase free water, 2.5uM random hexamers (Invitrogen, #100026484) and 10mM dNTP mix (Invitrogen, #55082, #55083, #55084, #55085) and heated at 65°C for 5 min and then cooled on ice for 1 min. After which SSIV buffer, 100mM DTT, RNaseOUT (Invitrogen, #100000840) and SSIV reverse transcriptase was added to the reaction, heated to 23 °C for 10 mis, incubated at 50-55 °C for 10 min and finally inactivated by heating at 80°C for 10 min. The cDNA was then stored at -20°C.
2.22 PCR

50ng of cDNA was used per PCR reaction using New England Biolabs taq (NEB M0273S). PCR reactions were set up according to the manufacturers’ protocol. Briefly, for 25µL reaction volume, 10X standard Taq reaction buffer was added in an X1 concentration, to which 200uM of dNTPs, 0.2µM of forward primer, 0.2µM of reverse primer (Table 9 details primers used in this thesis), 50ng template cDNA and 1.25 units/50µL taq was added to each reaction. Nuclease free water was added to make the reaction volume up to 25µL.

GoTaq Green (Promega, M7123) was also used for PCR reactions. Briefly, 10uL of GoTaq green, 0.2µM of forward primer, and 0.2µM of reverse primer was added to 50nM of cDNA. Nuclease free water was added to make the reaction volume up to 20uL. Primers were designed in house by the Wray lab group.

PCR products were separated by electrophoresis on an agarose gel composed of 1% or 2% agarose (Sigma-Aldrich, #A9539) dissolved in 1X TBE (Sigma-Aldrich, #93290) with 1X GelRed (Biotium, #BT41003) alongside a 100 base pair ladder (Bioline, #H4-617110A) at 75V for between 60 and 90 min. PCR products were visualised at 600nm using the Li-Cor Odyssey Fc system.

<table>
<thead>
<tr>
<th>PCR Primers</th>
<th>Name</th>
<th>Sequence 5’ to 3’</th>
<th>Programme</th>
<th>Product Length</th>
</tr>
</thead>
</table>
|             | 9-13 R Tau Forward | GTCAAGTCCAAGATCGGCTC | 94°C – 5 mins  
35 Cycles | 3R – 305bp  
4R - 398bp |
|             | 9-13 R Tau Reverse | TGGTCTGTCTTGGCTTTGGG | 94°C – 30 secs  
58°C – 15 secs  
72°C – 45 secs  
Final  
72°C – 10 mins |               |
<p>|             | 2-3 N Tau Forward | TGAACCAGGATGGCTGAGC | 94°C – 5 mins |               |</p>
<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Primer Sequence</th>
<th>Conditions</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-3 N Tau Reverse</td>
<td>TTGTTCATCGCTTCCAGTCC</td>
<td>35 Cycles</td>
<td>0N - 112bp, 1N - 199bp, 2N - 286bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94°C - 30 secs</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>55°C - 15 secs</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C - 45 secs</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C - 10 mins</td>
<td></td>
</tr>
<tr>
<td>2-3-4 N Tau Forward</td>
<td>TACGGGTTGGGGGACAGGAAACAT</td>
<td>94°C - 5 mins</td>
<td>0N - 253bp, 1N - 341bp, 2N - 428bp</td>
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<td></td>
<td></td>
<td>35 Cycles</td>
<td></td>
</tr>
<tr>
<td>2-3-4 N Tau Reverse</td>
<td>GGGGTGTCTCCAATGCCTGCTTTCT</td>
<td>94°C - 30 secs</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>60°C - 15 secs</td>
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<td>72°C - 45 secs</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>72°C - 10 mins</td>
<td></td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>CCATGGCACCCTCAAGGCT</td>
<td>94°C - 5 mins</td>
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<td></td>
<td></td>
<td>35 Cycles</td>
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<tr>
<td>GAPDH Reverse</td>
<td>GCCAGTAGAGGCAGGGATG</td>
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<tr>
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<td>60°C - 15 secs</td>
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<td>72°C - 45 secs</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C - 10 mins</td>
<td></td>
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<tr>
<td>Integration PCR Primer</td>
<td>ATCGTCAAAGCTGCACACAG</td>
<td>94°C - 2 min</td>
<td>666bp</td>
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<tr>
<td>(pEP4-SF2-oriP)</td>
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<td>35 Cycles</td>
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<td>94°C - 30 sec</td>
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</tr>
<tr>
<td>Integration PCR Primer</td>
<td>CCCAGGAGTCCCAGTAGTCA</td>
<td>55°C - 30 sec</td>
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<tr>
<td>Primer - Reverse</td>
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</table>
2.2.3 qPCR

Each reaction consisted of 10uL of SYBR (4472908, Thermo), 100nM of each forward and reverse primers, and nuclease free water was used to make each reaction a total volume of 18uL. Primers used for qPCR in this thesis are detailed in Table 10. A master mix for all wells utilising the same primer pairs was made for increased accuracy and homogeneity. 18µL of master mix was pipetted carefully in to each well, after which 100ng of each cDNA samples, in triplicate, was pipetted into the corresponding wells (2µL in this case to bring the total reaction volume to 20µL). cDNA from human brain was used as a positive control as well as H2O negative control. qPCR cycles were performed using an MX300P qPCR machine (Agilent). Melt curves were run for each primer set as well as agarose gels to determine a single product was being produced.

Analysis of the CT values produced was achieved by calculating the delta delta CT of each well against a control primer set. For example, 2N against total tau:

1. Delta CT calculation: 2N CT value - tTau CT value
2. Delta delta CT calculation: delta CT (sample) - control brain Delta CT
3. Fold change calculation – The power of 2, minus the above value

| MAP2 (1) | ATAGACCTAAGCCATGTGAC | 94°C – 10 min | 177 bp |

Table 9. PCR primers used in this study.

<table>
<thead>
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<th>Name</th>
<th>Sequence 5’ to 3’</th>
<th>Programme</th>
<th>Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP2 (1)</td>
<td>ATAGACCTAAGCCATGTGAC</td>
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<td>177 bp</td>
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<tr>
<td>Forward</td>
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<tr>
<td>Gene/Forward</td>
<td>Primer Sequence</td>
<td>Temperature Protocol</td>
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</tr>
<tr>
<td>-------------</td>
<td>-----------------</td>
<td>---------------------</td>
<td></td>
</tr>
</tbody>
</table>
| MAP2 (1) Reverse | AATCTTGACATTACCACCTC | 60°C – 60 sec  
72°C – 60 sec  
Final  
95°C – 60 sec  
55°C – 30 sec  
95°C – 30 sec |
| RPL18A Forward | CCCACAACATGTACGGGA | 94°C – 10 min  
40 Cycles  
95°C – 30 sec  
60°C – 60 sec  
72°C – 60 sec  
Final  
95°C – 60 sec  
55°C – 30 sec  
95°C – 30 sec |
| RPL18A Reverse | TCTTGGAGTCGTGGAACTGC | 180 bp |
| Total Tau (2) Forward | TGCAATAGTCTACAAACCAGT | 94°C – 10 min  
40 Cycles  
95°C – 30 sec  
60°C – 60 sec  
72°C – 60 sec  
Final  
95°C – 60 sec  
55°C – 30 sec  
95°C – 30 sec |
<p>| Total Tau (2) Reverse | CTTGTGGGTTTCAATCTTTT | 209 bp |</p>
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<th>Product Size</th>
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</thead>
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<td>0N (2) Forward</td>
<td>GTCGACTATCAGGTAACCTT</td>
<td>94°C – 10 min, 40 Cycles, 95°C – 30 sec, 60°C – 60 sec, 72°C – 60 sec</td>
<td>173 bp</td>
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<td>0N (2) Reverse</td>
<td>TGCTTTCTCAGCTTTCAG</td>
<td>Final, 95°C – 60 sec, 55°C – 30 sec, 95°C – 30 sec</td>
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<tr>
<td>1N Forward</td>
<td>CAACAGCGGAAGCTGAAGAA</td>
<td>94°C – 10 min, 40 Cycles, 95°C – 30 sec, 64°C – 60 sec, 72°C – 60 sec</td>
<td>68 bp</td>
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<tr>
<td>1N Reverse</td>
<td>GTGACCAGCAGCTTTCGTT</td>
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<tr>
<td>2N Forward</td>
<td>ACTCCAAACAGCGGAAGATGT</td>
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<td>159 bp</td>
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<tr>
<td>2N Reverse</td>
<td>GTGACCAGCAGCTTTCGTT</td>
<td>Final</td>
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Table 10. qPCR primers used in this study

<table>
<thead>
<tr>
<th></th>
<th>95°C – 60 sec</th>
<th>55°C – 30 sec</th>
<th>95°C – 30 sec</th>
</tr>
</thead>
</table>

2.24 Tau biosensor cell assay

HEK cells expressing the Tau RD P301S FRET biosensor (ATCC® CRL-3275™, were cultured in DMEM + GlutaMAX (Gibco, #31966-021) with 10% FBS (Gibco, #11573397) in T75 flasks. Every 3-5 days cells were split using trypsin (Gibco, #25200056) in a 1:10 ratio into a clean T75 flask. For experimental use, 115,000 cells were plated on autoclaved 13mm coverslips (VWR, #631-0148P) in 24 well plates (Thermo, #142485) and left to attach for 24h. The following day, after checking attachment was satisfactory, Lipofectamine 3000 (Invitrogen, #L3000-001) was used according to the manufacturers protocol, to transfect the Tau RD P301S FRET biosensor cells with 10ug of enCOR lysate, AD brain lysate or a negative control. Briefly, per reaction 1µL of Lipofectamine reagent was added to 25µL of Opti-MEM (11058021, Thermo), vortexed and left to incubate for 10 mins. Meanwhile in a new tube 25µL of Opti-MEM had 5µg of enCOR or human brain lysate added along with 1.5 µL P3000 reagent. Both tubes were mixed gently together and incubated at RT for 20 min. Media on the Tau RD P301S FRET biosensor cells was removed and replaced with fresh media before the Lipofectamine and protein solution was gently pipetted on to the cells. The media was not changed and after 72h cells were fixed with 4% PFA, stained with 4’,6-diamidino-2-phenylindole (DAPI) and mounted on to slides using fluorescent mounting medium. Slides were imaged on a confocal microscope (Zeiss LSM 710 equipped with a Zeiss mercury lamp, model HXP 120 C) at 405 and 488nm with normal detection, as well as solely at 405nm with detection changed for FRET signal / YFP at 525-550nm.

Data within the results chapters that has been statistically analysed has been highlighted. Said data was subjected to a Shapiro-Wilk normality test and a D’Agostino-Pearson omnibus normality test. Data sets that passed the normality test was subjected to a one-way ANOVA test. Data not passing the normality test was subjected to a nonparametric one-way ANOVA.
3 iPSC models of Alzheimer’s disease; generation of iPSCs and cerebral organoid models

3.1 Introduction

As discussed in the introduction of this thesis, 3D stem cell derived models offer unique opportunities for the study of neurological disorders. Having already been successfully implemented for research into developmental disorders (M. A. Lancaster and Knoblich, 2014; Qian et al., 2016; Watanabe et al., 2017; Setia and Muotri, 2019), many groups utilise these new models to investigate neurodegenerative disease. One of the interesting aspects of 3D models is they allow metabolites and secreted proteins to remain within the 3D culture, meaning they are not removed during media changes as they are during 2D culture. As one of the main pathologies of AD, the amyloid plaques, are extracellular, the nature of 3D culture has the potential to provide a more suitable model for the study of extracellular pathologies.

3.1.1 Amyloid precursor protein processing

Amyloid precursor protein (APP) is cleaved by alpha-secretase, beta-secretase, and gamma secretases (α, β, and γ-secretases), creating cleavage products of various lengths. Among these are the amyloidogenic Aβ species, generated by β and γ-secretase cleavage, that are prone to aggregate and eventually contribute towards amyloid plaques.
**Figure 15. Amyloid precursor protein (APP) processing.**

Simplified diagram of APP processing showing the roles of membrane secretases in Aβ production. A) APP processing resulting in longer more amyloidogenic Aβ species. B) APP processing resulting in non-amyloidogenic products. APP – Amyloid precursor protein. APPsα/APPsβ – APP secreted α/β. α-CTF (C83)/β-CTF (C99 – α/β carboxyterminal fragment. AICD - APP intracellular domain. Figure Cole and Vassar et al, with image under the creative commons license (Cole and Vassar, 2007).

APP is membrane bound and is cleaved by either α-secretase or β-secretase into alpha C-terminus fragments (αCTF) or beta C-terminus fragments (βCTF, also called C99). These fragments can both be cleaved further by γ-secretase, both producing the APP intracellular fragment (AICD), but also a secondary protein depending on the pathway. AICDs function is not entirely known although it has been linked to a transcription based role (Multhaup et al., 2015). Cleavage of α-CTF via γ-secretase produces, along with AICD, the P3 peptide. This fragment has been shown to be swiftly degraded and was once thought of as innocuous, however it has been linked to neurotoxic effects (Dulin et al., 2008). βCTF also creates ACID on cleavage but γ-secretase is not precise, meaning this reaction also gives rise to Aβ fragments through an amyloidogetic pathway (Figure 15). This final cleavage of β-CTF is imprecise and Aβ species of 38-49 amino acids in length can be created (Steiner et al., 2018).
longer species are more neurotoxic and prone to aggregation (Jarrett, Berger and Lansbury, 1993; Meisl et al., 2014). APP processing is summarised in Figure 15.

![Figure 15. APP processing](image)

**Figure 15. APP processing.**
Simplified diagram of the major cleavage sites on β-CTF in APP processing, giving rise to Aβ species. γ-secretase mediated production of Aβ49, Aβ46, Aβ43, and Aβ40 in red and Aβ48, Aβ45, Aβ42, and Aβ38 in blue.

Mutations in APP, together with the genes coding for the catalytic core of γ-secretase, PSEN1 and PSEN2, cause early onset familial AD (Kang et al., 1987; Levy-Lahad et al., 1995; Sherrington et al., 1995; Bekris et al., 2010). All known fAD mutation alter APP processing, resulting in a higher proportion, of longer amyloidogenic Aβ peptides (Szaruga et al., 2015).

3.1.1 Modelling Aβ pathology in iPSC-neurons

Mutations in APP, PSEN1 and PSEN2 are all causative of familial Alzheimer’s Disease and all are linked to altered APP processing (Kang et al., 1987; Hardy, 1991). Mutations in APP are clustered around the β and γ cleavage sites, and presenilin-1 or 2 forms the catalytic core of GSEC (Wolfe, 2019). Pathogenic mutations converge to alter APP processing, resulting in a higher ratio of longer, more amyloidogenic Aβ peptides to be produced (Zhao et al., 2004; Zhang et al., 2011). Mutations within APP favour processing along the amyloidogenic pathway, resulting in more total Aβ being produced (Ohshima et al., 2018). While mutations in PSEN1, generally, reduce processivity and increase the production of the longer and more hydrophobic Aβ species (Sun et al., 2017; Steiner et al., 2018).

iPSC derived neurons from fAD patients with mutations in APP (duplication, V717I), PS1 (A246E, L166P) and PS2(N141I) have been show to present an increased Aβ42:40 ratio and an increase in phosphorylated tau compared to control iPSC-neurons (Yagi et al., 2011; Yahata et al., 2011; Israel et al., 2012; Koch et al., 2012; Moore et al., 2015).
Several groups have generated iPSC models of fAD causative mutations and have investigated the impact on APP processing.

In these studies, Aβ species secreted into the media were collected and measured. Aβ accumulations have rarely been observed in 2D cultures, likely due to the removal of aggregation-prone species during media changes. One exception to this is Downs Syndrome neurons, which have increased Aβ levels due to trisomy of chromosome 21, where APP is located (Shi, Kirwan, Smith, MacLean, et al., 2012).

Altered APP processing has also been investigated within sporadic patient-derived lines, with mixed results. Some sporadic lines showing similarities to fAD neurons but others much more like control lines (Kondo et al., 2013). Israel et al, 2012, uncovered significantly increased levels of some pathological markers, such as Aβ1-40 and phosphotau T231 (via detection kits) as well as increased aGSK-3b levels (via total GSK-3b duplex kit) within fAD APP duplication iPSC (Israel et al., 2012). Interestingly these increased levels were also seen within a sAD patient iPSC neuronal line, but another sAD line failed to recapitulate these results. This shows the complexity of APP processing, and the myriad of mechanisms contributing to both fAD and sAD.

Few groups have shown amyloid plaques or tau fibrils within 2D or 3D iPSC neuronal cultures, however these experiments are usually conducted with the addition of exogenous or patient derived Aβ or tau protein seeds (Vazin et al., 2014; Nieweg et al., 2015; Reilly et al., 2017). Some 3D cultures have also shown plaques, however they often use ReN cell models or use duplicate gene cell lines (Choi et al., 2014; Kim et al., 2015; Gonzalez et al., 2018). ReN cells are an immortalised human neural progenitor cell line with the capability to differentiate in to neurons and glia (Donato et al., 2007; Choi et al., 2014). Although a powerful biological research tool, they harbour mutations which could affect outcomes as well as being highly proliferative.

As antibodies and levels of detection have improved, many studies in AD and other dementia patients have demonstrated the presence of tau, phosphotau and Aβ fragments within the CSF, and recently blood plasma (Hansson et al., 2019; Janelidze, Mattsson, et al., 2020). These advances increase the possibility of using these biomarkers as a diagnostic technique to differentiate between different clinical subtypes of dementias. Related to these patient centric findings, these proteins have also been discovered in media of iPSC derived neurons (Hu et al., 2018; Penney, Ralvenius and Tsai, 2019), paving the way for in vitro studies of these secreted proteins and their mechanisms. In contrast to plasma/CSF, iPSCs offer an insight into the earliest consequences of disease and the underlying effects of mutation as well as secreted proteins of interest.
3.1.2 Cerebral organoids to model neurodegenerative disease

Although 2D models have recapitulated the effects of fAD mutations on APP processing, they have largely failed to recapitulate Aβ aggregation, due to Aβ removal during media changes. The generation of cerebral organoids may allow the development and investigation of Aβ aggregates, as the Aβ may be trapped within the 3D tissue rather than released into the media.

Cerebral organoids differentiate according to intrinsic signalling and self-organisation, recapitulating the temporal stages and spatial patterning of neuronal development in manner similar to in vivo (Lancaster ref). Through developmental cues and spatial patterning, it is hypothesised that neurons within this model are more in-vivo like (Luo et al., 2016; Nascimento et al., 2019).

Cerebral organoids have already been successfully implemented in research into multiple developmental disorders including microcephaly, Zika virus, and developmental disorders (Qian et al., 2016; Watanabe et al., 2017; Setia and Muotri, 2019), with many groups are now using these new models to investigate neurodegenerative disease.

The development of 3D tissue-based models prompted many researchers to investigate whether these would be a good system to study Aβ accumulation.

One of the first 3D models of fAD used ReN cells overexpressing PSEN1 with the ΔE9 mutation or over expression of APP with both K670N/M671L(Swedish) and V717I (London) FAD mutations as well as the PSEN1 ΔE9 mutation (Choi et al., 2014). By suspending these cells in Matrigel to generate a 3D culture, they were able to observe robust extracellular Aβ deposits by staining with anti Aβ antibody 3D6, as well as formic acid extraction and immunoblot blot(Choi et al., 2014; Kim et al., 2015). Although exciting, it is important to note that these neurons are derived from an immortalised cell line, together with overexpression of multiple disease-associated genes. 3D Matrigel cultures using fAD iPSCs harbouring the PSEN1 A246E mutation have now also been shown to exhibit potential Aβ aggregation, via D54D2 antibody (Hernández-Sapiéns et al., 2020).

A limited number of studies have now used cerebral organoids to examine Aβ accumulation. COs were generated from iPSCs harbouring either a APP duplication, PSEN1 M146L, or PSEN1 A264E mutation (Raja et al., 2016). CO sections revealed robust Aβ aggregates when utilising the anti Aβ antibody D45D2. Furthermore, Aβ pathology could be significantly reduced by the use of β- and γ-secretase inhibitors (Raja et al., 2016). Work confirming Raja et al, 2016 results utilised iPSCs with the same mutations and generated COs (A246E in PSEN1 and APP duplication through DS) (Gonzalez et al., 2018). Aβ plaques were seen after 110 DIV and confirmed using two anti Aβ antibodies, 4G8 and 6E10 antibodies.
Impressive work from Lin et al, 2018, also uncovered Aβ plaques in iPSC derived COs harbouring APP duplication mutations via D45D2 antibody (Lin et al., 2018). However interestingly addition of APOE4 microglia-like cells marginally alleviating the Aβ aggregation, with APOE3 microglia-like cells significantly reducing Aβ positive aggregations within the organoids.

Overall, 3D cultures have shown early promise as a human in vitro model of fAD although further studies are required in this new research area, particularly to determine whether Aβ aggregation is robust and reproducible.

### 3.1.3 Chapter aims

We hypothesised that Cerebral Organoids would provide a novel human culture system for the investigation of Aβ processing, and that cerebral organoids are more likely to recapitulate Aβ deposition than 2D neuronal cultures. To investigate this, the aims of this chapter were as follows:

- **a)** To generate and characterise novel iPSC from fibroblast lines from patients with familial Alzheimer’s disease harbouring mutations in APP and PSEN1 utilising episomal reprogramming.
- **b)** Characterisation of newly created iPSC cell lines via RT-PCR, Karyotyping, and ICC.
- **c)** Differentiate iPSC lines (APP, PSEN1, and control) into 2D neurons and 3D cerebral organoids.
- **d)** Characterisation of 3D cerebral organoid cultures at 30 and 100 DIV.
- **e)** Investigate secreted and potentially deposited Aβ species within 2D and 3D models.
3.2 Results

3.2.1 Generation of iPSC with APP and PSEN1 mutations

In addition to iPSC with APP and PSEN1 mutations acquired from Stembannc (mutations and lines used within Table 5), additional iPSC lines were generated for use in this project. Skin biopsies were acquired from patients harbouring mutations in amyloid precursor protein (APP V717I, one patient) and presenilin 1 (PSEN1 R278I and PSEN1 Y115H, two patients) and used to establish fibroblast cell lines detailed in Table 5 (Wray et al., 2012).

Fibroblasts were reprogrammed using the Yamanaka factors and protocol as outlined in section 2.12 (Okita et al., 2013). iPSC colonies formed after 3-5 weeks in culture and were cultured in parallel both with and without a feeder layer of mouse embryonic fibroblasts (MEFs). Colonies exhibiting a clearly defined border, containing cells with a large nucleus to cytoplasm ratio, were picked using a P1000 pipette and expanded to create an iPSC clonal line. Each clone was named and numbered, with approximately 30 clones being generated from each reprogrammed fibroblast line.

![Biopsy](image1.png) ![Fibroblasts](image2.png) ![SOX2 c-MYC](image3.png) ![OCT4 KLF4](image4.png) ![iPSC Colony](image5.png)

**Figure 17.** Simplified schematic showing progression of biopsy to fibroblast culture to iPSC.

Skin biopsies were used to establish fibroblast lines (pictured middle) which were subsequently reprogrammed into iPSC via episomal reprogramming (left picture).

Clones that exhibited unusual morphology or spontaneous differentiation were discarded. Once stocks of each clone were frozen down, samples were taken to undergo genetic, karyotypic, and protein analysis for quality control.

Newly established iPSC clones were initially screened to ensure that integration of the episomal plasmids used for reprogramming had not occurred (Figure 19). This can lead to reactivation of the reprogramming factors and affect pluripotency and differentiation (Okita et al., 2013). DNA was extracted from each iPSC clonal line and PCR was used to highlight any potential remaining episomal sequence by amplifying a sequence only present within the vector itself. If the episome was integrated...
within the genome, a product at 666bp in size would be generated. Figure 18 shows screening of iPSC lines for episomal integration. iPSC samples in lane 3 shows definite episomal integration manifesting in a product at 666bps. Lanes 8 and 11 show possible products at 666bp again indicating possible integration. These clones were discarded and only clones definitively free of episomal integration were carried forward. In total, 40 clones were generated and screened, 6 displayed episomal integration and were therefore not taken forward for further analysis.

**Figure 18. Integration PCR for episomal integration within iPSC clones.**

Nascent iPSC lines were analysed by PCR to confirm the absence of episomal DNA. Each lane corresponds to an individual nascent iPSC clone, representative image. A PCR product at 666bp band indicates episomal integration within the genome of newly created iPSC lines. Episomal integration can be observed in lanes 3, 8 and 11. Samples run on a 2% agarose gel. PCR samples were diluted 1:6 with loading dye, with 10μL loaded per well. 100bp ladder used with bp size labelled on the left, all imaging was done on a Li-Cor imaging system.

Next, iPSC lines were fixed and stained for the pluripotency markers, OCT4 and SSEA4 using ICC. Representative images of and nascent iPSC clone from each patient are shown in Figure 19. All promising iPSC lines displayed punctate SSEA4 and nuclear OCT4 signals, confirming pluripotency marker expression. Finally, the karyotype of nascent iPSC lines was confirmed by G-banding, performed by the Doctors Laboratory, London. Figure 19 shows all lines exhibited a stable karyotype.
Figure 19. iPSC lines have a normal karyotype and express pluripotency markers
A-C) Karyotyping of iPSC line was performed by G-banding in all generated iPSC lines. D-F) Representative ICC images of each iPSC clone exhibiting the pluripotent antibody markers OCT4 in red and SSEA4 in green, with nuclear staining via DAPI in blue. Staining was carried out on three different iPSC passages for an n=3. Scale bar is 100µm.

3.2.2 Generation of Cerebral Organoids from control and fAD iPSC

COs were generated from four control iPSC lines and nine fAD iPSC lines (2 lines consisted of the same mutation but different iPSC clones) using the protocol described by Lancaster et al and summarised in section 2.11 in this thesis (Lancaster and Knoblich, 2014).

Figure 20 shows a simplified version of the protocol used.
Figure 20. Simplified schematic of the Lancaster cerebral organoid protocol.
Knockout Serum Replacement (KSR) media. Cerebral Organoid Differentiation Media (CODM). The top grey bar shows simplified main stages of organoid culture, with the bottom yellow to orange bar showing media changes through organoid culture. All media defined within the methods section of this thesis, chapter 2.

CO development was monitored by phase contrast microscopy (Figure 21). From 1 to 6DIV, dense embryoid bodies could be observed, after which differentiation to neuroepithelium was witnessed, as seen by clearing of the tissue edges from 12 DIV onwards. Embryoid bodies that failed to show neuroepithelium formation were discarded at this stage. At approximately 14 DIV, the COs exhibited small neural tube-like structures that increased in size greatly over the subsequent 10-12 days. From 25DIV onwards, the COs become too dense to image using the light microscope.

Figure 21. Morphological changes of cerebral organoids from day 1 to day 25.
Phase contrast images of COs from 1 to 25 DIV, demonstrating embryoid body formation (1-6DIV), formation of neuroepithelium (12-14DIV) and development of neural tube like structures, indicated by the black arrows (17-25 DIV). After 25 DIV onwards COs are too dense to visualise completely with the light microscope. Images taken using a phase contrast microscope utilising a camera down the lens. Day 1 to 20 take at X10, day 25 taken at X4 magnification.
3.2.3 Characterisation of cerebral organoids

To confirm successful differentiation of iPSC into the neuronal lineage, organoids at 30 DIV were fixed and serial sections were taken using a cryostat. Sections were stained for markers to confirm cortical and forebrain identity. These included immunostaining for Ki67 (proliferating cells), PAX6 (forebrain neuronal precursor cells), phospho-vimentin (radial glia), and TBR1 (deep-layer neurons). Figure 22 shows the successful differentiation of four control and six fAD iPSC lines into COs at 30 DIV.

The presence of Ki67, PAX6, phospho-vimentin, and TBR1 antibody markers confirms the successful differentiation to early cortical forebrain identity. Furthermore, distinct spatial locations of each of the markers revealed the self-organised structured nature of these 3D cultures, for example, the precursor marker PAX and post-mitotic neuronal marker TBR1 occupied distinct spatial locations, representing in vitro equivalents of the ventricular zone and cortical plate, respectively. Organoids that were not positive for these markers were deemed to be non-neuronal and discarded at this stage.

To further evaluate the differentiation of the cerebral organoids, and to assess their cellular composition at longer time points, COs were cultured to 100 DIV (Figure 23). Sections were taken in the same manner as the 30 DIV organoids.
Figure 22. Characterisation of 30 DIV Cerebral Organoids.
Figure 22. Continued, Characterisation of 30 DIV Cerebral Organoids.

Representative fluorescent images of COs generated from control and fAD iPSC lines after 30 DIV. Antibody staining confirmed the presence of proliferating cells (KI67 in green) and early-born deep-layer neurons (TBR1 in red) in the top row of images. The middle row of images show positive staining for the marker of forebrain identity (FOXG1 in red) and radial glia (phospho-vimentin in green). While the bottom row of images show positive staining for neural precursor cells (NPCs) (PAX6 in red) and again dividing cells (KI67 in green). Scale bar 200μm and each section was cut at 10μm thick.
Figure 23. Characterisation of 100 DIV cerebral organoids.
Figure 23. Continued, Characterisation of 100 DIV cerebral organoids.

Representative florescent images of COs generated from control and fAD iPSC lines after 100 DIV. Antibody staining confirmed the presence of early-born deep-layer neurons (TBR1 in red) and βIII-tubulin positive neurons (TUJ1 in green) in the top row of images. The middle row of images show positive staining for secondary progenitor cells (TBR2 in red) and layer 5 neurons (CTIP2 in green). While the bottom row of images show positive staining for forebrain identity (FOXG1 in red) and βIII-tubulin positive neurons (TUJ1 in green). Scale bar 250μm and each section was cut at 10μm thick.
Sections were stained for the pan-neuronal marker βIII-tubulin (Tuj1), the forebrain neuronal marker Foxg1 and the layer specific markers Tbr1 (layer 6) and Ctip2 (Layer 5). COs at 100 DIV were positive for these markers indicating correct differentiation to neuronal lineage. However, the levels and locations of expression were highly variable.

### 3.2.4 Amyloid-β secretome from 2D neurons and COs

To investigate the hypothesis that mutations within APP and PSEN1 affect the Aβ products being secreted, 2D iPSC derived neuronal cultures were kindly provided by colleague Dr Charlie Arber. COs from the same iPSC lines were grown alongside for comparative analysis, with the aim of comparing Aβ species within the media of both cellular models.

### 3.2.5 Optimisation of Aβ measurements from 2D neurons

2D cultures were generated, according to methods section 2.10, from iPSC lines with APP and PSEN1 mutations as well as controls. A subset of iPSC lines were used to optimise the measurement of Aβ, specifically APP V717I (from two individuals), PSEN intro 4 deletion (two clones from the same patient). Conditioned media samples were taken from 2D cultures at 100, 102 and 104 DIV and 200, 202 and 204 DIV. Three samples were taken for consistency, with 100 and 200 DIV taken to examine changes in secreted Aβ in aged cultures. The levels of Aβ38/40/42 contained in the conditioned media were measured by electrochemiluminescence (ECL) using a Meso Scale Discovery (MSD) V-PLEX Aβ peptide panel 1(6E10) kit, according to manufacturer’s instructions. ELISAs were performed by Dr Jamie Toombs and Dr Charlie Arber. Data was analysed and presented as Aβ ratios for normalisation.
Figure 24. Amyloid β peptide ratios within 2D neuronal culture conditioned media.

A) Aβ42:40. B) Aβ42:38. C) Aβ38:40. D) Aβ43:40. Concentration ratios of secreted Aβ42, Aβ40, Aβ38 measured using enhanced chemiluminescent (ECL) and Aβ43 enzyme-linked immunosorbent assay (ELISA) in 2D iPSC-derived neuronal conditioned media (CM). A–C Displayed an average percentage coefficient of variance (%CV) < 4% over 6 days and < 7% over 100 days for ratios. For D Aβ43:40 average %CV was 7.1% over 6 days and 20.7% over 100 days. Data is based on multiple independent inductions per line, specifically APP V717I-1 clone 1 (n = 3), APP V717I-1 clone 3 (n = 2), PSEN1 int4del clone 4 (n = 1), PSEN1 int4del clone 6 (n = 2), Ctrl1 (n = 1), Ctrl 2 (n = 1) and Shef6 (n = 2). With variability between total Aβ levels due to media volume differences, and the purpose of the experiment, to show ratio stability across samples, no statistics were run on these data. Figure from Arber and Toombs et al, 2019, with permission from the authors.

Assay results show secreted Aβ38/40/42 ratios to be stable in media from 2D cultures at both 100 and 200 DIV. Aβ ratios did not change between 100 and 200 DIV, pointing towards regulated and reliable Aβ production and secretion within cultures over time (Figure 24). Aβ42:40 levels were approximately
double in all fAD mutations compared to that of controls (Figure 24.A). These elevated levels were also tightly grouped showing high levels of consistency between mutation lines and the samples taken over time.

*PSEN1* mutations showed an increased Aβ42:38 ratio, almost double that observed in controls and the APP mutation (Figure 24.B). This indicates that the *APP V717I* mutation has minimal effect on the Aβ42:38 ratio.

While the *APP* mutations presented increased Aβ38:40 compared to control lines (Figure 24.C), *PSEN* mutations actually showed slightly reduced levels compared to controls, a potentially interesting trend that could be investigated with more n numbers. This result combined with the increased Aβ42:38 levels in the *PSEN* mutations, confirms that different AD-causing mutations alter the secreted Aβ profiles in diverse but predictable ways (Chévez-Gutiérrez et al., 2012).

Finally, the Aβ43:40 ratio was slightly increased in *PSEN1* mutation lines compared to controls and *APP* mutations, although these differences were small (Figure 24.D). *APP* mutations displayed very similar levels to the controls, potentially indicating that *APP* mutations do not affect changes to this amyloid beta pathway. Ratios were used in this analysis due to total amounts of Aβ being variable due to cell numbers and media volume. Due to this and the main purpose of this analysis to show consistent rations across time points and mutation lines, no stats were done on these data. However, stats are done in the following results chapter to compare 2D and 3D Aβ ratios.

### 3.2.6 Secreted Aβ from COs

We next compared Aβ profiles from cerebral organoid culture conditioned media alongside 2D (Figure 25) from the full panel of fAD mutation iPSC detailed in Table 5. COs and 2D cortical neurons were grown using the same iPSC lines harbouring *APP* and *PSEN1* mutations (*APP V717I*, *PSEN1 int4del*, *PSEN1 Y115H*, *PSEN1 M139V*, and *PSEN1 R278I*), along with the same controls. Each batch of organoid inductions was counted as a single n number, due to the fact multiple organoids are cultured pooled in a single dish. Media was collected at 100 DIV for each separate induction for analysis.

*APP* and *PSEN* mutant organoids showed concordant results with mutation matched 2D culture media. It should also be noted that overall levels of Aβ were lower in cerebral organoids than 2D cortical neurons.
Figure 25. Mutation-specific Aβ ratio differences in fAD iPSC derived neuronal cultures.

Conditioned media was collected at 100 DIV. Aβ peptide ratios shown - A: Aβ42:40, B: Aβ42:38, C: Aβ38:40, D: Aβ43:40, E: Aβ42:43, F: Aβ38:43. Figure from Arber and Toombs et al, 2019, with permission from the authors. 2D data were generated from multiple inductions per line, specifically APP V717I-1 clone 1 (n = 7), APP V717I-1 clone 3 (n = 3), APP V717I-2 (n = 2), PSEN1 Int4del clone 4 (n = 5), PSEN1 Int4del clone 6 (n = 5), PSEN1 Y115H (n = 6), PSEN1 M139V (n = 6), PSEN1 M146I (n = 3), PSEN1 R278I (n = 6). Control data were generated from the following inductions: Ctrl 1 (n = 5), Ctrl2 (n = 6), Ctrl3 (n = 7), Ctrl4 (n = 6), and SHEF6 (n = 4). 3D data consisted of two inductions of each line, except APP V717I-1 clone 3, SHEF6, and M139V for which no data is available. Significance levels: * = < 0.05, ** = < 0.01, *** = < 0.001.
In the media from both 2D and 3D cultures, the Aβ42:40 ratio was increased in samples carrying the fAD mutations to approximately twice that of controls. The PSEN1 R278I mutation exhibited the lowest increase in Aβ42:40 and PSEN Y115H the highest (Figure 25.A). An interesting observation is both APP and PSEN mutations increased the Aβ42:40 ratio. However, the specific mutations varied in their impact on this ratio, with some mutations showing more severe alterations that others.

The Aβ42:38 ratio is a recognised biomarker for γ-secretase processivity (Seppälä et al., 2010). In the media from COs, PSEN1 mutation lines showed a significant increase in this ratio when compared to controls (Figure 25.B). PSEN1 int4del and PSEN1 Y115H especially, exhibited similar increased Aβ42:38, whereas PSEN1 M139V, PSEN1 M146I, and PSEN1 R278I displayed decreasing changes versus non-AD. The APP V717I neurons showed a much reduced, but significant (P<0.01), increase in Aβ42:38. 3D cultures closely followed the results of their 2D counterparts, although as stated, variability within the 3D cultures was increased compared to 2D.

Fascinatingly, the Aβ38:40 ratio was able to differentiate between genotypes based on mutation status (Figure 25.C). When compared to non-AD cells, APPV717I Aβ38:40 ratio was significantly higher, whilst in PSEN1 lines against non-AD, the ratio was unchanged. A surprising result was recorded for PSEN1 int4del, PSEN1 M139V mutation lines where the Aβ38:40 decreased.

Ratios of Aβ43 to other Aβ species are not well studied, however these results showed that compared to control, all PSEN1 mutations, except PSEN1 M146I, displayed increased Aβ43:40 (Figure 25.D). Interestingly, 3D cultures seem to have an increased ratio, although the number of repeats are not high enough to conclude anything without further experimentation.

The Aβ42:43 ratios mostly match the results obtained for Aβ38:40 ratios (Figure 25.E & F). Showing a large increase in the APP V717I, and minimal increases in the other mutation lines when compared to control. Aβ38:43 ratios also showed the increase in APP V717I, but interestingly exhibited decreases among most cultures.

3.2.7 Comparison of Aβ profiles in iPSC and patient-matched post-mortem tissue

To determine how closely the in vitro systems recapitulate disease-associated changes, media and lysate Aβ profiles from the in vitro models were compared with CSF and post-mortem brain tissue from the same patient with an APP V717I mutation.
Aβ42:40 ratio was investigated in cell media (CM), 2D iPSC derived cell lysate (Lys), cerebral spinal fluid (CSF), and brain lysate (Brain) (Figure 26.A). Interestingly, the Aβ42:40 ratios were higher in cell media and patient brain sample than CSF. This is a well-known phenomenon as Aβ42 is depleted in patient CSF due to its deposition in plaques in the brain (Spies et al., 2012). The Aβ38:40 ratio was consistent across all samples, although it should be noted that Aβ38 in brain lysate was below the detection threshold (Figure 26.B).

To determine whether Aβ deposits were formed within COs, we stained iPSC-derived 2D neurons, and COs with the APP V717I mutation, together with matched post-mortem brain tissue from the same individual, with the antibody Aβ Dako M0872, which detects Aβ aggregates (Figure 26.C).

Figure 26. Comparison of Aβ profiles in neurons, COs, CSF and post-mortem tissue from the same individual

A) Aβ42:40 ratio across conditioned media (CM), 2D iPSC derived neuronal lysates (Lys), cerebral spinal fluid (CSF), and post mortem brain tissue homogenate (Brain). B) Aβ38:40 ratio across the same samples, Aβ38 levels were below the detection threshold in brain homogenate. C) Post-mortem tissue, 3D cerebral organoids and 2D iPSC neurons from the same patient stained for Aβ in red and MAP2 in green. Scale bar is 50µm with each section was cut at 10µm thick. Figure from Arber and Toombs et al, 2019, with permission from the authors.

Samples were co-stained for microtubule associated protein 2 (MAP2). Within the patient material, a dense signal from MAP2 is clearly visible, punctuated with amyloid beta positive inclusions, typical of
Alzheimer’s disease patient brain tissue (Figure 26.C, arrows). Within both the 2D neurons and COs, strong immunoreactivity for MAP2 is present, although showing different patterns due to the neuronal structure of the different models. In contrast to the patient tissue, no amyloid positive plaques were seen in any of the cultures grown, irrespective of the presence of an AD-causing mutation. For comparison of biomarkers in different tissues and fluids of the same patient, the sample size was limited to one. Therefore it was not possible to conduct tests of statistical significance between sample types.

3.3 Discussion

In this chapter, iPSC were successfully generated from patient fibroblasts harbouring APP and PSEN mutations APP V717I, PSEN1 R278I, and PSEN1 Y115H. These iPSC cultures underwent quality control prior to successful differentiation into cerebral organoids (Figure 21). In total, COs were generated from four control iPSC lines and seven fAD lines (two APP V717I carriers and five PSEN1 carriers with the following mutations: R278I, Y115H, int4del, M139V, and M146I) which were analysed for Aβ production.

Cerebral organoids are primarily differentiated with in vivo like intrinsic signalling rather than directed patterning as in 2D iPSC derived neuronal cultures (M. A. Lancaster and Knoblich, 2014). ICC of organoids at 30 DIV showed neuronal tube-like structures that stain positively for early neuronal markers, including phospho-Vimentin, FOXG1, and early born neuron marker TBR1. This early intrinsic signalling creates an in vivo like architecture to recapitulate human neuron development. Further characterisation of COs at 100 DIV revealed positive staining for the neuronal markers FOXG1 and βIII-tubulin. As discussed in section 3.2.3, COs can be highly heterogeneous, this will be further investigated later on in this thesis (4.2.2).

Variability within COs is partly down to one of the advantages of the model, that of reliance on their self-organising, intrinsic signalling, and in vivo like architecture. Dual SMAD inhibition results in directed differentiation of stem cells to be cortical neurons, allowing a more controlled and defined differentiation (Chambers et al., 2009). In the absence of patterning cues, cells are free to differentiate into different neuronal lineages (Lancaster et al., 2013; M. A. Lancaster and Knoblich, 2014). EB morphology also limits the reach of neural induction media, again allowing populations of cells within the EB to differentiate down unwanted, non-neuronal lineages due to a low surface area to volume ratio (Lancaster et al., 2017). These factors combined do allow the development of a more in vivo like
human neuronal model, however they also have the disadvantage of variability, something discussed and addressed later in this thesis.

The primary focus of this chapter was to compare and contrast the Aβ profiles generated by 2D neurons and cerebral organoids. APP cleavage occurs at the cell membrane and Aβ peptides are secreted extracellularly (O’Brien and Wong, 2011; Israel et al., 2012; Chen et al., 2017). Thus, in 2D culture models Aβ would be removed with each media change. In contrast, cerebral organoids provide a tissue-based in vitro system where it is hypothesised that secreted Aβ could accumulate within the organoid structure. As previous discussed, some groups have demonstrated Aβ aggregation within 3D neuronal cultures, while 2D neuronal cultures have largely not recapitulated this (Kim et al., 2015; Raja et al., 2016; Centeno, Cimarosti and Bithell, 2018; Gonzalez et al., 2018). This supports the idea that the tissue architecture in 3D models can “trap” Aβ, leading to aggregation and enabling these cultures to recapitulate AD brain pathology. This hypothesis could also be true for other 3D models that utilise over expression of fAD genes, or Ren cell type 3D models utilising differentiated immortal neural precursor cells.

Control, APP, and PSEN1 organoids were cultured in parallel with matched 2D cultures and extracellular Aβ proteins within the conditioned media were analysed. Similar Aβ profiles were observed for both 2D and 3D cultures, however total amounts of Aβ were lower in the conditioned media of cerebral organoids. This could either be due to Aβ retention within the organoid structure, or the fact that multiple organoids are co-cultured within the same culture dish, leading to pooled readouts of Aβ in a larger (and therefore more dilute) volume of media. The heterogeneous composition of organoids also means they may contain cell types that do not secrete APP, meaning less overall production when compared to 2D cultures. Another interesting hypothesis is that other cell types present within organoids, but not within patterned homogenous 2D cultures, could be helping to degrade Aβ species. For example the enzyme Neprilysin which has been implicated in Aβ degradation as well as being strongly expressed within the brain (Iwata et al., 2000; Baranello et al., 2015). The utilisation of Aβ ratios allows us to normalise for differences in overall amounts of Aβ and gain insights into qualitative alterations into APP cleavage in the context of fAD mutations. These qualitative shifts have been shown to be more important than overall Aβ levels, at least in the context of PSEN1 mutation (Szaruga et al., 2015).

In all mutations the Aβ 42:40 ratio was higher than controls. This is consistent with a disease profile, as Aβ 42 is more hydrophilic and aggregation prone both vivo and even in vitro (Tiiman et al., 2015; Chen et al., 2017).
PSEN1 mutations showed increased Aβ42:40 ratios compared with APP mutation cultures, while APP mutations showed increased Aβ38:40 ratios. These data show mutations alter Aβ ratios in predictable ways depending on the gene affected. With this knowledge and genetic sequencing, this could lead to mutation specific treatments for patients.

An increased Aβ42:40 ratios in mutation harbouring lines was consistently observed when compared to controls, demonstrating disease relevant APP cleavage within the 2D and 3D cultures, and demonstrating the value of 3D cerebral organoids as a beneficial model to investigate fAD mechanisms. Although the overall levels of Aβ were quite variable, the use of Aβ ratios normalises the data and reduces variability. The consistent Aβ ratios observed in all cultures supports the high regulation of APP processing with neurons.

We hypothesised that cerebral organoids would offer an opportunity to study Aβ deposition within the tissue structure. In order to do this, we stained 2D and 3D cultures with the APP V717I mutation, together with post-mortem brain tissue from a matched V717I case for Aβ using the antibody Aβ Dako M0872, which detects Aβ and highlights aggregates.

A robust Aβ deposition was observed in post-mortem brain tissues, however no aggregates were detected within 2D cortical neurons or 3D cerebral organoids. This was unsurprising due to many 2D and 3D papers reporting no aggregation in these models, however some 3D papers do report aggregates (Raja et al., 2016; Gonzalez et al., 2018; Kwart et al., 2019; Penney, Ralvenius and Tsai, 2019). Even with application of exogenous Aβ, groups show no aggregation although proteome changes have been described (Wu et al., 2019; Sackmann and Hallbeck, 2020).

The reasons for the lack of Aβ deposition in cerebral organoids are unknown, however this could be due to the fact that these are in vitro system that model early developmental time points, compared to the development of Aβ aggregates in the human brain occurring over decades (Masters et al., 2015; Parnetti et al., 2019). It is also possible that the critical concentration of Aβ required to start the aggregation process is not being reached. Future work could try to address this by:

1) Aging the cultures to extended time points
2) Utilising iPSC with APP duplications, which increase Aβ dosage as well as the ratio of longer Aβ species and have previously been shown to form aggregates in iPSC-derived systems (Choi et al., 2016; Gonzalez et al., 2018)
3) Inoculation of neural tissue with exogenous Aβ preparations to seed the aggregation process, as has been successfully used in vivo, from drosophila to murine models (Langer et al., 2011; Prado and Baron, 2012; Sowade and Jahn, 2017)
4) Exploring less variable 3D cerebral organoid models such as patterned brain region specific organoids. For example Pasca and Qian protocols (Qian et al., 2016; Sloan et al., 2018)

Introducing progerin mutations to iPSC derived neurons is another technique that has been tried as an artificial aging system to recapitulate aging and late onset diseases like AD and PD (Miller et al., 2013). Although aged phenotypes were detected, mainly dendrite degeneration and enlarged mitochondria, this approach impacts the physiological relevance of our model, but introducing a mutation unrelated to AD, making it harder to pin point important phenotypes and mechanisms from the model (Miller et al., 2013).

In summary, we successfully developed a 3D cerebral organoid model of fAD, and showed disease-relevant alterations in Aβ profiles but no Aβ deposition. As the main focus of this thesis was to develop a model of tau pathology, tau expression and splicing was further investigated within COs.
4 Investigating tau expression and splicing in cerebral organoids

4.1 Introduction

The use of 3D organoid cultures to model neurodegenerative disease is an area of expanding research (Arber, Lovejoy and Wray, 2017b; Centeno, Cimarosti and Bithell, 2018; Jensen and Teng, 2020). These self-organising, non-adherent cultures exhibit in vivo like architecture thanks to intrinsic signalling and extracellular matrix (ECM) support (Lancaster et al., 2013; Lancaster and Knoblich, 2014; Kelava and Lancaster, 2016).

4.1.1 3D culture and development

2D cultures are traditionally mono-cultures, consisting of mostly a single cell type isolated from tissue. Emerging 3D cultures utilising stem cells recapitulate developmental stages of the CNS, with analysis of organoids showing distinct similarities with early brain development (Amiri et al., 2018; Shi et al., 2020; Tanaka et al., 2020). Initial steps involve neuralectoderm formation, which is achieved without dual SMAD inhibition, allowing intrinsic signalling to guide cell fates and lineages. This leads to multiple neural tube-like structures being formed around the organoid itself. Within 2D cultures, these are called ‘rosettes’ and are formed while adhered to the plate surface, after which their architecture is broken up repeatedly to acquire neuronal cultures of the correct density (Shi, Kirwan, Smith, Robinson, et al., 2012).

Retention of these neural tube-like structures, and hence their architecture, allow for more in vivo-like development of the subventricular zone (SVZ). This area in neuronal development contains radial glia and is the starting point for neuronal migration, allowing cells to differentiate as they migrate away from the SVZ. Interestingly not only is the SVZ important in adult neurogenesis, it has also been implicated in neurodegenerative disease, therefore retaining this structure in 3D models could provide to be extremely valuable (Curtis, Faull and Eriksson, 2007).

Cellular migration away from the SVZ is towards the outer edge (basal surface) of the neural tube-like structure and allows the development of a cortical plate and marginal zone (M. A. Lancaster and Knoblich, 2014; Lancaster et al., 2017; García-Cabezas and Zikopoulos, 2019). These layers give rise to cortical neurons and as shown in Figure 27, this human cortical development is more complex than in other traditionally used models like rodents. Human neuronal development displays multiple distinct
layers of specialised cells, with the marginal zone itself having distinct characteristics over other species, mainly morphological differences that would be more viable within 3D culture (Tkachenko et al., 2016).

Figure 27. Neuronal dorsal cortical organisation in the developing mouse and human brain.

Mouse cerebral cortical development (A) showing apical radial glia (aRG) giving rise to intermediate progenitors (IPs), or to neurons directly, that propel themselves to the cortical plate (CP). However the developing human cerebral cortex is more complex (B), showing how the subventricular zone (SVZ) is divided into the inner SVZ (iSVZ) (a homologue to the mouse SVZ) and the outer subventricular zone (oSVZ). This oSVZ is colonised by outer basal radial glia (bRG) and basal intermediate progenitors (bIPs) which are highly proliferative and generate neurons, which migrate towards the cortical plate and marginal zone (MZ). Shading behind the different models indicate differences in the extra cellular matrix (ECM) composition. Diagram from Buchsbaum et al 2019. CP - cortical plate; IFL - inner fibre layer; iSVZ - inner subventricular zone; IZ - intermediate zone; MZ - marginal zone; oSVZ - outer subventricular zone; SP - subplate; SVZ - subventricular zone, VZ - ventricular zone. Reproduced/adapted with permission from Buchsbaum et al 2019, published in Development journal, (Buchsbaum and Cappello, 2019).
Traditional 2D cultures force cells to adhere to a two dimensional surface, losing some cell to cell interactions as well as correct 3D cell morphology. Utilising 3D cultures, we can hope to capture some of these unique human developmental characteristics and early cortical layer morphology. In the previous chapter, we analysed Abeta production from COs. In this chapter, we extend this work to analyse tau expression and splicing in COs.

As outlined previously, 2D neuronal cultures, although suited for a myriad of projects, retain fetal like phenotypes (Iovino et al., 2015; Livesey et al., 2016; Arber, Lovejoy and Wray, 2017b). Livesey et al 2016 conducted a comprehensive evaluation of the functional properties of human pluripotent stem cell (hPSC) derived neurons. Discovering them to be functionally reminiscent of immature cortical neurons. GABA receptors within hPSC neurons cultured for 5 weeks were shown to be mainly comprised of α2/3β3γ2 subunits, the most common GABA receptor composition expressed within the embryonic cortex (Olsen and Sieghart, 2009; Livesey et al., 2016). Expression levels of these receptor isoforms are similar to the developing cortex at 12–21 weeks post conception and aligns with other transcriptome-based studies (Stein et al., 2014; Pasca et al., 2015). This research also presents evidence of action potential firing characteristics of hPSC neurons being similar to embryonic, or at least early postnatal, rodent cortical neuronal primary cultures (Espuny-Camacho et al., 2013; Livesey et al., 2016).

### 4.1.2 MAPT splicing, models and developmental regulation

Central to the work of this thesis, it is important to determine the maturity of iPSC derived models with respect to tau splicing. Tau splicing is subject to complex developmental regulation with different species expressing different tau isoforms also (Goedert, Spillantini, Jakes, et al., 1989; Takuma, Arawaka and Mori, 2003).

During development, human neural tissue expresses only 0N3R tau, with adult human brain tissue expressing all six isoforms of tau (Goedert, Spillantini, Potier, et al., 1989; Goedert and Jakes, 1990; Wang and Mandelkow, 2015; Hefti et al., 2018). This is achieved by alternate splicing of the MAPT gene, further explained within the introduction (chapter 1.1). This switch to adult tau isoforms is not fully understood, however it appears to happen postnatally, soon after birth (Hefti et al., 2018).

In comparison rodents do show developmental regulation of tau, however 0N3R during gestation switches to 3 isoforms of 4R only tau postnatally (Takuma, Arawaka and Mori, 2003). However Hanes et al 2009 have suggested all 6 isoforms of tau to be present within adult rat brain, although with 3R tau isoforms being exceedingly low in abundance. Paired that with is the ratio differences of the
abundant 4R tau, showing an almost even split of 30%, 35%, and 35% tau containing zero, one or two N-terminus inserts respectively (Hanes et al., 2009). This is far from the ratios expressed within human adult brain, and from research in to MAPT splicing mutations tau ratios have shown to be important (Hutton et al., 1998). Other species have been looking at as a potential tau expression model, with Sharma et al 2019 looking at marmosets, unfortunately and surprisingly they harbour very similar expression to rodent models (Takuma, Arawaka and Mori, 2003; Sharma et al., 2019). Interestingly Deshpande et al 2008 reported 3R and 4R tau expression within human cortical neuron (HCN) cultures, originally gathered from donated 16 to 21 week human fetal brain tissue. Not only did the HCN cultures exhibit 3 and 4R mRNA, but also a robust tau protein expression after 40 DIV

HCNs harvested at 40 DIV and normal adult human brain exhibited a similar profile of tau isoforms as human brain samples. Although not as defined as the human brain samples, it is clear multiple isoforms are present indicating maturation of these HCNs from a tau splicing standpoint (Deshpande, Win and Busciglio, 2008). This would obviously be a useful model for investigating tauopathies, however ethical hurdles as well as access to this rare tissue means it is not a viable model for most studies.

Thus, the potential of iPSC-derived models to express and splice tau as seen in the adult human CNS has generated great interest. However, although there have been variable results between groups, iPSC-neurons mainly express 0N3R (fetal) tau. Even at extended time points in vitro, with other tau isoforms being detectable, 0N3R seem to be heavily predominant and therefore the expression profile of tau is not exactly physiologically similar as the human brain (Iovino et al., 2010; Ehrlich, A. L. Hallmann, et al., 2015; Sposito et al., 2015). Whole-genome gene expression comparisons of both human and fetal brain tissue against iPSC derived neurons confirmed these cells early fetal phenotype (Patani et al., 2012).

Research from two groups have also shown 10+16 MAPT mutations overriding developmental regulation of tau with the expression of 0N4R tau early in iPSC derived neurons. These papers also show at at 120 DIV, although 0N4R tau is present within the mutant neurons, the only other detectable tau isoform is 0N3R fetal tau (Ehrlich, A. L. Hallmann, et al., 2015; Sposito et al., 2015; Paonessa et al., 2019). Fetal tau being the only isoform within the control iPSC neurons. While Iovino et al 2015 has shown the MAPT splicing mutation N279K to not only disrupt developmental regulation of tau by expressing 0N4R, but also quicken maturation of iPSC neurons at least from a tau splicing standpoint. N279K neurons were shown to express at least 4 tau isoforms at 150 DIV compared to 150 DIV control iPSC neurons which still predominantly expressed only fetal tau (Iovino et al., 2015).
Other groups have shown 4R tau present within iPSC neurons not harbouring splice mutations. Sato et al 2018 used immunoprecipitation-mass spectrometry (IP-MS), finding 4R tau present within young 36 DIV iPSC neurons (Harada et al., 1994). However 4R tau levels were two orders of magnitude less abundant than common peptides, supporting data from other groups showing fetal tau overwhelmingly being the primary tau isoform in early iPSC neurons. Interestingly Sato et al 2015 also looked at the turnover of tau protein within control human CSF and iPSC derived neurons, discovering the half-life of tau from cultured neurons to be around 6.74 days compared to 17 – 29 days in the adult CNS (Harada et al., 1994).

With differing tau isoform expressions across studies, there is a need to refine these models in order to develop a human, neuronal system that can express tau isoforms at the correct stoichiometry, and at physiologically-relevant levels. Although iPSC-neurons with tau mutations have enabled the investigation of disease phenotypes such as MAPT splicing mutations and potential pathological tau phosphorylation, the lack of expression of all the tau isoforms means they are not ideal (Ehrlich, A. L. Hallmann, et al., 2015; Sposito et al., 2015; Verheyen et al., 2018). Although it is pertinent to note with the current lack of understanding regarding tau isoforms and aggregation it is unknown whether all isoforms, at least a physiological levels, are required for aggregation.

### 3D culture systems

It has been suggested by other groups that 3D culture and cerebral organoids (COs) mature faster than traditional 2D neuronal cultures. Previous work bolstering this hypothesis has shown evidence for spontaneous firing of action potentials and post-synaptic currents within 3D culture neurons disassociated after 60 DIV (Rigamonti et al., 2016). Organoids have also been shown to recapitulate gene expression of the developing neocortex (Camp et al., 2015). However from a tauopathy model point of view, maturation of neurons is heavily cantered around tau splicing, especially as human adult neurons display this desired switch from 3R to 4R tau postnatally (Hefti et al., 2018). Multiple papers have looked in to tau isoforms within certain 3D-differentiated cultures, some of which have shown to exhibit a dramatic increase in mature 4R tau isoforms.

A recent paper to detect 4R tau in iPSC derived neurons from Miguel et al 2019 utilised dual SMAD inhibition treated neuronal cultures, while also encasing the differentiated cells in to matrigel coated alginate capsules, creating a directed differentiation 3D culture (Shi, Kirwan and Livesey, 2012; Miguel et al., 2019). These cultures showed all 6 tau isoform mRNA at 25 weeks in culture, however 0N3R, fetal tau, was still the predominant MAPT mRNA at almost 80% of total tau mRNA. When tau protein
was analysed, multiple tau isoforms were present, however not all 6 isoforms were detectable at 25 weeks in culture (Miguel et al., 2019). Choi et al 2014 not only showed Aβ and tau pathology within their 3D neuronal cultures, but also multiple tau isoforms, although the immunoblots are not clear enough to identify specific tau isoforms. The group utilised a matrigel cell suspension culture, encasing cells in a layer of ECM, allowing the cells to adopt a much more in vivo like 3D morphology during differentiation and growth. Although this research utilised human neural progenitor cells (ReNcell) rather than iPSC. ReN cells are an immortalised human neural progenitor cell line with the capability to differentiate in to neurons and glia (Donato et al., 2007; Choi et al., 2014),

A heterogeneous culture environment has also been shown to be potentially advantageous to developing a suitable neuronal cell culture model. Astrocytes have been shown to release neurotrophic and neurite-promoting factors (Müller, Junghans and Kappler, 1995). Further to this, general astrocytic studies have used electron microscopy to show limited synapses develop in the absence of glial cells as well as playing significant roles in the formation, maturation, function and elimination of synapses (Ullian et al., 2001; Clarke and Barres, 2013)

For example co-culturing hPSC with rat primary astrocytes increases long-term culture survival, as well as the functional maturation of hPSC-derived neural cultures (Paavilainen et al., 2018). A similar outcome was described, including increased synapse formation, when looking at co-cultures in mouse models (Jones, Cook and Murai, 2012). Using a different approach, Downs syndrome (DS) iPSC derived astrocyte media was shown to inhibit ion channel maturation as well as synapse formation in vitro on control neural stem cells. Transplantation of the same DS astrocytes in to an immunodeficient mouse model showed inhibition of neurogenesis of the endogenous murine neural stem cells in vivo (Chen et al., 2014). Thus, we hypothesise that the 3D environment and heterogeneous composition of COs may accelerate the acquisition of mature tau splicing.

### 4.1.4 Chapter Aims

The aims of this chapter were:

a) To investigate MAPT expression and splicing in COs at the mRNA level using RT-PCR.

b) Conformation of MAPT expression changes using immunoblot and total tau antibodies at different time points.

c) Determine variability in tau expression between organoids using βIII-tubulin and total tau antibodies via immunoblot.
4.2 Results

4.2.1 Tau expression in 2D cortical neurons and 3D cerebral organoids

To investigate potential MAPT splicing differences between cerebral organoids (CO) and 2D neuronal cultures, COs were grown alongside 2D lines, culturing cultures using both methods up to 300 DIV. Control lines were exclusively used to study MAPT splicing within these cultures, with the goal of trying to model human brain tau levels as closely as possible and establish whether variability between organoids would limit their use as disease models.

Figure 28. Tau expression in 2D cortical neurons and 3D cerebral organoids.

PCR with primers spanning exon 10 were used to determine the expression of 3R and 4R tau in cerebral organoids and 2D cortical neurons at the time points indicated. RNA from human brain was used as a positive control. Bands corresponding to 3R tau (exon 10 negative) and 4R tau (exon 10 positive) are indicated. GAPDH was used as a control gene. Samples run on a 2% agarose gel. PCR samples were diluted 1:6 with loading dye, with 10uL loaded per well. Imaging was done on a Li-Cor imaging system.

Whole organoids (a single organoid per sample) and whole wells of 2D neuronal cultures (one well of a 6-well plate per sample) were lysed at ~50, 100, 200 and 300 DIV, followed by RNA extraction and RT-PCR. PCR primers spanning exon 10 of the MAPT gene were used, meaning two products are produced, with or without exon 10 (Table 9). The smaller product, indicating exon 10 exclusion (3R), can be seen at 305 base pairs, whilst the larger product, indicting exon 10 inclusion (4R), can be seen at 398 base pairs.

In cerebral organoids, 3R tau was robustly detected at the earliest time point of day 42, and in all other time points onwards (Figure 28). This result was expected as it is known that in the early development of the cerebral cortex, ON3R tau is the only transcript produced, and keeps being produced (although
in lesser amounts) in adult human brain (Hefti et al., 2018). A faint band corresponding to 4R tau could be observed at 100 DIV, potentially indicating the start of expression of other MAPT isoforms. 4R tau increased over time, showing a strong band at 200 DIV and a very robust band at 300 DIV in both control genotype COs. This is in contrast to 2D cortical neurons, where 4R tau was not detectable at 100 DIV. By 200 DIV a very faint band was detectable, however it was not until 300 DIV until that a clear PCR product was visible. As expected, adult human brain showed approximately equal levels of 3R and 4R tau isoforms. These results suggest that mature tau expression may occur earlier in cerebral organoids than in 2D cortical neuronal cultures. GAPDH was chosen as the control primer pair to show each cDNA sample was viable and was a genuine human tissue culture sample.

It should be noted that a faint third band of higher base pair numbers can be seen within all samples, including brain control. This was investigated earlier by Dr Nuria Seto-Salvia using tagged primer pairs, revealing both 3R and 4R products utilised tagged primers, while the third band did not. Hence this third band is a heteroduplex formed of PCR products only (data not shown). Heteroduplexes are PCR products that form as a result of cross-hybridization (Shore and Myerowitz, 1990; Thompson, Marcelino and Polz, 2002).

**4.2.2 Variability in tau expression in cerebral organoids**

Due to the intrinsic differentiation and self-organisation of organoids during development, there is a large amount of heterogeneity as the exact composition of cell types will vary between each organoid (Labialle et al., 2014). Many papers, as well as our study on Aβ, have shown variability within organoid models (Lancaster and Knoblich, 2014; Qian et al., 2016; Velasco et al., 2019). This highlights the variable levels of neuroepithelium being produced in the early stages of organoid culture, and therefore variable levels of neurons being produced per organoid, especially as culture time increases.

With this in mind, and as well as much of our analysis of tau is at the protein level by western blotting, heterogeneity in tau protein levels within 2D and 3D cultures was investigated. Three individual control iPSC lines were differentiated in parallel into 2D cortical neurons and 3D COs. Three independent inductions per line were carried out, and protein lysates were generated at 80 DIV. One well per 2D induction was lysed, each lysate for the 3D samples consisted of a single organoid. Western blots for total tau are shown in Figure 29.A (2D) and Figure 29.B (3D). Total tau and B-III tubulin antibodies used for this blot were very specific, although some background noise on this blot was viable. All original, uncropped, blots are available on request.
Figure 29. Western blot investigating variability of COs.
A) 2D iPSC derived cortical neurons from three independent control iPSC lines (Ctrl1, Ctrl2, Ctrl3) and three independent inductions. B) 3D cerebral organoids from three independent control iPSC lines (Ctrl1, Ctrl2, Ctrl3) and three independent inductions. Both membranes were probed for total tau (Dako Tau), astrocytes (GLAST), B-III tubulin (Tuj1) and a loading control (GAPDH). All organoid samples taken at 80 DIV. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left.
Immunoblotting for total tau revealed consistent levels of tau between lines and between inductions in 2D cultures (Figure 29.A). In contrast, large amounts of variability in tau levels was detected within COs, with the greatest variability between independent iPSC lines, rather than between organoids derived from the same iPSC line (Figure 29.B). As tau is predominantly expressed by neurons, we hypothesised that this variability in tau levels could be a result of variable neuronal content between organoids. In all samples analysed, a single band was observed, suggesting that at this time point both 2D cultures and 3D COs express only 0N3R tau. We next used the neuronal marker βIII-tubulin and the astrocytic marker GFAP to assess the cell type composition of the cultures. βIII-tubulin levels were relatively consistent in 2D cultures across inductions and control lines (Figure 29), however the β-III tubulin levels in cerebral organoids were highly variable. β-III tubulin levels appeared to correlate with tau levels, suggesting that the variable tau levels could be a result of variable neuronal levels between organoids. Interestingly, despite the variable neuronal content of the COs, GFAP labelling suggested that glial content seemed to stay consistent in both 2D and 3D cultures. The loading control GAPDH shows equal loading between samples.

After densometric analysis both total tau (Figure 30.A) and βIII-tubulin (Figure 30.B) expression are shown to be much more variable within the 3D cultures when compared to 2D when normalised to GAPDH. Confirming overall more consistent neuronal composition. GLAST expression (Figure 30.C), interestingly, was equally variable between 2D and 3D models, when normalised to GAPDH, potentially showing similar gliogenesis. Total tau normalised to βIII-tubulin (Figure 30.D) shows much less variability, helping confirm that tau expression is down to the neuronal component of the cultures, rather than other cell types. No statistics were performed on this data due to the high variability present within the CO data, as well as this experiments primary aim was to simply demonstrate variability.
Figure 30. Quantification of 2D and 3D immunoblots.

Levels of tau, βIII-tubulin and GLAST in 2D and 3D control cultures, calculated via by densometric analysis. A) Total tau over GAPDH, relative to control 1. B) βIII-tubulin over GAPDH, relative to control 1. C) GLAST over GAPDH, relative to control. D) Total tau over βIII-tubulin, relative to control 1. Stats were done, multiple t tests, not significant.

Due to this biochemical, and earlier ICC characterisation of the COs, a new and alleged improved upon model was pursued to further this project, details of this model are explained within the next chapter of this thesis.

4.2.3 Tau expression and splicing in long-term CO cultures

Although substantial variability in tau levels was observed in COs at 100 DIV (Figure 28), given that we observed splicing maturation at the RNA level we decided to carry out a protein analysis of tau isoform expression in long-term CO cultures. In order to characterise tau in COs were maintained in culture and harvested at 100, 200 and 300 DIV and then analysed for total tau by western blot (Figure 31).
Figure 31. Tau protein levels in 100, 200 and 300 DIV COs.

COs from three independent control iPSC lines (Ctrl1, Ctrl2, Ctrl3) at 100, 200 and 300 DIV. Membranes were probed for total tau (Dako Tau), β-III tubulin (TuJ1) and a loading control (GAPDH). Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left.

Substantial variability in tau levels can be observed within COs at all time points. For example, at 100 DIV CO lines, showing low tau levels in control 1 and 2. At 200 DIV, low levels of tau were detected in all organoids examined. The presence of a single band (approximately 50kDa) at early time points likely corresponds to the fetal tau isoform (0N3R). At 200 DIV, faint bands of higher molecular weight were visible within control 1, suggesting the presence of other tau species, probably tau isoforms as seen at the RNA level in Figure 28. By 300 DIV, all three control samples show a smear for total tau, indicating the presence of either additional tau isoforms or tau that has been modified post-translationally. Neuronal content of the samples was next analysed via immunoblot utilising the TUJ1 antibody for βII-tubulin.

βII-tubulin, a neuron specific protein, is variable between organoids suggesting variable numbers of neurons per culture. Within the 100 DIV organoids βII-tubulin is highly variable, however interestingly at longer time points, 200 and 300 DIV, this variability reduces in a similar way to total tau, as shown in the quantification (Figure 32). It also reveals more βII-tubulin present at longer culture times, with both βII-tubulin and total tau showing more expression at 300 DIV (Figure 31).

Although an issue, this variability isn’t detrimental in this experiment looking specifically for evidence of multiple tau isoforms. GAPDH was used as a control to confirm equal loading.
Densometric analysis was conducted (Figure 32) by combining results from the three control organoids per time point. The data shows variable βIII-tubulin levels when normalised to total cells within the organoid using GAPDH (Figure 32.A). βIII-tubulin does increase at the later time point, however it is unclear why this would be the case. D100 COs show very high variability, potentially showing how other cell lineages can differentiate within these cultures. A downward trend of tau expression is seen when tau expression is normalised to βIII-tubulin (Figure 32.C). This is potentially due to early born neurons making up large portions of the organoid in the first 100 DIV, while post-mitotic neuronal populations stay relatively constant over extended culture times, allowing glial and other cell populations to increase over time (De Anda et al., 2016). Other undesired lineages might also be contributing towards the cellular population, something shown in the original Lancaster paper (Lancaster et al., 2013). However, when looking at total tau, using GAPDH to normalise, there is a clear increase from 100 to 300 DIV (Figure 32.B). This points to high neuronal content within the organoids but also tau expressed in other cells types could be contributing to this increase, for example glial cells. Although glial cells produce small amounts of tau, as they proliferate throughout these extended culture times these cells would add tau expression to these total organoid lysates. Despite the trends seen in this blot quantification, when statistical analysis was run on these samples, no statistical differences are shown between any time points. This is primarily down to the variability of the organoid samples. However from the immunoblot itself, it is clear that addition tau isoforms are viable at later time points, something investigated further within the thesis.
Figure 32. Quantification of control COs at 100, 200 and 300 DIV.
Comparison of protein expression within WT CO lysates from immunoblots, samples ranging from 100 DIV to 300 DIV. A) TUJ expression normalised to βIII-tubulin expression. B) Total tau expression normalised to βIII-tubulin expression. C) Total tau expression normalised to GAPDH expression.

4.2.4 Tau isoform expression in COs at 300 DIV

Although multiple tau species were observed at 300DIV in COs, this could be due to the presence of multiple tau isoforms or tau that has been subject to PTMs such as phosphorylation. To confirm the presence of multiple isoforms of tau within the 300 DIV COs, lysates were dephosphorylated using lambda protein phosphatase (λPP) (section 2.19) to remove most phospho-groups from the proteins as described previously. Tau has 85 known potential phosphorylation sites, and is highly phosphorylated within the developing brain (Hefti et al., 2019). This phosphorylation alters the molecular weight of tau on immunoblots, meaning tau appears as a smear, rather than clear isoform specific bands.

Dephosphorylation was therefore necessary to visualise tau isoforms present within organoid samples. Western blots of dephosphorylated samples to total tau with two antibodies, DAKO and HT7, revealed multiple bands corresponding to multiple tau isoforms were present in all samples (Figure
A downwards shift was observed in the samples, (from approximately 50kDa to 46kDa) treated with lambda phosphatase, confirming removal of phosphate groups and reduction in molecular weight. A recombinant tau ladder consisting of all six tau isoforms was included, however the resolution of the samples was not sufficient to allow assignment and quantification of specific protein isoforms. βIII-tubulin was consistent between samples suggesting equal neuronal content, and so the variability in tau banding patterns suggests variability in isoform expression between COs. Some control samples do display potentially more tau isoforms than other control samples, again illuminating the variability in tau splicing between organoids. B-actin was used as a loading control.

**Figure 33. Lambda Protein Phosphatase treated and untreated 300 DIV CO control samples.**
Protein samples run without Lambda PP treatment (-) and with Lambda PP treatment (+). Total tau probed using Dako tau and HT7 antibody. βIII-tubulin (Tuj1) was used to confirm neuronal content and B-actin used as loading control. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used as well as a tau isoform ladder, with kDa markers on the left.
In this chapter, tau expression and splicing were analysed COs to determine whether the acquisition of mature tau splicing occurred quicker in 3D cultures, compared to what has previously been observed in 2D.

iPSC were differentiated in parallel into 2D and 3D cultures and MAPT splicing was analysed at the RNA and protein levels. At the mRNA levels, 4R tau isoforms could be detected from 100 DIV in COs, compared with 200 DIV in 2D cortical neurons (Figure 28). This suggests a more rapid maturation of MAPT splicing in 3D at least at the RNA level, and is consistent with contemporary reports in alternative 3D culture systems (Choi et al., 2014; Zhang et al., 2016; Miguel et al., 2019).

In spite of this promising result, and investigation of tau expression and splicing at the protein level was challenging due to the variability observed between organoids. Variability in key proteins such as βIII-tubulin, tau, and GFAP were shown to be consistent across 2D cultures, both across control lines and inductions. While 3D COs were shown to have increased variability across the same neuronal markers. With inconsistent βIII-tubulin, especially at earlier time points, indicating less neuronal cells as part of the culture as a whole, thus making analysis and comparison difficult. In line with this was variable total tau levels, which presents an issue when the model is destined to be used as a tauopathy model. Interestingly, based on immunoblots and analysis of GLAST expression within 2D and 3D cultures (Figure 30.C), glial cell populations seem to be similar as well as being quite consistent (Figure 29).

This can be partly be explained due to protocols for 2D cultures using directed differentiation by dual SMAD inhibition, exposing the monolayer of cells to patterning factors which will uniformly direct them towards a forebrain cortical neuron identity (Shi, Kirwan and Livesey, 2012). Regarding COs variability, one reason for the decreased neuronal populations within COs is their surface area to volume ratio (M. A. Lancaster and Knoblich, 2014; Lancaster et al., 2017). Neural induction media is only able to penetrate the outer cells of the EB, meaning cells closer to the centre of the EB are free to differentiate in to other cells types (Camp et al., 2015; Quadrato et al., 2017; Qian, Song and Ming, 2019). Antibody ICC staining of early organoids by the Lancaster group have shown other undesirable lineages developing within their organoids. These cell types can become predominant with the organoids, masking the investigation of neuronal proteins.

However, in spite of this variability we investigated whether mature tau splicing could be observed at extended time points in COs. Control organoids at 100 DIV showed only the presence of a single tau isoform using the methods detected. However, by 200 DIV low levels of multiple tau isoforms were detected.
observed. By 300 DIV this switch is much more robust, providing preliminary data to suggest these heterogeneous and self-organising cultures express more than fetal tau at extended culture points. This was confirmed by protein dephosphorylation, demonstrating that the additional bands that were observed are splice variants rather than phospho variants. Although this was the first investigation of tau splicing within COs, our results are consistent with Choi et al 2014 and Hye Kim et al 2015 which showed multiple tau isoforms expressed within their 3D and ECM supported models, although utilising ReN cells (Choi et al., 2014; Kim et al., 2015). These results are also supported by Miguel et al 2019, showing ECM iPSC derived neurons expressing multiple tau isoforms by week 25 in culture (Miguel et al., 2019). However these studies not only use other 3D culture methods, not specifically organoids, but also do not show tau isoforms to be comparable to adult human brain.

The reasons why mature tau expression occurs more rapidly in 3D are unknown, however it may be due to the 3D nature of organoids, achievable native cell morphology, and heterogeneous cell population may account for this increased maturation speed (Yakoub, 2019). Studies have shown increases neuronal survivability when co-cultured with astrocytes as well as neurotrophic factor release (Müller, Junghans and Kappler, 1995; Paavilainen et al., 2018). Astrocytes also cover their own overlapping domains, with each domain covering potentially up to 2,000,000 human tripartite synapses, something more likely to be recapitulated within a 3D culture (Robertson, 2013). With neurons harbouring many synapses, and astrocytes domains covering these synapses, there is potential for this increased interaction in a 3D and morphologically relevant space for faster maturation (Clarke and Barres, 2013). Astrocytes have also been implicated as a primary driving force in tripartite synapse formation (Farhy-Tselnicker and Allen, 2018). In conjunction with astrocyte interactions, cell to cell interactions in general have been shown to be advantageous for cellular groups to collate and organise (Mueller et al., 2020).

Although these initial results were promising, due to the high variability observed and the fact that most of the planned analysis of tau relies on bulk cell, biochemical readouts, it was decided to alter the organoid differentiation protocol to an alternative method designed to reduce variability (Lancaster et al., 2017). This work is described in the next chapter (Section 5).
5 **MAPT** expression and splicing in engineered cerebral organoids

### 5.1 Introduction

In the previous chapter, tau expression and splicing in COs was analysed. Although this preliminary data was encouraging, it also revealed considerable amounts of heterogeneity between COs, particularly in biochemical analysis. Therefore, it was decided to address this variability by setting up a complementary organoid protocol developed to reduce this heterogeneity, based on Lancaster *et al.* (2017) (Lancaster *et al.*, 2017).

#### 5.1.1 Approaches to reduce variability within COs

Variability within COs has been described previously, and is largely thought to be a result of variable neuronal content, stemming from variable amounts of neuroectoderm generation (Lancaster *et al.*, 2017). This is due in part to the low surface area to volume ratio of embryoid bodies within induction media (Lancaster *et al.*, 2017).

There are many other variables that could contribute to the variability of these 3D organoid cultures, including starting cell populations and optimisation of media at different stages. Other sources of variation come from diffusion limits causing cell death and eventually necrotic cores within the organoids due to their size, shape and lack of vasculature (Lancaster *et al.*, 2013). The development of approaches to reduce this variability is an area of investigation for several groups. Giandomenico *et al.*, (2019), developed methods for organoid slice cultures, which retain a 3D environment, but with a reduction in the size to a 300µM slice, thus allowing better oxygen and nutrient diffusion (Giandomenico *et al.*, 2019). A second group also developed organoid slice cultures, showing increased neuronal survival due to better diffusion (Qian *et al.*, 2020). They also demonstrated sustained neurogenesis and as well as formation of more defined cortical plate layers via TBR1, CTIP2, and SATB2 staining.

Other groups have developed approaches for the vascularisation of organoids, with both murine implantation and human epithelia cell co-culture methods investigated (Mansour *et al.*, 2018; Cakir *et al.*, 2019). Additional methods have included patterning factors at the EB stage, reducing the amount of cells that differentiate down unwanted lineages, for example dual SMAD inhibition to generate organoids with a forebrain identity (Qian *et al.*, 2016). This successfully reduces variability, however
this technique also reduces the amount of NPCs, leading to less proliferation and therefore much smaller organoid cultures (Qian et al., 2016; Sloan et al., 2018).

Other approaches to reduce variability include the addition of patterning factors, such as widely used dual SMAD inhibitors. Addition of these inhibitors drive more iPSCs towards forebrain identity, lowering the amount of undesired lineages (Qian et al., 2016). This in addition to bioreactors, allow increased control over cell fate as well as the culture conditions of the organoids. Bioreactors can be set to very specific rotation speeds, sometimes multiple speeds per plate, allowing different rotations to be experimented with (Qian et al., 2016, 2018).

### 5.1.2 Engineered cerebral organoids

Engineered cerebral organoids (enCORs) combine bioengineering with organoid generation to reduce heterogeneity (Lancaster et al., 2017). The inclusion of polylactic-co-glycolic acid (PLGA) scaffolds provide a structure for iPSC to adhere to, and increase the surface area of the initial embryoid bodies. The increased surface area leads to more cells being exposed to neural induction media, and therefore more neuralectoderm being produced and less cells following undesirable lineages (Lancaster et al., 2017).

### 5.1.3 MAPT Splicing

The overall aim of this work was to develop a system to investigate MAPT splicing and determine how splice-site mutations in MAPT affect tau expression and splicing and tau protein. To do this a series of previously described isogenic lines containing both splice-site and coding mutations in MAPT were used (Verheyen et al., 2018). The 10+16 mutation destabilises a vital stem-loop structure in intron 10, increasing exon 10 inclusion and generating a 2-6 fold increase in 4R tau (Hutton et al., 1998; Grover et al., 1999; Connell et al., 2005). Patients harbouring this mutation usually develop frontal temporal dementia (FTD) at 50 years of age, on average, with brain pathology including cortical neurofibrillary tangles comprised mainly of 4R tau isoforms (Lantos et al., 2002; Tsuboi et al., 2003). Previous work with this mutation in 2D cortical neurons has shown it can override the developmental regulation of MAPT (Sposito et al., 2015; Verheyen et al., 2018). Fetal tau (0N3R) is usually only expressed during development, however 0N4R tau is also expressed when this mutation is present. iPSC derived neurons from patients harbouring this mutation have been shown to express 4R tau alongside 0N3R fetal tau (Iovino et al., 2010, 2015; Sposito et al., 2015).
Here, iPSC with mono and biallelic inclusion of the 10+16 mutation were utilised. Sposito et al. 2015 demonstrated expression of ON4R tau in developing 10+16 mono allelic neurons (Sposito et al., 2015). Previous work from Verheyen et al. 2018 confirmed increased 4R tau expression in iPSC derived neurons harbouring the 10+16 bi-allelic mutation, showing a dose-dependent increase in ON4R tau when compared to the mono allelic (Verheyen et al., 2018). The lines used for this study were engineered using zinc finger technology, with the 10+16 and P301S mutations inserted on to a WT background. This allows isogenic lines, helping reduce variability and shoring up experimental conclusions by hopefully taking genetic variability out of the results.

Additionally, we utilised a further iPSC line containing both the 10+16 and P301S mutations in a bi-allelic form. This P301S mutation, unlike the 10+16, is a coding mutation which changes a proline at position 301 to a serine. First reported in 1999, onset is usually early in life, 25 – 35 years of age, with changes in mood, behaviour, and memory. However, other symptoms seen in patients can vary, with some patients showing significant motor defects and seizures (Bugiani et al., 1999; Sperfeld et al., 1999). Pathology within these patients usually presents as atrophy in the frontocortical region and hence significant neuronal loss. Tau aggregations of extensive hyperphosphorylated tau are observed within both neurons and glia (Bugiani et al., 1999; Rohrer et al., 2011).

Pathogenic mutations at proline 301, for example P301S and P301L, have been shown to be more prone to aggregation (Falcon et al., 2015). An elegant study showed P301L, P301S, and P301T mutations to be more aggregation prone when compared to 12 other tau mutations (Strang et al., 2018). Interestingly, the sole mutation that showed similar aggregation properties to 301 mutations was at serine 320 (S320F), a region close to the P301 mutation, pointing to an important region of tau regarding aggregation. (Strang et al., 2018).

Although this combination of mutations wouldn’t occur naturally in patients, this ‘double bi-allelic’ mutation provides a useful experimental tool that will not only drive 4R tau expression, overriding developmental expression, but as the P301S mutation is located in exon 10, it will result in the expression of mutant tau within our system.

5.1.4 MAPT splicing and pathology in organoids

We wanted to investigate whether mature tau splicing would be accelerated in an organoid model, providing a more physiologically relevant in vitro system and exacerbating in vitro phenotypes.
Several reports have suggested that 4R tau expression occurs earlier in 3D culture systems. Early work in a neuroblastoma model, using differentiated SHSY-5Y cells cultured in 3D and with a Matrigel ECM, showed increased 4R tau expression both at the mRNA and protein level (Agholme et al., 2010).

Choi et al., 2014 showed increased 4R tau expression at both the RNA and protein level in ReN cells differentiated over 3 weeks, when they were cultured in 3D suspended in Matrigel (Choi et al., 2014). These findings were replicated by Hye Kim et al in 2015, however assignment of tau isoforms by utilising λ-phosphatase to remove the phospho groups would have allowed better assignment of specific tau isoforms (Hanger et al., 2002; Kim et al., 2015).

Gonzalez et al 2018 not only showed their iPSC derived organoids to exhibit Aβ pathology at 110 DIV utilising both 6E10 and 4G8 anti-Aβ antibodies, but also NFT-like pathology using Gallyas silver staining. This tau staining was confirmed to be composed of tau via AT8 (S202) and PHF1 (S396 and S404) positive antibody staining (Gonzalez et al., 2018). However, comprehensive analysis of tau isoforms was not conducted, and tau immunoblots run with PHF1 antibody seem to only display a single tau band, however without a total tau antibody it is difficult to confirm this (Gonzalez et al., 2018).

Similarly, Miguel et al 2019 used human iPSC derived neurons, however utilising Matrigel coated alginate capsules to encase the neurons as a 3D model. Pathology was not investigated within this study however multiple tau isoforms were shown to be expressed and investigated in detail. After 25 weeks in culture, cultures expressed all 6 isoforms of MAPT mRNA, however almost 80% of expressed MAPT mRNA was still 0N3R transcript, which is fetal tau. When protein expression of tau was analysed, there was also dominant expression of 0N3R, although with dephosphorylation treatment, 3 other tau isoforms were identifiable, 0N4R, 1N3R, and 1N4R albeit at low levels. Both 2N splice variants, 2N3R and 2N4R, are not detectable, probably down to the exceptionally low levels of their respective mRNAs (Miguel et al., 2019).

The work in this chapter describes the development and characterisation of enCORs as a 3D neuronal model to investigate the regulation of tau splicing and its disruption by MAPT mutations. Furthermore, we investigate the presence of early markers of tau pathology using an in vitro assay to detect seed-competent tau species.
5.1.5 Chapter aims

Taken together, this work suggests that 3D culture environments may accelerate mature tau splicing. We therefore hypothesised that the in vivo like architecture, cell morphology and multicellular composition of enCORs could allow expression of more mature tau isoforms, but with reduced heterogeneity compared to the COs analysed in Chapter 4.

a) Generate iPSC derived engineered cerebral organoids from isogenic lines containing the MAPT 10+16 splice mutation together with the P301S coding mutation.

b) Characterise engineered cerebral organoids at 30 and 100 DIV as well as investigate variability within this new model.

c) Investigate MAPT expression and splicing in enCORs with the MAPT 10+16 mutations utilising RT-PCR, qPCR, and immunoblot.

d) Investigate whether seed competent tau species are present within enCORs using a tau biosensor assay and confocal microscopy.
5.2 Results

5.2.1 Generation of engineered cerebral organoids

To address the variability seen within the COs, the updated Lancaster protocol for engineered cerebral organoids (enCORs) was adopted (Lancaster et al., 2017). A simplified schematic of this protocol is shown in Figure 34.

Figure 34. Simplified engineered cerebral organoid (enCOR) protocol diagram.

Knockout Serum Replacement (KSR) media. Improved Differentiation Media (IDM). The top grey bar shows simplified main stages of organoid culture, with the bottom yellow to orange bar showing media changes through organoid culture. All media defined within the methods section of this thesis.

After cell counting, 18,000 iPSC were seeded directly onto 8-15 small (0.5-2mm) fibres (Figure 35, Day1), allowing iPSC to aggregate around the scaffold rather than in to a dense spherical EB as in previous organoid protocols (Figure 35.Day 2). As previously discussed, this exponentially increases the EBs surface area to volume ratio compared to the standard EBs.

Figure 35. Generation of engineered cerebral organoids (enCORs).

Phase contrast images of enCORs during initial differentiation stages. Day 1) PLGA fibres within a single cell suspension of iPSC. Day 2) iPSC cells aggregate around the fibres, with some cell death. Day 6) Elongated embryoid body. Day 12) Matrigel embedded enCOR after induction showing increased volume of bright neuroectoderm tissue (arrow). Day 17) Neural-tube like structures form along the scaffolds within an enCOR (arrow). Images taken using a phase contrast microscope.
On day 1, single iPSC cells were dispersed amongst PLGA fibres placed within a round-bottomed plate well (Figure 35, Day 1). By day 2, iPSC cells have attached and aggregated onto these fibres. At 6 DIV (Figure 35, Day 6) the scaffolded EBs have increased in density compared to day 2, with stem cells adhering around the PLGA fibres, induction media was added at this time point. After 12 DIV (6 days in induction media), clearing of neuroepithelium tissue could be observed along these branches (Figure 35, Day 12 arrow) as well as the main body of the scaffolded EB. Due to the increase in surface area to volume ratio provided by the PLGA fibres, robust neural tube-like structures (Figure 35, D17 arrow) had been formed after 17 DIV, again both along the branches as well as the main scaffolded EB structure. Due the density of the enCOR cultures after 17 DIV, it was no longer possible to image using the light microscope.

5.2.2 Characterisation of early stage enCORs

enCORs were generated from iPSC with WT, 10+16m, 10+16bi and 10+16bi/P301Sbi genotypes. enCORs were harvested at 30 DIV for quality control to confirm successful differentiation into the neuronal lineage (Figure 36). Samples were taken, fixed, and sectioned on a cryostat as previously carried out on the COs.
Figure 36. Characterisation of 30 DIV enCORs.

A) Immunohistochemical analysis of 30 DIV enCORs using antibody markers to confirm neuronal differentiation. The top row of images indicating the presence of neural pre-cursor cells (NPCs) (PAX6 in red) and Proliferating cells (Ki67 in green). The middle row of images show positive forebrain identity (FOXG1 in red) and βIII-tubulin positive neurons (βIII-tubulin in green). The third row of images show positive staining for deep layer, early born neurons (TBR1 in red) and radial glia (Phosphovimentin in green). B) Enlarged image of 30 DIV enCOR neural tube-like structure, exhibiting phospho-vimentin staining (upper arrow, green positive cells) as well as TBR1 positive migrating neuronal cells (lower arrow, red positive outer layer cells). All sections taken at 10µm on a cryostat, with a scale bar shown for 200µm.
At 30 DIV, phospho-vimentin staining showed the presence of radial glia in the ventricular zone (Figure 36.B, upper arrow), and the elongated morphology of these cells through a densely packed precursor layer supports the occurrence of interkinetic nuclear migration of the cells during asymmetrical cell division. Pax6, a marker of forebrain precursor cells, was also present in all organoids examined, together with Ki67, a marker of proliferating cells which is a highly expressed by neuronal precursors. enCORs exhibited robust expression of the forebrain marker FOXG1 and pan-neuronal marker βIII-tubulin could be observed in all genotypes (Figure 36), demonstrating successful differentiation into the neuronal lineage. Finally, the glutamatergic, deep-layer neuronal marker TBR1 was present and showed a spatially distinct localisation from the precursor markers. Tbr1 positive neurons are the first post-mitotic neurons to be formed during corticogenesis, showing that this process has started in enCORs after 30 DIV (Hevner et al., 2001).

It is important to note that the PLGA fibres do hydrolyse by around 60-70 DIV (Lancaster et al., 2017), leaving no trace of the scaffolds. However, within Figure 36.B you can see the fibres auto-fluorescing in the top right of the image due to these sections being taken at 30 DIV. In this instance, for characterisation, this does not affect the outcome, however, if enCORs were to be used at lower DIV for immunoblot or image analysis, they may alter or hamper results.

### 5.2.3 Characterisation of mature enCORs

Further characterisation of enCORs was performed at 100 DIV, which represents the end of neurogenesis and therefore is when the formation of neurons within the enCORs is predicted to be complete (Figure 37). Samples were taken and prepared for staining in the same manner as the 30 DIV panel.
Figure 37. Characterisation of enCORS at 100 DIV.

A) Immunohistochemical analysis of 100 DIV enCOR slices using antibodies to investigate neuronal content. The top row of images shows confirmed staining for upper layer neurons (BRN2 in red) and βIII-tubulin (βIII-tubulin in green). The second row of images show positive staining for deep layer neurons (CTIP2 in red) and forebrain-specific markers (FOXG1 in green). The third row of images show positive staining for glutamatergic neurons (VGLUT in red) and a pan-neuronal marker (MAP2 in green). B) Large mage of 100 DIV enCOR showing pan-neuronal marker βIII-tubulin (βIII-tubulin in green) staining large sections of the enCOR with BRN2 (in red) marking cortical layer 2 and 3 neurons within (arrow). All sections taken using a cryostat at 10 µm with scale bar of 200µm.
Staining with the pan-neuronal markers Tuj1 and Map2 showed a high neuronal content in all enCORs examined, demonstrating an increase in neurogenesis between 30 – 100 DIV. Staining was present throughout the sections examined, although the intensity was highest at the outer edge of the enCORs suggesting that the highest concentration of neurons is at the exterior of the enCOR in contact with the media. Next, regional identity of the neurons within the enCORs were determined using markers of forebrain (Foxg1), as well as layer specific markers Ctip2 (deep-layer 5 and 6) and Brn2 (upper layer, 2 and 3). Positive staining for these three markers was present in all enCOR analysed, supporting the presence of glutamatergic, cortical neurons from both deep and upper cortical layers. Finally, the glutamatergic identity was further confirmed by positive staining for the glutamate transporter VGLUT1 (Figure 37). Together, these results confirm successful generation of enCORs from WT and tau mutation iPSC, and suggest that the mutations included in this study do not affect neuronal differentiation.

5.2.4 Variability in total tau protein levels in enCORs at 100 DIV

As previously demonstrated (Chapter 3), a major barrier to the use of COs was the high variability in tau protein levels, both within and between induction batches. Prior to embarking on an in-depth expression of tau splicing changes, we first set out to determine whether this variability was reduced in enCORs. We harvested enCORs from three independent batches/inductions for western blot, with one organoid from each batch constituting a single sample (Figure 38.A). High levels of tau were observed in all enCORs examined, and quantification of total tau levels showed little variability between genotypes or batches (Figure 38.B, C, and D). βIII-tubulin levels were also consistent across all enCORs examined, suggesting lower inter and intra batch variability of neuronal content within the enCOR model. β-actin was used as a loading control.
Figure 38. Quantification of enCOR variability.
A) Three independent batches of enCORs, comprising all four genotypes, were analysed by western blot at 100DIV. Membranes were probed for total tau and β-III tubulin (TUJ1) and β-actin (β-actin) B) Total tau normalised to β-actin, over control samples. C) β-III-tubulin normalised to β-actin, over control samples. D) Total tau normalised to β-III-tubulin, over control samples. All values are relative to their normalised control. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. Statistics were run with no significant differences between batches or genotypes.

5.2.5 Astrocytic content of enCORs at 100 DIV

In addition to assessing the neuronal content of enCORs, we also assessed the astrocytic content of enCORs at 100 DIV using the astrocytic markers GLAST1 and glial fibrillary acidic protein (GFAP) (Figure 39). GLAST was observed in all enCORs analysed with quantification of the immunoblots (Figure 39.B & C) demonstrating consistent gliogenesis to have taken place across enCOR genotypes by 100 DIV, with no statistical differences in GLAST levels between genotypes or batches. This confirms that by
100 DIV, gliogenesis had taken place and astrocytes are present in enCORs as well as neurons. Western blotting for GFAP, although levels of GFAP showed high variability between samples. Further, multiple immunoreactive bands were observed with the GFAP antibody in WT, 10+16m and 10+16bi genotypes, suggesting either the presence of GFAP cleavage fragments or that these samples may be expressing multiple GFAP isoforms or cleavage products (Kamphuis et al., 2012; Moeton et al., 2016).

Interestingly within all three 10+16bi/P301Sbi enCORs, GFAP expression was decreased, and a higher exposure showed the absence of the multiple bands detected in other genotypes. Densometric analysis of GFAP revealed significant differences between WT and 10+16bi/P301Sbi enCORs as well as 10+16bi and 10+16bi/P301Sbi enCORs (Figure 39). Although no statistically significant difference was shown between the remaining genotype (10+16m) and 10+16bi/P301Sbi, there is a trend for the double bi-allelic enCORs to have reduced GFAP compared to the mono-allelic. β-actin was used as to confirm equal protein loading across all samples.

![Western blot images](image-url)

**A**

- All Blue Ladder
- WT
- 10+16m
- 10+16bi
- 10+16bi/P301Sbi

**B**

- GLAST / Bactin

- WT
- 10+16m
- 10+16bi
- 10+16bi/P301Sbi

**C**

- GFAP

- WT
- 10+16m
- 10+16bi
- 10+16bi/P301Sbi

***

*
Figure 39. Astrocytic content of enCORs at 100 DIV.
A) Three independent batches of enCORs for all genotypes, WT, 10+16m, 10+16bi and 10+16bi/P301Sbi analysed by western blot at 100 DIV. Membranes probed for glutamate aspartate transporter 1 (GLAST) and glial fibrillary acidic protein (GFAP). GFAP shown at two exposures to highlight the smaller bands beneath the primary band. Bactin was used as a loading and sample control. B) Densometric quantification of GLAST normalised to total β-actin in 100 DIV enCORs. C) Densometric quantification of GFAP normalised to total β-actin in 100 DIV enCORs. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. Prism software was used for statistical analysis by way of unpaired t-test. *p < 0.05, ** = < 0.01, *** = < 0.001.

5.2.6 Astrocytic markers in WT enCORs during extended culture

We further explored the differences observed in the astrocytic marker GFAP by examining GFAP and GLAST expression between 50 – 300 DIV using western blot (Figure 40). This would allow us to determine whether the variability in GFAP reflects early variability in astrocytic content of enCORs, whether the astrocytic component of enCORs changes over time, or whether GFAP is a marker of reactive astrocytes rather than total astrocytic load.
enCORS were harvested at the time points indicated for analysis of astrocytic content. A) Membranes were probed for GLAST and glial fibrillary acidic protein (GFAP). β-actin was used as a loading and sample control. B) Quantification of GLAST normalised to total β-actin relative to WT. C) Quantification of GFAP normalised to total β-actin relative to WT. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. Prism software was used for statistical analysis by way of One-Way ANOVA. *p < 0.05, ** = < 0.01, *** = < 0.001.

GLAST expression was observed in all enCORS at all time points, with a non-significant increase visible at 200 and 300 DIV. In contrast, GFAP expression was extremely low within the 50 DIV enCORS, but increased over time with maximum levels observed at 200 and 300 DIV. As seen previously, multiple
bands were observed which could indicate the presence of either cleavage fragments or multiple isoforms. The increase in GFAP levels potentially suggests the increased presence of a proportion of activated astrocytes (Kamphuis et al., 2012, 2014). β-actin was used as the loading control and for the normalisation of GFAP and GLAST expression for quantification. Even though equal amounts of protein were loaded, β-actin levels showed some variability. However, as each sample being normalised within the same lane on the same blot, we proceeded with quantification as this would take into account this variability in the normalisation.

When normalised to total β-actin GLAST is not significantly different between any of the time points when analysed using a one-way ANOVA. However, there is a clear trend showing low levels of GLAST in 50 and 100 DIV enCORs, with expression increasing and remaining constant from 200 DIV onwards. When also normalised to β-actin, GFAP expression seems to follow a similar pattern to GLAST, with 50 and 100 DIV enCORs have little GFAP activity. However, from 200 DIV there is a significant increase in GFAP expression (\( p<0.0001 \)) compared to 50 and 100 DIV, which is also observed in 300 DIV enCORs.

Together, these data suggest an increase in astrocyte content during extended culture, and hint at a potential increase in astrocyte activation state. It should be noted that this gel did have running issues, however due to sample availability, and astrocytes not being the main focus of this project, it was not rerun. The gel issues do not affect the visualisation or the quantification of this immunoblot.

### 5.2.7 Astrocyte morphology and location with enCORs

In addition to levels of astrocytic markers, we used ICC to perform a preliminary investigation astrocyte morphology at 100 and 300 DIV samples (Figure 41, n=1). At 100 DIV, enCORs showed positive staining for GLAST and GFAP, confirming gliogenesis and the presence of astrocytes within enCORs at this time point, which is consistent with other studies (Yakoub, 2019). Staining also showed typical astrocytic distribution and morphology of branching processes with a high surface area to volume ratio (Zhou, Zuo and Jiang, 2019). This cell morphology is possible within the organoid potentially due to the architecture and 3D ECM of the model (Rodríguez-Arellano et al., 2016).

GLAST and GFAP staining was also observed in enCORs at 300 DIV. GLAST staining was more diffuse than GFAP than appeared to be moderately non-specific. It was not optimised further as this was not the main focus of the project. GFAP staining labelled cells throughout the slice, and in comparison to 100 DIV these cells showed extended processes similar to mature astrocytes. Co-staining of βIII-tubulin and GFAP was carried out, this co-staining distinctly showed separate positive signals for the two cell types.
5.2.8 **Tau expression and splicing in enCORs throughout development and maturation**

We next performed a thorough investigation of tau expression and splicing in enCORs sampled throughout development, specifically at 50, 100, 200 and 300 DIV. Data is presented in the following sections separated by time point.

5.2.9 **MAPT expression and splicing in enCORs at 50 DIV**

RT-PCR was used to assess tau expression and splicing in enCOR at 50 DIV (Figure 42). Organoids from WT, 10+16m, 10+16bi and 10+16bi/P301Sbi were analysed from three independent batches to account for any variability between inductions. To examine the inclusion of exons 2 and 3, exon...
spanning primers located in exons 1 and 5 were used. These primers generate three products based on either no exon being included in the transcript (0N tau, 112bp), inclusion of exon 2 alone (1N tau, 199bp), or exon 2 and 3 together (2N tau, 286bp). Exon 10 inclusion was examined using primers spanning exons 9 to 13, producing two products, either including exon 10 (4R tau, 398bp) or excluding exon 10 (3R tau, 305bp) within transcripts. All PCR products were separated by electrophoresis on 2% agarose gels.

Figure 42. *MAPT* splicing in enCORS at 50DIV

enCORS from three independent inductions were analysed by RT-PCR to determine 0/1/2 N tau and 3/4R tau expression with enCOR cDNA utilising primers spanning A) exons 2 and 3, resulting in three products corresponding to 0N, 1N and 2N tau isoforms and B) exon 10, resulting in two products corresponding to 3R and 4R tau isoforms. H2O used as the negative control, and whole brain cDNA as the positive control. C) GAPDH was used as control. Samples run on a 2% agarose gel. PCR samples were diluted 1:6 with loading dye, with 10uL loaded per well. 100bp ladder used with bp size labelled on the left, all imaging was done on a Li-Cor imaging system. N=3 independent inductions per iPSC line.

Adult human brain cDNA was used as a positive control, and as expected three bands corresponding to 0N, 1N and 2N were observed, with 0N and 1N approximately equal intensity and 2N being the least abundant PCR product. In contrast, a single band was observed in enCORS at 50 DIV from all genotypes, suggesting only 0N tau is expressed, corresponding to fetal tau. We next analysed exon 10 inclusion. RNA from human adult brain displayed two bands of equal intensity, corresponding to equal
levels of 3R and 4R tau transcripts, as expected. A single band corresponding to 3R tau was observed in WT enCORs, supporting a predominant expression of the fetal tau isoform at this time point, similar to what was observed in our earlier data on COs (cross ref to figure). In contrast, in enCORs, with both the mono allelic and biallelic 10+16 mutation, two bands corresponding to both 3R and 4R tau were detectable in all enCORs analysed. This was also the case in enCORS with the 10+16/P301bi genotype. The 4R band in the bi-allelic 10+16 mutation enCORS appeared to be a greater intensity than the mono-allelic, suggesting a dose-dependent effect of this mutation leading to more 4R transcript being produced. These results are consistent with other studies that have revealed fetal tau splicing within iPSC- neurons, and consistent with studies showing that the 10+16 mutation can override developmental regulation of MAPT splicing (Sposito et al., 2015).

5.2.10 Tau protein levels and splicing in enCORs at 50 DIV

To assess whether multiple tau isoforms were present at the protein level we generated protein lysates from enCORs for analysis by western blot (Figure 43). As previously, these were first treated with λ-phosphatase to enable bands to be assigned definitively as splice variants rather than phospho-tau species. Immunoblots on samples treated and untreated with λ-phosphatase were performed and probed with total tau antibody Dako tau and phosphotau antibody PHF1.
Figure 43. Tau immunoblot of lysates from 50 DIV enCORs with λ-phosphatase treatment.

Protein samples from enCORs generated from WT, 10+16m, 10+16bi, 10+16bi/P301Sbi, treated (+) or untreated (-) were analysed by western blot for total tau and phosphotau (pS396/S404). β-actin used as loading control. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used as well as a tau isoform ladder, with kDa markers on the left. Representative blot where n=1, to demonstrate tau isoforms expression.

In all samples, there is a clearly visible downward shift following λ-phosphatase treatment, consistent with removal of phosphate groups and reduction in molecular weight. Immunoblotting with a phospho-specific antibody to phosphorylated S396/S404 shows the same downward shift, very minimal to zero signal could be detected in the treated samples. Total tau immunoreactivity showed a diffuse, broad band in untreated samples, but distinct bands following dephosphorylation that could be assigned as specific isoforms by comparing to recombinant tau ladder. In WT enCORs, a single band could be seen after dephosphorylation, corresponding to 0N3R tau when compared to a recombinant tau ladder. In contrast, a doublet of bands could be observed in all samples where the 10+16 mutation is present. This demonstrates expression of both 0N3R and 0N4R tau is expressed in the mutant lines, confirming our RT-PCR results (Figure 42) and showing that the 10+16 mutation drives early expression of 4R tau at the protein level. β-actin was used as a loading control.

5.2.11 MAPT expression and splicing enCORs at 100 DIV

We next repeated this analysis in enCORs harvested at 100 DIV (Figure 44). RT-PCR was performed as described previously, to assess the presence of 0, 1 and 2N tau, and 3R and 4R tau levels.

As observed in organoids at 50 DIV, 0N tau was expressed in all enCORs at 100 DIV and was the predominant N-terminal splice variant. However, the majority (11 out of 12 examined) of samples also showed low but detectable expression levels of 1N tau transcripts, providing evidence for the expression of additional MAPT isoforms. Further, the expression of 2N tau was also observed in 2 out of 12 enCORs (Figure 44, Batch 2, WT and 10+16 biallelic genotypes).

In both mono and bi allelic 10+16 mutation enCORs, 4R tau was detected in all samples examined, similar to what was observed at 50 DIV. In contrast to what was observed at 50 DIV, a faint band corresponding to 4R tau could be observed in two WT organoids (Batch 2 and 3), potentially indicating earlier maturation of tau splicing in enCORs. GAPDH was used as a loading control.
Figure 44. MAPT splicing in enCORs at 100 DIV.

Tau splicing was assessed in enCOR samples at 100 DIV using primers spanning exons 2 and 3 (0N, 1N, 2N) or primers spanning exon 10 (3R, 4R) of MAPT. 0N, 1N and 2N tau were detected as bands of 112 bp, 199 bp and 286 bp respectively. 3R and 4R tau were detected as products of 305 bp and 398 bp respectively. RNA from human brain tissue was used as a positive control. Samples run on a 2% agarose gel. PCR samples were diluted 1:6 with loading dye, with 10uL loaded per well. 100bp ladder used with bp size labelled on the left, all imaging was done on a Li-Cor imaging system. N = 3.

5.2.12 Tau protein levels and splicing in enCORs at 100 DIV

We next used dephosphorylation followed by immunoblot to confirm which tau isoforms were present at the protein level (Figure 45). Two total tau antibodies were used to confirm the presence of any additional tau isoforms. As observed earlier, 0N3R was the predominant tau isoform in enCORs of all genotypes. As previously, the presence of the 10+16 mutation also resulted in expression of 0N4R.
Figure 45. Tau immunoblot of lysates from 100 DIV enCORs with λ-phosphatase treatment.

Protein samples from enCORs at 100DIV run without λ-phosphatase treatment (-) and with λ-phosphatase treatment (+) were analysed by western blot with two total tau antibodies, probed using Dako Tau and HT7 antibody. β-actin used as loading control. N=3 inductions per genotype, representative image shown. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used as well as a tau isoform ladder, with kDa markers on the left. Representative blot where n=1, to demonstrate tau isoforms expression.

Other tau isoforms (i.e. 1N and 2N) variants could not be detected, despite them being detectable at the RNA level. This suggests either a delay between the appearance at the RNA level and translation into protein expression, or that these isoforms are present but below the limit of detection by western blot.

5.2.13 MAPT expression and splicing in enCORs at 200 DIV

Given that the appearance of additional tau isoforms was observed at 100 DIV, we continued our longitudinal analysis of MAPT splicing at the longer time point of 200 DIV (Figure 46).
0N tau was still the predominant transcript within these 200 DIV enCORs. However, 1N tau could again be observed in 9 out of 12 enCORs, and 2N tau could also be detected in 4 out of 12 enCORs. The appearance of N-terminal splice variants was variable but did not appear to correlate with presence of the 10+16 mutation. Interestingly, no 1N or 2N tau expression was detected in the 10+16bi/P301Sbi mutant enCORs, potentially pointing to an unexpected effect of the P301S mutation on N-terminal splicing.

As previously, all enCORs with the 10+16 mutation showed both 3R and 4R expression. However, several samples now showed a 4R signal which was more intense than the 3R signal, suggesting an excess 4R as observed in patient brain (e.g. 10+16bi, batch 3). 4R expression could also be observed in all WT enCORs, confirming this trend of 4R tau being expressed earlier within these cultures when compared to 2D iPSC derived neurons (Sposito et al., 2015). GAPDH was used as a housekeeping gene, with H20 the negative control and human brain RNA as the positive control.

Figure 46. MAPT splicing in enCORS at 200 DIV.

MAPT splicing in enCOR samples at 200 DIV using primers spanning exons 2 and 3 (0N, 1N, 2N) or primers spanning exon 10 (3R, 4R) of MAPT. 0N, 1N and 2N tau were detected as bands of 112 bp, 199 bp and 286 bp respectively. 3R and 4R tau were detected as products of 305 bp and 398 bp respectively. RNA from human brain tissue was used as a positive control and water as the negative control. Samples run on a 2% agarose gel. PCR samples were diluted 1:6 with loading dye, with 10uL loaded per well.
100bp ladder used with bp size labelled on the left, all imaging was done on a Li-Cor imaging system. 

\( N = 3 \).

### 5.2.14 Tau protein levels and splicing in enCORs at 200 DIV

To determine whether multiple tau isoforms were present at the protein level at 200 DIV, immunoblots were performed on dephosphorylated lysates as described previously, and probed for total tau (Figure 47). Due to some of the additional isoforms appearing at low levels, two exposures are shown for clarity.

**Figure 47. Tau protein expression in enCORS 200DIV.**

enCORs from WT, 10+16 monoallelic, 10+16 biallelic and 10+16 and P301S biallelic genotypes were harvested at 200 DIV and analysed by western blot. Lysates are shown treated with (+) and without (−) \( \lambda \)-phosphatase in order to remove phosphate groups from proteins. Total tau was used to confirm protein tau isoforms present (Two exposures shown) and beta actin was used as a loading control. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used as well as a tau isoform ladder, with kDa markers on the left. Representative blot where \( n=1 \), to demonstrate tau isoforms expression.
Within WT enCORs, multiple isoforms of tau were clearly visible, especially following dephosphorylation (Figure 47, high exposure). Alignment with the recombinant tau ladder suggests expression of 0N3R, 0N4R, 1N3R, 1N4R, and 2N3R. Due to the difficulty of tau immunoblots 2N4R might be expressed, however further experiments would be needed to confirm this.

Within 10+16m enCORs, at low exposure, two predominant bands can be seen, corresponding to 0N3R and 0N4R tau isoforms. Faint bands at a higher molecular weight could also be observed, and at higher exposure these are revealed to be additional tau isoforms, likely 1N3R and 1N4R. A similar pattern of tau splicing was observed in the bi-allelic 10+16 enCORs, and, as expected. The 0N4R band showed higher intensity compared to the 10+16 mono allelic enCORs, suggesting a dose-dependent effect on 4R tau expression.

Finally, 10+16bi/P301Sbi enCORs show a similar pattern of tau isoform expression to the 10+16 only enCORs, showing that the presence of the P301S mutation does not alter the effect of the 10+16 mutation on tau splicing. Taken together, these results show that by 200DIV, enCORs have started to acquire adult tau isoforms.

5.2.15 MAPT expression and splicing in enCORs at 300 DIV

Finally, enCORs cultured to 300 DIV were analysed for tau expression and splicing at both the RNA and protein levels (Figure 48).

As observed at previous time points, 0N was the predominant N-terminal isoform in the majority of enCORs. 1N transcripts were now also clearly observed in all samples, showing a consistent maturation of MAPT splicing across all genotypes. 2N tau was only faintly detected in 4 out of 12 enCORs, consistent with it being the least predominant variant. GAPDH was used as a loading control. Brain cDNA was used as the positive control, showing all bands as expected, while all bands were absent from the H2O negative control.

Next, we investigated the expression of 3R and 4R tau. A doublet of bands could be observed in all enCORs, suggesting both 3R and 4R tau were present in all genotypes, including WT. In WT enCORs, 3R continued to be the predominant band, although 4R was now expressed at high levels. In bi-allelic 10+16 enCORs, the 4R tau expression was higher than observed in other genotypes, showing that this mutation in the bi-allelic form drives an excess of 4R transcription. Another interesting observation is the apparent equal levels of 3R and 4R within the 10+16bi/P301Sbi enCORs. Since this genotype also contains the 10+16 bi-allelic mutation, you would expect the 3R/4R ratio to be similar to the 10+16bi
allelic line, however the intensity of 4R tau within these enCORs seems to be reduced. As this is qualitative data this finding will be investigated further with qPCR (Figure 55).

![Batch 1 Batch 2 Batch 3](image)

Figure 48. MAPT Splicing in 300 DIV enCORS.

MAPT splicing in enCOR samples at 300 DIV using primers spanning exons 2 and 3 (0N, 1N, 2N) or primers spanning exon 10 (3R, 4R) of MAPT. 0N, 1N and 2N tau were detected as bands of 112 bp, 199 bp and 286 bp respectively. 3R and 4R tau were detected as products of 305 bp and 398 bp respectively. RNA from human brain tissue was used as a positive control and water as the negative control. Samples run on a 2% agarose gel. PCR samples were diluted 1:6 with loading dye, with 10uL loaded per well. 100bp ladder used with bp size labelled on the left, all imaging was done on a Li-Cor imaging system. N = 3.

5.2.16 Tau protein levels and splicing in enCORs at 300 DIV

We next confirmed these findings using western blotting on dephosphorylated lysates as previously (Figure 49). The WT enCORs showed expression of multiple tau isoforms, with a very similar splicing pattern to what was observed at D200.

Within the 10+16 mono-allelic enCORs the 0N4R tau band shows strong expression, and this band is now the most predominant isoform, consistent with this mutation driving an excess of 4R tau.
However the ON3R tau expression is still robust, showing that some splicing of exon 10 can still occur. Similar to the WT enCORs, additional tau isoforms are visible, likely corresponding to 1N3R and 1N4R. Again, the intensity of the upper band of the doublet (1N4R) is higher than the lower band (1N3R), due to the effects of the mutation on exon 10 inclusion.

10+16 bi-allelic enCORs show the presence of the same tau isoforms as 10+16 mono allelic enCORs. However, the predominance of 4R tau species (0N4R and 1N4R) is much higher in this sample, with the 0N3R and 1N3R bands now representing minor species. This is consistent with the presence of a biallelic splice mutation, which will one would hypothesise will drive exon 10 inclusion to near completion. It seems that 4R tau is highest within this genotype, and this ratio dysregulation continues to later time points. Interestingly there is a strong band corresponding to 1N4R tau, mirroring the mono-allelic enCORs. This suggests that at this extended time point the 4R isoforms become predominant over 3R tau in the presence of the 10+16 splice mutation, mirroring what is seen in patient tissue.

Figure 49. Tau immunoblot of lysates from 300DIV enCORS with λ-phosphatase treatment.
Lysates treated with (+) and without (-) λ-phosphatase in order to remove phosphate groups from proteins. Total tau was probed for using Dako tau antibody to confirm protein tau isoforms present (Two exposures shown). Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was
loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used as well as a tau isoform ladder, with kDa markers on the left. Representative blot where n=1, to demonstrate tau isoforms expression.

Interestingly the 10+16bi/P301Sbi enCORs display a different tau splicing pattern to the 10+16bi enCORs. Specifically, the 0N isoforms are still predominant in this genotype, with apparently equal levels of 3R and 4R. This suggests a potential transcriptional or post-translational effect of the P301S coding mutation on tau splicing. The high weight tau band corresponding to 1N4R tau is again visible within this genotype as a strong band, although less prominent than in previous mutation genotypes. B-actin was used as a loading control.

5.2.17 Mature MAPT splicing is observed in multiple organoid protocols

We next wanted to determine whether the acquisition of mature tau splicing was protocol-specific or would be conserved across multiple organoid models. We therefore investigated tau splicing in forebrain organoids, also known as dual-SMAD (DS) organoids, at 300 DIV. Forebrain organoids were cultured in collaboration with Argyro Alatza, according to the Qian et al. 2016 protocol which has been described within the method section of this thesis (Qian et al., 2016).
Figure 50. MAPT splicing in forebrain organoids at 300 DIV.

MAPT splicing in forebrain organoid samples at 300 DIV using the ‘2-3-4’ primers spanning exons 2 and 3 (0N, 1N, 2N) or primers spanning exon 10 (3R, 4R) of MAPT. 0N, 1N and 2N tau were detected as bands of 253 bp, 341 bp and 428 bp respectively. 3R and 4R tau were detected as products of 305 bp and 398 bp respectively. RNA from human brain tissue was used as a positive control and water as the negative control. Samples run on a 2% agarose gel. PCR samples were diluted 1:6 with loading dye, with 10uL loaded per well. 100bp ladder used with bp size labelled on the left, all imaging was done on a Li-Cor imaging system. Samples and analysis performed by Argyro Alatza.

MAPT splicing was analysed at the RNA level as previously described in 3 forebrain organoids per genotype. N-terminus splicing 0N is still the predominant N-terminal isoform that is expressed, but 1N transcripts were detected in all organoids analysed. 2N tau isoforms were also detected in at least 5 out of the 12 samples, although levels were very low.

C-terminal splicing shows 3R and 4R tau is present in WT forebrain organoids as well as samples with the splicing mutation (Figure 50). The 4R band intensity in the DS organoids with the splicing mutation was higher in most samples, suggesting an excess of 4R transcripts.

5.2.18 Mature tau isoforms are expressed at the protein level in forebrain organoids

To investigate tau protein isoform expression within patterned forebrain organoids, immunoblots were run on dephosphorylated samples treated with λ-phosphatase (Figure 51).

Similar to results seen in the enCORs, all forebrain organoids showed the expression of multiple tau isoforms at the protein level by 300 DIV. All samples show a downward shift following dephosphorylation, and a resolution of bands into distinct protein isoforms. WT forebrain organoids show expression of multiple tau isoforms: four distinct bands are clearly visible, corresponding to 0N3R, 0N4R, 1N3R and 1N4R when compared against a recombinant tau ladder. 0N3R tau is still the most dominant tau isoform expressed.

10+16 mono-allelic forebrain organoid samples show the same pattern of 4R tau expression as other organoid models. 0N4R tau is the dominantly expressed isoform, which differs from the WT forebrain organoids. 1N4R tau is also more heavily expressed than in WT organoids.

Similarly, the bi-allelic forebrain organoids show an increased expression of 0N4R tau in a similar pattern of tau isoforms seen in enCORs. 0N4R tau and 1N4R tau are both present at higher levels.
compared to the mono-allelic forebrain organoids, together with reduced levels of 3R tau isoforms. β-actin was used as a loading control, helping prove equal loading of 10ug for all samples.

Figure 51. Tau of 300 DIV forebrain organoid lysates with λ-phosphatase treatment.

Lysates treated with (+) and without (-) λ-phosphatase in order to remove phosphate groups from proteins. Total tau was probed for using Dako tau antibody to confirm protein tau isoforms present. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used as well as a tau isoform ladder, with kDa markers on the left. Representative blot where n=1, to demonstrate tau isoforms expression. Samples grown and run by Argyro Alatza.

5.2.19 MAPT splicing in multiple organoid protocols resembles adult human brain at the RNA level

The data so far suggest that, in multiple organoid models, a mature splicing of tau is acquired at an earlier time point than what is observed in 2D cortical neurons. We therefore confirmed this by assessing tau expression and splicing at 300 DIV in 2D cortical neurons, enCORs, DS organoids and human brain in a single experiment.

RT-PCR was performed as described previously. Primers used for exon 10 were the same as previous experiments, however alternate N terminus primers were used, producing products of 253 (0N), 341 (1N) and 428 (2N) base pairs (Primers in Table 9).
Figure 52. MAPT splicing in 2D iPSC neurons, forebrain organoids, and enCORs at 300 DIV.

MAPT splicing in three iPSC derived neuronal models at 300 DIV using the ‘2-3-4’ primers spanning exons 2 and 3 (0N, 1N, 2N) or primers spanning exon 10 (3R, 4R) of MAPT. 0N, 1N and 2N tau were detected as bands of 253 bp, 341 bp and 428 bp respectively. 3R and 4R tau were detected as products of 305 bp and 398 bp respectively. RNA from human brain tissue was used as a positive control and water as the negative control. Samples run on a 2% agarose gel. PCR samples were diluted 1:6 with loading dye, with 10uL loaded per well. 100bp ladder used with bp size labelled on the left, all imaging was done on a Li-Cor imaging system. Samples and analysis performed by Argyro Alatza. Forebrain samples provided by Argyro Alatza.

As can be seen in Figure 52, in 2D cortical neurons, predominant expression of 0N tau was detected, accompanied by a faint band corresponding to 1N tau. No 2N transcripts were observed in the 2D cortical neurons. However, within both DS organoids and enCORs, there is robust expression of 0N and 1N isoforms, together with a clear 2N transcript expression, although 2N represents a lower proportion of total tau transcripts. In human brain, all three N-terminal variants can be detected, with 1N representing the most predominant species, followed by 0N and then 2N. Thus, both enCOR and DS organoid models recapitulate tau splicing more closely than 2D cortical neurons cultures.

3R and 4R tau transcripts were next examined, and all cultures show robust expression of both C-terminal isoforms, with bands of approximately equal intensity. This Indicates mature neuronal tau splicing within these neuronal cultures, similar to adult human brain. GAPDH was used as a housekeeping gene.
5.2.20  **Tau expression and splicing in multiple organoid protocols resembles adult human brain at the protein level**

To confirm whether the tau splicing profiles observed in the different models at the mRNA level were also present at the protein level, equivalent protein samples were dephosphorylated and analysed by immunoblot to total tau (Figure 53).

Total tau within 2D neuronal cultures still displays predominantly lower molecular weight tau isoforms. The most abundant of these being that of fetal tau (0N3R). Although fetal tau is the predominant isoform in these 2D iPSC derived neuronal cultures, 0N4R can be readily detected, but at lower levels. Higher molecular weight tau isoforms are harder to distinguish as well as being expressed at lower levels. A smear could be observed at higher molecular weights, likely corresponding to additional tau isoforms being present, but in smaller amounts. It was difficult to assign specific isoform identities due to the lack of resolution in this sample. Both DS organoids and enCORs show a similar pattern of tau isoform expression.

Dephosphorylation resulted in the appearance of discrete bands rather than a smear as seen in the untreated samples. DS organoids exhibited increased levels of 0N3R and 0N4R tau compared to 1N3R and 1N4R. A similar profile was also observed in enCORs. Finally, in human brain six bands could be observed. The 0N3R, 0N4R, 1N3R and 1N4R showed the strongest expression, and only faint bands corresponding to 2N3R and 2N4R could be seen, consistent with the 2N isoforms being the least abundant in human brain. β-actin was used as a loading control.
Figure 53. Tau immunoblot of 300 DIV 2D, forebrain and enCOR lysates.

Tau isoform protein expression was assessed by immunoblot in lysates from 2D iPSC derived cortical neurons, forebrain organoids, engineered organoids, and post-mortem human brain. All cell lysates harvested were 300 DIV and samples run with (+) and without (-) prior λ-phosphatase treatment. Total tau was assessed using DAKO tau antibody and β-actin was used as a loading control. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used as well as a tau isoform ladder, with kDa markers on the left.

5.2.21 qPCR analysis of N-terminal splicing in enCORs

To confirm the analysis of enCOR RNA which indicated that multiple tau mRNA isoforms were present, quantitative PCR was performed on enCOR samples from all genotypes.

Primers were designed for the N-terminal of the gene where exon 2 and 3 are alternative spliced (Table 10). The levels of each N-terminal isoform were analysed in enCORs at 100, 200 and 300 DIV (Figure 54). All graphs show each isoform relative to total MAPT expression, followed by normalisation to the control brain run on each qPCR 96 well plate.
Figure 54. Quantitative analysis of 0N, 1N and 2N expression in enCORs
A) 0N tau mRNA expression in enCORs with genotypes WT, 10+16m, 10+16bi and 10+16bi/P301Sbi, at 100, 200, and 300 DIV. B) 1N tau mRNA expression in enCORs at 100, 200, and 300 DIV. C) 2N tau mRNA expression in enCORs at 100, 200, and 300 DIV. All samples are represented as expression relative to MAPT, normalised to control human brain. One-Way ANOVA was run. Results are expressed as fold difference compared to human brain RNA, with SEM used for the error bars.

0N MAPT transcripts were shown to be high in 100 DIV enCORs (Figure 54.A), an expected result due to early developing neurons producing only 0N3R fetal tau isoform. However, 0N expression remained high throughout 200 and 300 DIV, suggesting that 0N isoforms account for a large fraction of total tau within enCORs even at extended time points. enCORs have higher 0N expression when compared to the human brain sample, however this control brain sample is adult human brain rather than fetal brain, which was not available to us for this analysis but which we would expect to have a more similar profile to enCORs. In WT enCORs, 0N seems showed a trend to decrease from 100 to 200 DIV, however there is then an increased trend to 300 DIV, although these changes were not significant and variable. Both mono and biallelic 10+16 enCORs show little change in 0N tau isoform expression across time points. However, interestingly, the double biallelic mutation enCORs show a steady but small increase
in 0N tau transcript expression, although variability is an issue and these results were not significant. None of these trends shown here were significant after statistical analysis, also the levels of 0N tau appeared quite variable and more repeats would be necessary to be able to show conclusive changes in 0N ratios over time.

1N expression in all enCORS was detectable, but at lower levels than control human brain expression (Figure 54.B). However this analysis confirmed the presence of 1N tau from 100 DIV, suggesting enCORs start producing mature MAPT isoforms earlier than other iPSC models. WT enCORs show relatively constant expression of 1N tau between 100 - 300 DIV. A similar expression pattern is observed in the mono allelic 10+16 mutation enCORs. Interestingly in both the 10+16 bi-allelic and 10+16bi/P301S bi-allelic enCORs, levels of 1N transcript are lower than WT or mono-allelic carrying enCORs at the 300 DIV time point. An increased number of samples are needed to confirm this. Further to these results, 10+16bi/P301Sbi enCORs show very low levels of 1N (with a single outlying sample at 200 DIV) when compared to other genotypes across all time points, highlighting the variability in tau splicing.

2N expression for all samples was below the control human brain sample (Figure 54.C), and variability for 2N MAPT expression was less variable than anticipated considering pervious RT-PCR results (Figure 48). WT enCORs show an increase in 2N in a time dependent manner, with 300 DIV enCORs showing the highest expression. 10+16 mono-allelic enCORs also show an increase in expression of 2N over time. In a similar manner to 1N expression, 10+16 bi-allelic shows an increase in expression from 100 to 200 DIV, however a sharp decrease is seen at 300 DIV. 10+16/P301S bi-allelic enCORs also seem to follow 1N expression patterns, with low expression across all time points bar a single outlying data point.

5.2.22 Quantitative analysis of exon 10 expression in enCORs

For analysis of exon 10 inclusion, RNA samples isolated from enCORs at 50, 100, 200 and 300 DIV were sent to Washington University, St Louis, USA for analysis in collaboration with Dr Celeste Karch using TaqMan qPCR which was already optimised in their lab, according to the protocol described in the methods chapter of this thesis. Due to sample limitations and losses during the 300 days of culture, only two WT enCOR RNA samples were available to use, however these were from independent inductions. qPCR was first run using a TaqMan probe for all isoforms of tau to verify levels of overall tau within enCORs as well as to confirm the mutations do not influence total tau levels. MAP2,
expression was used as a housekeeping gene to calculate the mean normalised expression (MNE) values (Figure 55).

Figure 55. Quantitative analysis of 4R tau transcripts
A) Total tau mRNA expression from enCORs with genotypes WT, 10+16m, 10+16bi and 10+16bi/P301Sbi, at 50, 100, 200, and 300 DIV. Represented as mean normalised expression (MNE) using MAP2 as the normalising value per sample. Prism software was used for statistical analysis by way of one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001. B) 4R tau mRNA expression over MAP2 expression from enCORs at 50, 100, 200, and 300 DIV. Represented as mean normalised expression (MNE), normalised to MAP2 levels. Prism software was used for statistical analysis by way of one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001. C) 4R tau mRNA expression over total tau expression from enCORs with genotypes at 50, 100, 200, and 300 DIV. Represented as mean normalised expression (MNE), normalised to total tau levels. Prism software was used for statistical analysis by way of one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001.
Total tau expression within control enCORs was relatively consistent although an unexpected decrease in total tau was seen at 200 DIV (Figure 55.A). This was not significant and likely just reflects variability between enCORs and may suggest that these particular enCORs had a lower neuronal content. Stable tau expression was observed across all other genotypes with no statistically significant differences in total tau levels between the genotypes. However, a statistically significant increase in total tau was observed in the 10+16bi/P301Sbi enCORs 50 DIV and 100 DIV (P=0.0331) and 50 DIV and 200 DIV (P=0.0042) enCORs. This suggests that the presence of two mutations within these enCOR cultures could result in an upregulation in tau expression.

Next, 4R was quantified in enCORs using an isoform specific Taqman probe, which was normalised to MAP2. In WT enCORs (Figure 55.B), there is a gradual increase in 4R tau until 300 DIV where expression of 4R decreases, although this was not statistically significant. It also needs to be noted that the n number for the 300 DIV WT enCORs is only two, not three as in all other data points presented. However, this clearly shows early but low levels of 4R tau transcript expression from 100 DIV in WT enCORs, and robust expression of 4R tau isoforms from 200 DIV onwards, confirming previous RT-PCR results as well as corroborating the protein analysis time line shown in this thesis (Chapter 5.2.21).

10+16 mono-allelic enCORs show a small increase in 4R tau over time up until 200 DIV, with a large increase in 4R tau at 300 DIV. 10+16 bi-allelic also shows an increase over time in 4R tau, more robust increases per time point are seen compared to mono-allelic enCORs, and the data culminates in a high expression level of 4R tau at 300 DIV. Due to the presence of the 10+16 mutation being bi-allelic within this cell line, it is understandable that 4R tau levels will be much higher than in wild type or the mono-allelic enCORs. 10+16 biallelic enCORs seem to be expressing 4R tau at increased levels at early time points, although by 300 DIV expression levels of 4R tau are similar to the mono-allelic counterpart. 4R tau expression levels between 50 and 300 DIV were statistically significant (P=0.0488).

10+16bi/P301Sbi enCORs show an increase in 4R tau over time, with 300 DIV enCORs expressing the most 4R tau. However, interestingly the expression of 4R mRNA is much lower in these enCORs than in the bi-allelic, and even WT enCORs. When comparing 50, 100, and 200 DIV against the 300 DIV sample, all results were all statistically significant (P= 0.0016, 0.0024, and 0.0129 respectively). All comparisons between genotypes were not statistically significant.

Next, 4R tau was normalised to total tau to determine what proportion of total tau within our enCOR samples consisted of 4R transcripts (Figure 55.C). Similar patterns of expression were observed as seen when data was normalised to MAP2, consistent with both MAP2 and tau being neuronal proteins. No significant differences between genotypes were found, however there were significant differences at different time points within genotypes.
4R tau was low in WT enCORs at 50 DIV with a small increase at 100 DIV, however there is a large increase in 4R mRNA expression from 100 to 200 DIV, potentially pointing to a key moment in neuronal maturation during these time points from a MAPT splicing standpoint. At 300 DIV, control enCORs show a decrease in 4R mRNA expression, this could be due to an increase in expression in 1N and 2N 3R transcripts and thus an increased diversity of tau isoforms at this time point.

enCORs with the 10+16m genotype show a time dependent increase in 4R mRNA up to 300 DIV. Interestingly this expression is lower than WT up until 300 DIV, where expression is higher, although also with increased variability. This indicates that in this mutant line, 4R tau is being over-produced and overriding developmental cues. 4R tau expression in 50 and 100 DIV enCORs are significantly different when compared to 300 DIV samples within this genotype (P=0.0376 and 0.0291 respectively).

In a similar manner, 10+16bi enCORs show an increase in 4R tau expression in a time dependent manner. These enCORS show increased 4R mRNA expression at 100 DIV compared to all other genotypes and at 300 DIV. The MNE for these enCORS is much higher than all other genotypes, although there is also more variability within this genotype. Due to this bi-allelic mutation producing native 4R tau and driving excess 4R tau, it is understandable to see 4R tau expression increasing to higher levels over extended time periods. There was a significant increase in 4R tau expression between 50 and 300 DIV within this genotype (P=0.0488).

Finally, the enCORs harbouring the 10+16bi/P301Sbi double mutation show a slower increase in 4R tau mRNA over time. Expression levels are generally lower in this genotype than in the other enCOR samples. Even by 300 DIV, these mutant enCORs only produce around 25% of the 4R tau mRNA compared to the 10+16 biallelic enCORs. Interestingly biallelic enCORs are very consistent compared to other genotypes, with 4R tau expression within this genotype at 50, 100 and 200 DIV being significantly different than 300 DIV enCORs (P=0.0053, 0.0005, and 0.0010 respectively).

5.2.23 Detection of tau pathology in enCORs

Our data so far support a pathological pattern of tau expression, with an excess of 4R tau in the presence of the MAPT 10+16 mutation. We next we set out to examine whether any pathological tau species were present in our enCOR cultures, using an established tau biosensor assay. This assay consists of HEK cells overexpressing the human tau microtubule binding domain (MBD) containing the P301S mutation, tagged to either CFP and YFP domains (Holmes et al., 2014). Addition of seed-competent tau species will promote self-association and aggregation of tau, and the fluorescent tags allow for visualisation of these aggregates, either by excitation and detection of CFY directly or by
Förster resonance energy transfer (FRET), exciting CFP and detecting emission from the YFP tag due to close proximity energy transfer from one to the other. This assay has been previously used for the detection of early tau pathology (Takeda et al., 2016).

HEK biosensor cells were transduced with 10μg of 300 DIV enCOR lysates from all genotypes using lipofectaime, explained in detail in section 2.24. AD brain lysate was transduced as a positive control as well as solely lipofectamine as a negative control. It should be noted that recombinant heparin incubated P301S tau protein could have been an additional rigorous positive control, however this was unavailable. However, it has been shown that recombinant tau appears to aggregate in a different manner and structure than pathological human tau (Zhang et al., 2019)
Figure 56. Assessment of seed-competent tau species in enCORs using a tau biosensor aggregation assay.

Fret signal of Tau RD P301S FRET Biosensor (ATCC® CRL-3275™) cells after 72 hrs transfection of 300 DIV lysates from WT/WT, WT/10+16, 10+16/10+16 and 10+16bi/P301Sbi enCORs, imaged via confocal microscopy. AD lysate and lipofectamine only was also transfected as controls. AD lysates show traditional tau aggregates. Cells transfected with 10+16bi/P301Sbi lysates show tau aggregates, albeit with a lower efficiency than the AD control. All other lysates show no measurable seeding potential. Representative images show, n = 6. Scale bar is 50µm
Cells were grown for 72 hours, after which they were fixed and analysed for tau aggregation using confocal microscopy to detect the FRET signal observed by exciting CFP and detecting emission from YFP (Figure 56).

Biosensor cells transduced with AD brain lysate showed a high proportion of tau aggregation (Figure 56, arrows). No detectable aggregates were observed in the negative control, as well as cells transduced with WT, 10+16m and 10+16bi lysate. However, in biosensor cells transduced with the 10+16bi/P301Sbi enCOR lysate, rare aggregation events were observed. This suggests that seed-competent tau species are present in enCORs with this genotype, although this would require further investigation, optimisation and quantification, which was prohibited due to the COVID-19 shutdown.

5.3 Discussion

In this chapter, enCORs from an isogenic series of iPSC were successfully generated with the following MAPT genotypes: WT, 10+16 monoallelic, 10+16 biallelic, and 10+16 biallelic / P301S biallelic.

After confirming successful generation of enCORs, a thorough longitudinal analysis of tau expression and splicing in this system was performed. Finally, a preliminary analysis was performed with the aim of identifying whether pathological forms of tau were present within this model.

The overall aim of this thesis was to develop a 3D cerebral organoid model of tauopathy. As our earlier data showed high variability in COs, we wanted to establish a new protocol that would minimise this variability. Several methods have been described to achieve this including suspension cultures and alginate capsule cultures, detailed within section 1.12.

enCORs were chosen as the neuronal model, as the provision of a scaffold reduces variability whilst still enabling intrinsic differentiation to occur, providing a culture system directly comparable with the COs. Differentiation into enCORs was successful across all genotypes, this was confirmed by high levels of neuronal content in multiple ICC images at 100 DIV, showing positive BRN2, βIII-tubulin, FOXG1, VGLUT1, and MAP2 staining.

Interestingly, VGLUT1 antibody positive staining (Figure 37) suggests synaptic development and expression of glutamate transporters within the enCORs. Previous studies have also shown VGLUT1 and 2 to be predominantly co-expressed in adult neurons, with VGLUT2 being dominantly expressed in developing neurons (Wojcik et al., 2004). ICC analysis of both proteins within enCORs would enable comparison between genotypes, but also against other cell models, for example 2D neuronal cultures. Using specific software like Volocity by Quorum Technologies, 3D volumetric data could be established.
from z-stacks taken on a confocal microscope, allowing a detailed comparison of VGLUT1 vs VGLUT2 expression, as well as comparison between models. This data paired with western blot analysis of total VGLUT 1 and 2 protein normalised to a neuronal marker such as βIII-tubulin would give a robust comparison and an indication of neuronal maturity.

The expression of these markers does not necessarily mean the synapse are functional, and this is an important area for further investigation. Sood et al 2019 have shown enhanced maturation of neurons when placed in fetal brain derived ECM, pointing to 3D architecture, presence of ECM, and ECM components being important in neuronal maturation. This was accomplished by gene expression and secretome profiling, but importantly also by calcium signalling and spectral analysis. Spectral analysis is examination of events or measurements across a time series, trying to characterize the relationships between actions and measurements, for example calcium signalling within a 3D model (Sood et al., 2019).

As cryostat sectioning and subsequent immunofluorescent analysis utilises a section from within the 3D enCOR, we complemented this analysis with western blot data of βIII-tubulin and total tau. This was to further gain insight into the neuronal content of the whole organoid and how much heterogeneity between enCORs exists. This data showed consistent neuronal and tau content, confirming these enCORs to be much more homogenous and more suited to bulk analysis than COs, as described previously (Lancaster et al., 2017). This was observed across all genotypes, showing that the 10+16 and P301S mutations do not effect neuronal differentiation, as previously shown in 2D (Sposito et al., 2015). Similar findings were seen with a directed-differentiation protocol designed to give forebrain organoids (Qian et al., 2016), showing that there are multiple ways to reduce heterogeneity.

In addition to examining neuronal content, the astrocytic content of enCORs throughout differentiation was also briefly explored. The astrocytic marker GLAST expression was present in all enCORs at 100 DIV, demonstrating the presence of astrocytes during enCOR development. This was present in enCORs of all genotypes, suggesting that gliogenesis is not impaired by the MAPT mutations studied in this work. ICC was also used to briefly investigate the morphology of astrocytes, which showed in vivo like morphology with the cells having the ability to create a three-dimensional shape (Freeman, 2010). However, more in-depth 3D imaging would is needed to confirm astrocyte morphology within these models, though previous work shows maturation of astrocytes within 3D cultures (Sloan et al., 2017).

Thus, enCORs could provide a suitable model for the study of neuronal-astrocyte interactions and their relevance to tauopathy. Tau pathology has been shown in astrocytes as well as work showing
glial cells contribute to neurodegeneration (Leyns and Holtzman, 2017; Reid et al., 2020). An intriguing paper from Richetin et al., (2020), shows accumulation of tau in astrocytes of the dentate gyrus in AD patients. Further investigation using a murine model found overexpression of 3R tau and altered mitochondrial dynamics and function within these astrocytes. This in turn, it is reported, reduced adult neurogenesis within their murine model (Richetin et al., 2020). Having access to a human in-vitro model, allowing astrocyte and neuronal interaction in an in-vivo manner is an exciting avenue of exploration for tauopathy and astrocytic research. As well as being an appropriate model for astrogliosis and astrocytic scaring, something explored in other early 3D models (East, Golding and Phillips, 2009).

An induction of GFAP expression was seen in enCORs over time, although this was variable between genotypes. Interestingly, we observed a significant decrease in GFAP within 10+16bi/P301Sbi enCORs. As GFAP is a marker of reactive astrocytes, this suggests fewer reactive astrocytes within these enCORs (Li et al., 2019). This could be due to the presence of excess 4R tau that also harbours the P301S mutation. However, this result fails to correlate with current research, as many papers have reported reactive astrocytes around Aβ plagues in AD, as well as other NDD (Nagele et al., 2003; Phatnani and Maniatis, 2015). Including specifically GFAP isoform expression seen in AD and Downs syndrome patient brains (Hol et al., 2003).

Further work to investigate astrocytes within enCORs could involve looking at other newly discovered reactive astrocyte markers and comparing with current GFAP results. For example Barber et al, 2020 showed CD49f as a reliable marker for human reactive astrocytes (Barbar et al., 2020). Other work suggests reactivity of astrocytes increases with age in murine samples, something that would be interesting to relate to human astrocytes (Clarke et al., 2018). GFAP and CD49f cellular localisation within 2D and 3D models utilising high resolution microscopy, STORM or light sheet imaging would also be incredibly interesting both across time points as well as across genotypes. Impressive work by Dekkers et al, 2019, has shown high resolution imaging of organoid cultures is possible (Dekkers et al., 2019).

Having established that enCOR are a suitable model for bulk analysis by biochemistry, MAPT splicing was investigated in all genotypes at 50 – 300 DIV. Tau splicing is developmentally regulated in the human brain, with developing neural tissue only expressing 0N3R tau until soon after birth, where the postnatal brain switches to expressing all 6 isoforms (Hefti et al., 2018). As expected, at early time points, (50 DIV) WT enCORs only expressed 3R tau and 0N tau mRNA, something shown in previous work in 2D iPSC derived neurons as well as being true in developing fetal brain (Sposito et al., 2015; Hefti et al., 2018). This confirms the fetal identity of our early time point
enCORs, similar to what has been shown with other organoid studies (Gonzalez et al., 2018; Miguel et al., 2019).

In contrast, 4R tau expression was detected within enCORs with the 10+16 mutation. This verifies the MAPT splicing mutation is able to override developmental regulation as has previously described in 2D iPSC derived neurons (Sposito et al., 2015). This same phenotype of early 4R expression has been shown independently by Iovino in iPSC derived neurons harbouring another MAPT splicing mutation, N279K (Iovino et al., 2015).

Expression of 4R tau could be seen in WT enCORs from 100 DIV and increased at 200 and 300 DIV, potentially showing a faster maturation than their 2D counterparts as previous papers have reported, from a MAPT splicing standpoint (Zhang et al., 2016; Sood et al., 2019). Although impressive previous work from Miguel et al, 2019, has shown all 6 isoforms within 3D WT neuronal cultures, a few important differences should be noted. Firstly, although all 6 isoforms are detectable, almost 80% of the mRNA present at 25 weeks is 0N3R (fetal) tau. Tau protein levels at 25 weeks are still heavily fetal, with no 2N tau being detectable. Finally the 3D model itself is patterned, where iPSC cells are treated early in the protocol using dual SMAD inhibitors (LDN-193189 and SB431542) before encapsulation in a matrigel and hydrogel microcapsules following Alessandri et al, (2016), protocol (Alessandri et al., 2016).

In contrast enCORs presented in this thesis do show detectable levels of higher molecular weight tau (2N3R and 2N4R), although at low levels. It is also difficult to make out individual bands, something that would need to be optimised going forward, potentially through high resolution immunoblotting.

In addition, fetal tau expression within enCORs was shown to be reduced, particularly when compared to 2D iPSC derived neuronal cultures. With 0N3R, 0N4R, 1N3R, and 1N4R tau isoforms showing a balanced expression, comparable to human brain. Immunoblot data points to 300 DIV WT enCOR tau isoform expression being more representative of human brain tau expression than other previously examined iPSC derived neuronal models. Also, due to 4R tau in WT human brain being expressed postnatally, this switch is an interesting indicator that in-vitro neurons are maturing and developing in a similar fashion to human in-vivo neurons (Hefti et al., 2018).

This expression was confirmed using PCR as well as qPCR, with large increases in 4R tau mRNA detected at 200 DIV. In enCORS with the 10+16 mutation, 4R tau showed subtle but earlier 4R tau mRNA expression, increasing steadily until 300 DIV. Bi-allelic enCORs showed a robust and clear increase in 4R tau mRNA, increasing steadily from 50 to 300 DIV. Interestingly 10+16bi/P301Sbi enCORs showed overall low levels of MAPT mRNA when compared to other genotypes. This could be
linked to the transcripts within this genotype having a coding mutation and going through nonsense mediated decay (NMD), while splicing mutations alone do not. There is little research on MAPT mutations and their effect on mRNA stability, with limited research showing NMD effects the aggresome (Caillet-Boudin et al., 2015; Park et al., 2020). It would be interesting to investigate how the P301S mutation affects RNA stability.

A similar developmental regulation of N-terminal splicing corroborates our findings, as only fetal tau (0N3R) is detectable at early time points. 0N mRNA for all genotypes is very similar across time points due to 0N isoforms being over 50% of the tau in adult human brain, and exclusively expressed in fetal brain (Hong et al., 1998; Wang and Liu, 2008). From 100 DIV onwards, levels of 1N and 2N tau increase, showing maturation of MAPT splicing, and therefore of neuronal populations within these organoid cultures. Hefti et al 2018 robustly show 1N and 2N isoforms increase in expression postnatally as well as suggesting MAPT is a neuronal maturation marker given its relationship with axonal growth and expression times (Hefti et al., 2018). In a notable paper, Hefti et al, 2018, use computational analysis of large transcriptomic datasets totalling 502 samples to investigate MAPT splicing. This comprehensive look at tau isoforms compares post mortem human fetal, paediatric and adult brain tissue utilising qPCR and immunoblots. Our data correlates with this hypothesis.

MAPT and its developmental regulation is hypothesised to related to neuron development, for example axonal growth ends on the 20th postnatal day in the mouse cerebellum, after which only a limited number of new tau molecules are synthesised (Vilá-Ortiz et al., 2001). This points to fetal tau having a specific role within neuronal development, after which more stabilising tau molecules are required for neuronal maturation. Although tau knockout mice display no developmental or NDD defects, deficits in migration have been observed, again pointing to an important role in fetal tau in neuronal development (Fuster-Matanzo et al., 2009).

No N-terminal exons are expressed during development. N-terminal exons have been shown to control the formation of MT bundles, potentially more important when neurons need to develop longer more permanent axons and dendrites, areas of the cell that are rich in tau (Rosenberg et al., 2008). It has also been hypothesised that they help with spacing between microtubules, again within axons and dendrites (Chen et al., 1992). Interestingly, 1N and 2N transcripts in both 10+16bi and 10+16bi/P301Sbi enCORs both decrease suddenly at 300 DIV, while the other genotypes increase or plateau. This, combined with low levels of 4R MAPT mRNA, potentially points to bi-allelic mutations and/or coding mutations affecting mRNA regulation or degradation, which seems to be occurring as the neurons mature. There is literature around the truncation of tau protein and the general regulation of MAPT RNA, however more work is needed in to the potential regulation or degradation...
of MAPT RNA when mutations are present and at different time points (García-Sierra, Mondragón-Rodríguez and Basurto-Islas, 2008; Caillet-Boudin et al., 2015; Welden et al., 2018).

Although mRNA didn’t equate to clear protein expression until 200 DIV, a similar pattern was observed with increasing tau protein isoforms with increasing DIV. Showing 4R, and other tau isoforms, being expressed at detectable levels within WT enCORs. 4R tau is clearly visible in the mutant lines from early time points (50 DIV), again showing the developmental overriding effect of this mutation (Sposito et al., 2015). Taken together, this data supports the hypothesis of enCORs increased maturation rate compared to that of 2D iPSC derived neuronal models, at least from a MAPT splicing standpoint. This supports what has been shown previously regarding maturation in electrical activity, response to depolarization and calcium signalling (Rigamonti et al., 2016; Sood et al., 2019).

Although both RT-PCR and qPCR were utilised for N and C-terminus splicing, both techniques performed in this thesis do not give information about the whole transcript. They measure each isoform separately as well as only measuring the section of the mRNA containing the target area. Long range PCR since its inception in 1992 has improved drastically, now enabling over 30 kb products to be made, all by modifying the polymerases (Barnes, 1992; Jia et al., 2014). Utilising long range PCR would allow us to analyse whole transcripts present and get more information on individual relative isoform abundances.

Furthermore, due to the similar molecular weights of the different tau isoforms, it is difficult to quantify the relative abundance of each individual isoform. This could be achieved using alternative approaches, such as high resolution automatic immunoblot systems (e.g. Peggy Sue™) (Harris, 2015), isoform specific antibodies (Liu and Götz, 2013; Liu et al., 2016), or quantitative mass spectrometry (Mair et al., 2016; Zetterberg, 2018). Although other groups have found aggregations within 3D neuronal models, our investigations revealed no definitive Aβ plaques or tau tangles (Choi et al., 2014; Kim et al., 2015; Gonzalez et al., 2018). In order to investigate whether pathological tau was present, we used a biosensor seeding assay. This assay has previously been used to show lysates of brain tissue that have not yet developed NFTs can still seed tau pathology (Kaufman et al., 2017).

Although no seeding was observed with WT or 10+16 mutation alone, however 300 DIV 10+16bi/P301Sbi lysate did result in a low level of seeded aggregation. Further development of this result was not possible due to the lab shutdown, but ongoing work aims to optimise and quantify this results using FACs sorting to quantify cells with aggregates and enriching the samples for seed-competent tau by lowering centrifugation speeds to 3000 g (av) and using a PBS based buffer, as shown within Takeda et al, 2015 (Takeda et al., 2015).
Despite this, these results suggest that seed competent seeds are present within 3D organoid models. The identity of the seed-competent species in enCORs is not known, but previous papers have suggested that high molecular weight tau species are more seed competent than smaller tau species (Dujardin et al., 2018). Interestingly a recent paper from the same author, Dr Simon Dujardin, shows remarkable patient-to-patient heterogeneity in seed-competent tau as well as some PTM sites being associated with enhanced seeding activity (Dujardin et al., 2020). All sAD tau aggregates were shown to be entirely reactive to either only AT8 antibody (phosphotau S202) or both AT8 and Alz50 (misfolded tau). Interesting MAPT mutation tau aggregates contained these immune reactive species, but also tau only immunoreactive to the misfolded tau Alz50 antibody, suggesting specific tau species. Further investigations showed different spreading patterns for the different immunoreactive tau species, with spreading experiments done in rat brain. These tau species spreading techniques are something potentially applicable to this human neuronal enCOR model for future projects.

Finally, it has been shown that aggregates from human brain can induce seeding within murine models, even within non-transgenic mice, and it would be very interesting to compare possible seeding from enCORs alongside patient material (Langer et al., 2011; Guo et al., 2016). Seeding of tau aggregation in organoids by the introduction of exogenous seeds has not yet been reported, and would be interesting to investigate in future work.
6 Phosphorylation states of tau in enCORs

6.1 Introduction

In the previous chapters we have focussed on the expression and splicing of tau in our CO and enCOR system. In addition to this, tau is subject to extensive post-translational modification (section 1.3), of which phosphorylation is the best characterised.

6.1.1 Phosphorylation of tau

Phosphorylation of tau has been implicated in both the physiological regulation of tau protein function, and hyperphosphorylation of tau is a hallmark feature of FTD and other tauopathies (Johnson and Stoothoff, 2004; Trushina et al., 2019). The longest tau isoform, 2N4R, has 80 serine and threonine residues together with 5 tyrosine residues, allowing 85 possible phosphorylation sites. Thus, almost 20% of the largest tau molecule has the potential to be phosphorylated (Goedert, Spillantini, Jakes, et al., 1989).

The phosphorylation of tau is known to regulate its function. Tau is classified as an intrinsically disordered protein (IDP), and as such tau contains intrinsically disordered regions (IDRs), which lack distinct, three-dimensional structures within their cellular environments (Dyson and Wright, 2005; Trushina et al., 2019). Phosphorylation of within IDRs can alter various intracellular interactions and regulate the properties of IDRs (Noble et al., 2013; Fealey et al., 2018; Trushina et al., 2019). Over 20 years ago it was shown that phosphorylation of tau reduced its ability for it to bind to microtubules, an important characteristic for a dynamic microtubule protein (Bramblett et al., 1993; Busciglio et al., 1995). For example, phosphorylation of T231 causes tau to undergo conformational change, this tau structure reduces this tau structures affinity for microtubule binding (Lu et al., 1999).

The KXGS motifs are important regions in tau for the regulation of it function, particularly S262 in MTBR1. The KXGS is located in each MTBD in tau but also in MAP2 MTBDs, and has been shown to regulate microtubule binding via phosphorylation of this motif (Ozer and Halpain, 2000). In addition to microtubule binding, phosphorylation of S422 of tau appears to regulate caspase dependent cleavage of tau (Guillozet-Bongaarts et al., 2006).

This phosphorylation can affect a wide range of characteristics of tau protein, including its conformation within the cell. As tau is an IDP it is naturally unfolded, however its phosphorylation at S199, S202, and T205 encourages the N-terminus to move away from the C-terminus domain. Similarly
phosphorylation at S396 and S404 also encourages separation of the two terminal domains. However when all of these phospho-sites are utilised (S199, S202, T205, S396, and S404) it brings both tau terminal regions together, allowing tau to adopt a ‘paperclip’ or ‘hairpin’ conformation (Jeganathan et al., 2008). This opens up the MTBR region of the usually disordered tau structure, and even allowing tau to adopt cis and trans isomers through phosphorylation of key proline motifs (Lu et al., 1999; Hamelberg, Shen and McCammon, 2005; Jeganathan et al., 2006, 2008). Cis-trans isomerism has been shown to be somewhat regulated by proline kinases, with tau isomer conformation balance potentially being important in early AD (Nakamura et al., 2012).

Tau is mainly confined to axons in neurons, however work by multiple groups have shown that phosphorylation may also to some degree control localisation of tau within neuronal cells. Mandell et al back in 1996 demonstrated a phosphotau gradient, at least phosphorylation at S199 and serine S202, with dephosphorylated tau being more abundant at the distal regions of the axon (Mandell and Banker, 1996). Other phospho-sites, such as S202 and T205 are also shown to potentially alter the localisation of tau. Kobayashi et al showed AT8 positive tau (which recognises tau phosphorylated at S205 and T205) accumulation within dendritic spines, with increased accumulation on application of glutamate (Kobayashi et al., 2017). Interestingly, the relationship between phosphorylation and localisation also possibly crosses over with early disease phenotypes. For example application of Aβ oligomers has been shown to enhance phosphorylation at Serine 262 and 356 and subsequently transfer tau to dendrites (Zempel et al., 2010). It has also been shown that tau phosphorylation can affect axon transport and degradation (Rodriguez-Martin et al., 2013).

6.1.2 Tau phosphorylation and disease

Tau phosphorylation has been most extensively studied for its importance in tau pathology within neurodegenerative diseases (Noble et al., 2013). Tau becomes hyper-phosphorylated at specific residues, allowing it to more easily permanently detach from microtubules as well as aggregate in to fibrils (Ksiezak-Reding, Liu and Yen, 1992; Avila, 2006; Gong and Iqbal, 2008; Šimić et al., 2016). Over 45 phosphorylation sites have been identified on tau isolated from AD brain, and it has recently been shown that specific phospho-tau signatures result in different filamentous structures and can be associated with clinical severity in AD (Hanger et al., 2007; Wesseling et al., 2020). Additionally, tau PTMs have recently been associated with distinct tau fibril species as well as clinical heterogeneity and AD progression rates (Arakhamia et al., 2020; Dujardin et al., 2020).
Interestingly, many of the phosphorylation sites identified in tauopathy are the same sites observed in control brain and known to regulate tau function (Figure 57) (Šimić et al., 2016).

Figure 57. Tau protein diagram showing phosphorylation sites.

Possible and known phosphorylation sites on the longest isoform of tau protein, 2N4R. Sites shown are categorised as either found with the normal adult human brain (Blue), in the AD brain (Red), or in both (Green). Antibodies utilised to detect these epitopes are also show (Purple), as well as sites that need further characterisation (Black). Diagram from Goran Šimić et al., 2016.

The phosphotau phenotype is also high during neuronal development. Arguably fetal tau has been shown by some studies, during neuronal development, to be phosphorylated to a similar degree to PHF tau in AD brain lysate (Brion et al., 1993; Kenessey and Yen, 1993). For example 7 moles of phosphate per mole of protein has been reported, which is 2-4 times the level of normal adult tau. Thus, it is important to consider whether tau phosphorylated is truly pathological or a reflection of the developmental status of young / developing cellular models. This suggests that it could be the relative stoichiometry of phosphorylation, rather than specific sites that is important in disease. Diane Hanger from Kings College London has collated a very useful document of known tau phospho-sites (Diane Hanger, 2020).
Questions have been raised about how this correlation might be related to hyperphosphorylated disease relevant tau species, and how developing brain tissue is able to contend with these high levels of tau phosphorylation (Yu et al., 2009; Jovanov-Milošević et al., 2012; Hefti et al., 2019). Interestingly hibernating rodents, specifically arctic ground squirrels, have been shown to have high levels of tau phosphorylation (Su et al., 2008). Six sites (S199, T205, S214, S262, S396, and S404) were demonstrated to have hyperphosphorylation, however summer active squirrels were shown to have the lowest tau phosphorylation. This suggests neurons have the capacity to cope with high phosphotau levels, as well as possibly reversing this, raising questions about high phosphorylation within disease.

6.1.3 Tau phosphorylation in iPSC derived neurons

It is therefore of interest to determine whether disease-associated tau phosphorylation is recapitulated in iPSC models of tauopathy. Multiple groups have investigated tau phosphorylation within iPSC derived neurons, looking at both familial AD (fAD) and sporadic AD (sAD) patient lines (Arber, Lovejoy and Wray, 2017a). Increased tau phosphorylation was detected among both fAD and surprisingly sAD neurons when compared to controls. However, with neurons only grown to 42 DIV, tau phosphorylation would be predicted to be high regardless due to fetal tau expression, hence longer time points are needed (Ochalek et al., 2017).

Of more relevance to this thesis, multiple reports have examined tau phosphorylation in iPSC-neurons with MAPT mutations. Using the same iPSC lines used in this thesis but differentiated in 2D, Verheyen et al, (2018), phosphorylation within iPSC derived 2D neurons harbouring the 10+16 mutation. Increased levels of phosphorylation at S396/S404 and T181 were observed when compared to control neurons at both 65 and 80DIV (Verheyen et al., 2018). Other studies have looked at a variety of MAPT mutations in iPSC derived neurons (10 + 16, P301L, N279K, and V337M), showing increases in tau phosphorylation (Ehrlich, A. L. Hallmann, et al., 2015; Iovino et al., 2015; Paonessa et al., 2019). Some of these increased phospho-sites are fetal in nature (Hefti et al., 2019), however S202 and T205 were also increased, phospho-sites associated with pathological tau in tauopathies (Alonso et al., 2004; Wang et al., 2013). Lines harbouring multiple MAPT mutations (N279K, P301L, and 10+16) also had high levels of phosphorylated tau, however despite the presence of three mutations, no pathology in the form of tau tangles was reported (García-León et al., 2018).

Sato et al, 2018, examined phosphotau turnover using stable isotope labelling kinetics (SILK) with quantitative mass spectrometry. This revealed several phosphorylated tau peptides in iPSC-neurons, and interestingly the turnover of phosphorylated tau was faster in iPSC neurons than non-phosphorylated tau (Sato et al., 2018). This suggests unique processing of tau once phosphorylated.
6.1.4 Chapter aims

As we have previously observed disease-associated splicing of tau together with the detection of seed-competent species (Chapter X), we hypothesised that tau would be hyperphosphorylated within enCORs harbouring the 10+16 and P301S mutations.

To investigate this, the aims of this chapter are as follows:

a) Examine tau phosphorylation via immunoblot at S199, T181, S202 and T205, S396, and S396 and S404 by immunoblotting at 100 DIV and 300 DIV, utilising phosphotau-antibodies.

b) Densometrically and statistically analyse immunoblot results and compare phospho epitopes across genotypes.

6.2 Results

To investigate tau phosphorylation levels in enCORs, we analysed tau phosphorylation at S199, T181, S202 and T205, S396, and S396 and S404 by immunoblotting. The sites were selected due to the availability of specific phospho tau antibodies. We analysed tau phosphorylation at two different time points during enCOR development and elongated culture, in WT and MAPT mutation enCOR. The time points selected were 100 DIV (end of neurogenesis) and 300 DIV (when the switch to mature tau splicing has occurred). We hypothesised that by 300DIV, as neurogenesis is complete the developmental phosphorylation of tau would be lower and therefore pathological hyperphosphorylation would be more apparent. Samples from 3 individual enCORs at each time point were analysed. Antibodies recognising phosphorylated and total tau were used, alongside β-actin for normalisation. Total tau for each blot was measured using either HT7 (mouse) or DAKO (rabbit), depending on the species of the phospho antibody used.
6.2.1  **Tau phosphorylation at S202 and T205**

Tau phosphorylation at S202 and T205 was analysed using the phospho specific antibody AT8 in enCOR samples taken at 100 (Figure 58) and 300 DIV (Figure 59). Figure 58 shows tau phosphorylation at the AT8 epitope at 100 DIV. Total tau levels, when normalised to β-actin, were relatively consistent between the mutation genotypes (Figure 58.B). However, WT enCORs however express significantly higher levels of total tau compared to the other genotypes. Total tau expression between inductions were consistent. AT8 levels were variable across genotypes in comparison to total tau (Figure 58.C). However, when normalised to total tau (Figure 58.D), no significant differences in tau phosphorylation were observed between genotypes. However, the AT8 antibody shows reduced labelling and high variability between samples so this quantification should be interpreted with caution. Unfortunately the lab shutdown due to COVID meant this analysis could not be repeated.

![Figure 58. Phosphorylation of pS202/pT205 in enCOR at 100DIV.](image)

A) Immunoblot on 100 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (AT8), tTau (Dako tau) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed. B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of Phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical
analysis was performed using one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001.

AT8 levels in 300 DIV enCOR samples were next analysed (Figure 59). When total tau levels are normalised to β-actin, total tau was lowest in WT enCORs and highest in 10+16bi/P301Sbi enCORs, a reverse of the trend from 100 DIV samples. However, it should be noted that this doesn’t take into account the neuronal content as assessed previously using BIII tubulin. Very low immunoreactivity with AT8 was observed, and this blot is shown at high exposure in order to visualise the bands (Figure 59.A). With this in mind, it is important to treat the quantification of AT8 with caution given the high background that can also be observed. When AT8 is normalised to β-actin no significant differences were seen in any genotypes (Figure 59.C). Similarly, when phosphotau was normalised relative to total tau, no significant differences in AT8 were observed between genotypes (Figure 59.D).

**Figure 59.** Phosphorylation of pS202/pT205 in enCOR at 300DIV.

A) Immunoblot on 300 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (AT8), tTau (Dako tau) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed. B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of Phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical
analysis was performed using one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001.

### 6.2.2 Tau phosphorylation at T181

To investigate phosphorylation at T181, immunoblots were run and probed with the pT181 phospho specific antibody AT270 (Li and Cho, 2019). Lysates from 100 and 300 DIV enCORs were analysed from all four isogenic lines.

**Figure 60** shows total tau and pT181 levels at 100 DIV. No significant difference in total tau levels between genotypes was observed when normalised to β-actin. In contrast to the weak and variable signal observed with AT8, a strong immunoreactivity for AT270 was observed in all samples. This could reflect higher phosphorylation levels at this site, or increased sensitivity of the AT270 antibody. No significant differences in AT270 levels were observed when normalised against either β-actin or against total tau (**Figure 60.C & D**). However, AT270 levels did appear to be slightly higher in WT enCORs.

**Figure 60.** Phosphorylation of pT181 in enCOR at 100DIV.
A) Immunoblot on 100 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (AT270), tTau (Dako tau) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were
analysed. B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of Phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical analysis was performed using one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001.

Figure 61 shows total tau and pT181 levels in enCORs at 300DIV. Again, no significant differences in total tau levels were observed between genotypes (Figure 61.B). In contrast to 100 DIV, a much lower reactivity with AT270 was observed across samples. However, in a similar way to 100 DIV, no significant difference in AT270 levels was observed between genotypes, when normalised to either β-actin or total tau (Figure 61.C & D).

**Figure 61.** Phosphorylation of pT181 in enCOR at 300DIV.

A) Immunoblot on 300 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (AT270), tTau (Dako tau) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed. B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of Phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical
analysis was performed using one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001.

6.2.3 Tau phosphorylation at S199

Figure 62 shows tau phosphorylation at S199 in enCORs at 100 DIV.

When normalised to β-actin, WT enCORs show significantly increased total tau expression in WT enCORs when compared to other genotypes at 100 DIV (Figure 62.B). A strong immunoreactivity was also observed in all samples for pS199. The levels of phosphorylated S199 were also increased in WT enCORs when normalised to β-actin, however this increase is not significant (Figure 62.C). When pS199 tau is normalised to total tau, there is no significant difference between the genotypes (Figure 62.D).

**Figure 62.** Phosphorylation of pS199 in enCOR at 100DIV.

A) Immunoblot on 100 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (S199), tTau (HT7) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed. B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of Phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical
Analysis was performed using one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001.

Figure 63 shows total tau and pS199 levels in enCORs at 300 DIV. When normalised to β-actin, no significant difference in total tau expression is observed (Figure 63.B). pS199 levels were highest in the samples where total tau signal was highest, however no significant differences were observed between genotypes when normalised to total tau (Figure 63.D).

**Figure 63. Phosphorylation of pS199 in enCOR at 300DIV.**

A) Immunoblot on 300 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (S199), tTau (HT7) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed. B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of Phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical analysis was performed using one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001.
6.2.4  Tau phosphorylation at S396

To investigate tau phosphorylation at S396, immunoblots of 100 (Figure 64) and 300 (Figure 65) DIV enCOR lysates were probed with total tau and S396 antibodies.

Figure 64 shows total tau and pS396 levels at 100 DIV. Densometric analysis of total tau normalised to β-actin again shows significantly higher levels of total tau in WT compared to the other three genotypes (Figure 64.B). This is also observed for S396 levels when normalised to β-actin, suggesting that phosphorylation levels at this epitope reflect levels of total tau (Figure 64.C). When normalised to total tau, levels of pS396 appear similar between genotypes, although pS393 levels in WT enCORs were significantly lower than in 10+16 biallelic enCORs (Figure 64.D). Increased sample numbers would be needed to confirm this whether this change is robust.

Figure 64. Phosphorylation of pS396 in enCOR at 100DIV.
A) Immunoblot on 100 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (S396), tTau (HT7) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed.  B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical
analysis was performed using one-way ANOVA with Tukey’s post hoc test. \( *p < 0.05, ** = < 0.01, *** = < 0.001. \)

Figure 65 shows total at and pS396 tau in enCORs at 300 DIV. Again, total tau varied across genotypes and was highest in WT enCORs when normalised to \( \beta \)-actin (Figure 65.B). This pattern was repeated when looking at S396 phosphorylation (Figure 65.C). Interestingly the pattern of total tau and serine 396 phosphorylated tau here at 300 DIV is the inverse of the 100 DIV enCORs. It should be noted that this blot and antibody set did contain higher variability between organoid batches when compared to other experiments. When pS396 levels were normalised to total tau levels, there was no significant difference between the genotypes (Figure 65.D).

**Figure 65. Phosphorylation of pS396 in enCOR at 300DIV.**

A) Immunoblot on 300 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (S396), tTau (HT7) and \( \beta \)-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed. B) Densometric quantification of total tau normalised to \( \beta \)-actin. C) Densometric quantification of Phosphorylated tau normalised to total \( \beta \)-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical analysis was performed using one-way ANOVA with Tukey’s post hoc test. \( *p < 0.05, ** = < 0.01, *** = < 0.001. \)
6.2.5  **Tau phosphorylation at S396 and S404**

To further investigate S396, as well as S404 phosphorylation, we used the PHF1 antibody which recognises tau doubly phosphorylated at both sites and is commonly used as a marker for PHF tau (Li and Cho, 2019). PHF1 levels were assessed in enCORs at 100 DIV (Figure 66) and 300 DIV (Figure 67).

When normalised to β-actin, total tau levels were significantly increased in WT enCORs compared to other genotypes (Figure 66.B). A robust signal for PHF1 was detected in all enCORs, and similar to total tau, an increased signal was observed in WT enCORs compared to the other genotypes when normalised to β-actin (Figure 66.C). This is similar to what was observed in Figure 64, when pS396 was analysed, demonstrating concordant results between the two antibodies. When PHF1 signal is normalised to total tau, we observed an increase in PHF1 in WT enCORs (Figure 66.D).

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**Figure 66. Phosphorylation of pS396/pS404 in enCOR at 100DIV.**

A) Immunoblot on 100 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (PHF1), tTau (Dako tau) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed. B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of Phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical
Analysis was performed using one-way ANOVA with Tukey’s post hoc test. *p < 0.05, ** = < 0.01, *** = < 0.001.

Figure 67 shows total tau and PHF1 levels in enCORs at 300 DIV. No significant differences in total tau (Figure 67.B) or PHF1 (Figure 67.C) were observed after normalising to β-actin, however, as observed previously in these samples, total tau appears to be lower in WT enCORs, potentially reflecting reduced neuronal content. No significant differences were observed in PHF1 tau levels when normalised to total tau (Figure 67.D). However, qualitative differences could be observed in the banding pattern on the PHF1 western blot. 10+16bi/P301Sbi samples appear to have increased 0N3R and 0N4R S396 and S404 phosphorylated tau than the other samples. This potentially suggests mutation specific/isoform specific differences in phosphorylation.

Figure 67. Phosphorylation of pS396/pS404 in enCOR at 300DIV.
A) Immunoblot on 300 DIV enCOR protein samples from WT, 10+16m, 10+16bi, and 10+16bi/P301Sbi enCORs probed for phosphotau (PHF1), tTau (Dako tau) and β-actin. Samples run on a 4-12% gradient polyacrylamide gel. 10µg of protein was loaded for each sample, diluted up to 20µL with LDS buffer. Precision Plus all blue ladder used, with kDa markers on the left. N=3 enCOR for each genotype were analysed. B) Densometric quantification of total tau normalised to β-actin. C) Densometric quantification of phosphorylated tau normalised to total β-actin. D) Densometric quantification of phosphorylated tau normalised to total tau. Error bars show standard error of mean. Statistical
**Discussion**

In this chapter, tau phosphorylation in enCORs was analysed at a selection of epitopes, specifically S199, T181, S202 and T205, S396, and S396 and S404 by immunoblotting. As these epitopes are associated with hyperphosphorylation of tau in disease, it was hypothesised that they would be increased in enCORs with *MAPT* mutations. As tau is known to be highly phosphorylated in development (Yu *et al.*, 2009), we opted to analyse two time points: 100 DIV, which corresponds to the end of neurogenesis (Shi, Kirwan, Smith, Robinson, *et al.*, 2012), and 300 DIV, when we have shown enCORs to have a tau splicing profile similar to adult human brain (Figure 53).

In our analysis, we saw a consistent increase in total tau levels in WT enCORS at 100 DIV when normalised to β-actin. This was statistically significant in all but one analysis. In previous chapters when assessing the robustness of enCORs, we shows that total was consistent with neuronal content of both CO and enCOR when normalised to the neuronal specific marker βIII-tubulin. In this chapter it was not possible to use βIII-tubulin as a loading control as it is the same molecular weight as tau, and our western blotting system only allows for labelling in two channels.

Therefore, the differences in total tau observed do not take into account differences in neuronal context within the enCORs. However, this could also indicate a downregulation of tau expression within the mutant enCORs, as we observed previously at the mRNA levels using qPCR (*Figure 55*), where reduced tau expression was observed in 10+16bi/P301Sbi samples. However, 10+16 mono and biallelic enCORs did not exhibit changes in tau expression so this in unlikely to explain the results observed at the protein level.

Another possibility is protein degradation, as both 4R and phosphotau have been shown to be degraded more rapidly in iPSC-neurons when compared to 3R and non-phosphorylated tau species (Sato *et al.*, 2018). The theory of increased protein levels and turnover is supported by studies showing no significant upregulation of *MAPT* mRNA expression within AD brains (a secondary tauopathy) compared to controls (Loring *et al.*, 2001; A. Boutajangout *et al.*, 2004). While looking specifically at patients with FTD, groups have shown reduced tau protein in patient brains but overall no reduction of *MAPT* mRNA levels (Zhukareva *et al.*, 2001; D’Souza and Schellenberg, 2005). This could mean that tau expression in the mutation enCORs may remain stable at the mRNA level, however the turnover of tau protein remains high, reducing the total tau within cells.
However, reduced soluble protein levels in patient brain tissue could also be down to tangles sequestering free tau protein into the aggregates. This would lead to less free soluble tau available in samples, as sequestered tau would only be present in the insoluble tau fraction (Greenberg et al., 1992).

At 300 DIV, enCORs showed an increase in total tau from WT to 10+16bi/P301Sbi in three of the five blots (Figure 59, Figure 61, & Figure 65). The remaining two showed no difference between genotypes. These data need to be interpreted carefully, as with the 100 DIV total tau results, as due to blotting limitations, β-actin was used as the normalising protein, rather than βIII-tubulin, which would have been preferable as it would allow normalisation to neuronal content.

However, an increase in total tau in a step-wise manner across three of the five blots is still intriguing and would be something to investigate in the future, using βIII-tubulin as the normalising protein.

Similarly, we also saw increased levels of phosphorylated tau in WT enCORS at 100 DIV when normalised to β-actin. However, it is important to note that many of these changes were lost following normalisation to total tau. This suggests increases in tau phosphorylation levels likely reflect the amount of total tau present in a particular sample. This is important to consider when assigning disease-relevant changes in phosphorylation, as it is the stoichiometry of phosphorylated that becomes altered in disease, therefore normalisation to total tau is an important part of this analysis.

Overall, we did not see any consistent differences in the pTau epitopes examined between genotypes. This is likely due to the developmental nature of the model and the fetal identity of neurons within organoids (ref). Thus tau is likely to be highly phosphorylated anyway, as observed in fetal brain (Yu et al., 2009). Other papers have shown tau phosphorylation to be increased in iPSC neurons harbouring 10 + 16, P301L, N279K, and V337M MAPT mutations (Ehrlich, A.-L. Hallmann, et al., 2015; Iovino et al., 2015; Paonessa et al., 2019). This is in contradiction to findings presented here, where no real significant difference has been shown. However, many variables come into play regarding tau phosphorylation, with neuronal maturity playing a large role due to the high levels of tau phosphorylation present in developing neurons (Brion et al., 1993; Yu et al., 2009).

Some work has shown reduced tau phosphorylation within iPSC neurons expressing an R406W mutated MAPT gene (Nakamura et al., 2019). This is interesting as Nakamura et al., 2019, cultured these neurons as a CO for 30 days at first, with dissociation taking place and plating to a 2D culture at 30 DIV, with analysis at 60 DIV. Potentially the beginning 3D element of this protocol altered phosphorylation of tau, although without further work it is impossible to say. The most probable cause for this is the mutation itself, which could potentially be responsible for less tau phosphorylation by
inducing conformational changes that inhibit kinase access to the tau molecule, although further work would be needed to confirm this hypothesis.

Our findings could also be due in part to the fact the western blotting is semi-quantitative and potentially not the best approach for an accurate assessment of differences in PTMs. This is highlighted by differences observed between antibodies directed at the same sites of phosphorylation. Both PHF1 and pS396 antibodies share recognition of pS396, although PHF1 requires double phosphorylation of pS396 and pS404. PHF1 showed higher phosphorylation in WT enCORs at 100 and 300 DIV, while S396 showing significantly more phosphotau within 10+16bi enCORs at 100 DIV and an even distribution at 300 DIV. This potentially shows S404 phosphorylation could be significantly different in enCORs. The multiple splice variants and PTMs within tau make analysis by western blot difficult due to the many possible tau variants present within cells. Future analysis of total tau within enCORs would highly benefit from high resolution band separation by instruments such as the Peggy Sue™ system (Harris, 2015). This would increase accuracy regarding quantification of tau, although awkward due to phospho-groups increasing tau proteins molecular weight.

For higher resolution phosphotau analysis, the use of the ‘Phos-Tag’ or ‘phospho-tau bar code’ technique pioneered by Eiji Kinoshita et al in 2005, since honed for tau protein by Kimura et all in 2018, would be ideal (Kinoshita et al., 2006; Kimura et al., 2018). This technique allows you to visualise clear bands for specific tau phosphorylation sites along a protein, and has been successfully used to look at FTD mouse brain and human cells showing loss of phosphorylation at serine 404 (Kimura et al., 2016).

Enzyme-linked immunosorbent assay, or ELISAs, have been an important feature of biochemistry for decades since their advent in 1972 (Engvall and Perlmann, 1972). ELISAs detecting total tau and especially phosphotau have been invaluable when investigating phosphotau variants as potential biomarkers for NDD (Yamamori et al., 2007; Janelidze, Stomrud, et al., 2020). This method of phosphotau detection would increase the accuracy and specificity of any phosphotau data from enCORs.

Comprehensive identification and quantification of tau phosphorylation could be obtained using mass spectrometry. A tau based mass spectrometry-based technique called FLEXITau, allows for PTMs to be analysed along the tau protein molecule (Mair et al., 2016). We recently prepared samples for mass spectrometry analysis in collaboration with Washington University, according to the protocol published by Sato et al (Sato et al., 2018), unfortunately due to the lab shutdowns it was not possible to obtain the data in time for this thesis. Extending this work, it would be interesting to analyse the turnover of tau in WT vs. MAPT mutation neurons utilising stable isotope labelling and mass
spectrometry, as described previously for control iPSC-neurons (Sato et al., 2018). This would allow us to assess whether tau turnover is different within 2D and 3D iPSC cultures, at late vs. early culture time points, as well as highlighting the effects of MAPT mutations on the half-life of tau.

The study of PTMs to tau can give insight into its regulation in the cell. Recently, it has been shown that the half-life of phosphorylated and non-phosphorylated tau differs, with a faster turnover of phosphorylated tau species. Two groups have shown specifically that PHF1 reactive phosphotau has a shorter half-life than native tau, as well as demonstrating phosphotau building up to three times the level of native tau when autophagy is inhibited within neurons (Chesser, Pritchard and Johnson, 2013; Rodríguez-Martín et al., 2013). However, other groups have shown phosphotau can directly inhibit proteolysis, although this study examines hyperphosphorylated tau rather than tau phosphorylated at physiological levels (Poppek et al., 2006).

In summary, although no disease associated changes in tau phosphorylation were observed in our analysis, the experiments performed in this chapter were preliminary and further analysis of tau PTMs in enCORs is needed.
7 Discussion

7.1 Summary of findings

In this thesis, multiple iPSC-derived organoid culture models implemented and characterised for the investigation of Aβ and tau. These were compared to iPSC-neurons grown in 2D, to determine the utility of 3D organoids as in vitro models for AD and FTLD-tau.

Aβ processing and secretion was examined in cerebral organoids and 2D cortical neurons from iPSC with the following genotypes: R278I, Y115H, E280G, int4del, M139V, and M146I PSEN1 mutations, and V717I APP mutation. Additionally, we were able to compare 2D neurons, cerebral organoids and post-mortem brain tissue from the same individual with the APP V717I mutation.

Cerebral organoid cultures were set up within our laboratory according the protocol described by Lancaster et al (Lancaster et al., 2013; Lancaster and Knoblich, 2014). Characterisation of these organoids showed positive staining for PAX6 (forebrain neuronal precursor cells), KI67 (proliferating cells), phospho-vimentin (radial glia), and TBR1 (deep-layer neurons). As well as exhibiting neural tube like structures with inner radial glia, and migrating and differentiating TBR1 positive early born neurons (Chapter 3.2.2).

To assess whether Aβ processing was altered in our models, we used ratios of Aβ42:40, which are widely used to indicate an increase in the relative levels of longer Aβ peptides. Aβ42:40 were increased in cultures from all APP and PSEN1 examined, at 100 and 200 DIV. Further, mutation specific effects on APP processing were observed, for example PSEN1 mutations had increased Aβ42:38 ratios, while APP mutations showed ratios similar to control (Chapter 0).

Similar Aβ profiles were observed in 2D neuronal cultures and 3D cerebral organoids, providing replication of our data across multiple models. Aggregation of Aβ together into plaque-like pathology was not found within our patient iPSC derived 2D or 3D cultures. These results showed that cerebral organoids could be successfully generated in the laboratory for the first time and that organoids from fAD patients recapitulate disease associated alterations in APP processing.

The main focus of this thesis was to develop organoids as a model to study tauopathy. Marked variability of neuronal content between COs was shown, especially between batches, which makes biochemical analysis difficult (Section 4.2.2). However, a preliminary analysis of tau splicing and isoform expression in the early CO model showed increased maturity from a tau splicing standpoint when compared to 2D iPSC derived neuronal cultures (Section 4.2.4).
To reduce heterogeneity and optimise the organoid system for further analysis of tau expression and splicing, I developed a second organoid model with the aim of reducing variability; engineered cerebral organoids (enCORs). enCORs differ from COs due to the use of PLGA scaffolds, allowing an elongated EB, which increases surface area and led to increased neural ectoderm formation. enCORs showed positive staining for PAX6 (forebrain neuronal precursor cells), Ki67 (proliferating cells), phospho-vimentin (radial glia), and TBR1 (deep-layer neurons) at 30 DIV, with increased neural tube like structures in the sections analysed. 100 DIV enCORs consistently displayed FOXG1 (forebrain), as well as layer specific markers CTIP2 (deep-layer 5 and 6) and BRN2 (upper layer, 2 and 3), as well as increased TUJ1 (βIII-tubulin/neuronal) staining (Section 5.2.2 and 5.2.3). Immunoblots for 100 DIV enCORs were also less variable than COs, displaying consistent levels of βIII-tubulin and tau across genotypes and across batches (Section 5.2.4).

enCORs were subsequently generated from isogenic iPSC lines harbouring the 10+16 monoallelic, 10+16 biallelic, and 10+16 biallelic with a P301L biallelic mutations in MAPT, along with the isogenic WT control. This confirmed that early expression of 4R MAPT isoforms was present in 3D models with this mutation, as has been shown in 2D models (Iovino et al., 2014; Sposito et al., 2015). Importantly, an accelerated maturation of MAPT splicing was observed in the enCORs compared to 2D models, both N-terminal and C-terminal splicing associated with later developmental stages was observed at the RNA and protein levels. Tau protein isoform patterns in enCORs revealed a similar pattern to human brain, supporting our hypothesis that enCORs provide a physiologically relevant system for the study of tau expression and splicing and its disruption by MAPT mutations.

Due to time and material constraints, the presence of tau pathology couldn’t be extensively explored in enCORs. No differences in the phosphorylation status of tau was found, at least at the epitopes examined. However, a preliminary analysis showed the presence of seed-competent within 300 DIV enCORs harbouring 10+16 and P301S biallelic mutations at 300 DIV. The seed competent tau species remains to be identified.

7.2 Aβ generation in COs

Utilising 2D and 3D iPSC derived neurons, ratios of Aβ species secretion revealed consistent differences between mutations in both APP and PSEN1, with Aβ42:40 increased in both compared to control, Aβ42:38 being increased in PSEN1 mutations only, and Aβ38:40 being increased in only APP mutation cultures (section 3.2.5). Results confirm other work done on fAD APP processing, showing increases in longer Aβ species depending on the mutation present (Hecimovic et al., 2004). Aβ plaques
have not yet been shown in 2D, however plaques within 3D organoid cultures have been reported (Gonzalez et al., 2018). Gene dosage mutations within this study, such as APP duplications, have shown significant Aβ aggregates. This suggests that a critical concentration of Aβ may be required for aggregation to occur, and this is reached when gene dosage of APP is altered but not by point mutations in APP or PSEN1.

Understanding the Aβ profiles generated by specific mutations, as well as profiles of sporadic AD patients, could lead the way to treatments via explaining the mechanisms behind Aβ aggregation and neurodegeneration. Over 20 years ago, murine models showed immunisation against aggregation prone Aβ species was possible (Schenk et al., 1999). With positive results in mice, trials were conducted in humans, with pathological evaluation happening 14 years later in 2019 (Bayer et al., 2005; Nicoll et al., 2019). 14 of 16 AD patients showed some evidence of plaque removal, and 2 patients showing very sparse or no detectable plaque pathology. With the combination of 2D and 3D investigations into APP processing and specific Aβ species secretion, more targeted immunisation and antibody therapies could be explored. Especially 3D co-cultures, allowing the addition of immune cells like microglia to examine changes in these secretion profiles. These models, alongside other traditional models such as mice, allow the exploration into ways of reducing specific species of Aβ, or addressing a specific Aβ species ratio imbalances.

For example, antibody based therapies such as Aducanumab, which is able to bind to aggregated forms of Aβ, but not monomeric forms. Trials in both mice and humans have shown reductions in Aβ plaques, with the antibody currently under review by the FDA for approval as an AD treatment (Sevigny et al., 2016). 3D models potentially allow another way of looking at these kinds of therapy and their effectiveness.

Other avenues of AD therapy that have been explored are β site APP cleaving enzyme 1 (BACE-1) and γ-Secretase inhibitors (Maia and Sousa, 2019), which would enhance APP processing towards the less amyloidogenic pathways and therefore reduce Aβ generation. Unfortunately secretase inhibitors have also been associated with cognitive worsening, such as Atabecestat, showing that much more research in to these drugs effects are needed on human models. (Novak et al., 2020). For example Tarenflurbil was investigated as a γ-secretase inhibitor for the treatment of early AD (Green et al., 2009). Trials and research was eventually stopped due to low inhibition activity but also low CNS penetrance (Golde et al., 2013). This penetrance issue is potentially something that could have been investigated if choroid plexus organoids were available, as the Lancaster group have shown potential CNS drug penetrance data using that model (Pellegrini, Bonfio, et al., 2020)
Investigations into mutated APP pathways are needed to further understand disease mechanisms, and will be fundamental in guiding further trials into γ-secretase inhibitors or, more likely, secretase modulators. Modulators allowing secretases to interact with their other targets, unhindered, while affecting Aβ production in therapeutic ways (Haapasalo and Kovacs, 2011). Overall, these data demonstrate the value of not only iPSC derived 2D cultures, but also 3D cultures when investigating both WT and dysfunctional cellular APP / protein processing.

### 7.3 Reducing heterogeneity in 3D models

COs were found to variable between batches regarding total neuronal content, hampering efforts to compare COs with 2D cultures, as well as between CO genotypes. This observed variability has also been reported within the literature and is thought to be due to inner areas of EBs not becoming neural ectoderm, partly down to the low surface area to volume ratio of spherical EBs (Lancaster et al., 2013; Camp et al., 2015; Quadrato et al., 2017; Qian, Song and Ming, 2019). We established enCORs in the lab as a way to reduce heterogeneity. Analysis with ICC, western blot and PCR all showed enCORs to be less variable than COs, an important result when planning future projects (chapter 4.2).

In spite of this reduced heterogeneity, there are still limitations that need to be addressed, such as the absence of vasculature which would increase oxygen diffusion and the lack of microglia (discussed in section 1.12). Further, imaging in this thesis was performed on sections and imaging of whole organoids has advanced significantly in recent years. Whole organoid imaging allows analysis of certain areas of the organoid as well as reducing bias, as sections are not relied upon. In addition to single whole organoid imaging, more high throughput analysis techniques are becoming more sensitive, some even allowing single cell resolution (Kiemen et al., 2020).

In addition to incorporating multiple cell types, rapid progress towards the development of assembloids, which permit the interaction between different regions is being explored. Assembloids for corticostriatal and corticomotor pathways have been developed (Andersen et al., 2020; Miura et al., 2020). With the predictable spread of tau pathology described by Braak through brain regions as well as the more mechanistic spread from neuron to neuron (Braak et al., 2006; Vogel et al., 2020), these complex models provide opportunity for looking at disease progression and selective vulnerability.

Current analytical methods are heavily reliant on whole organoid biochemical analysis, cross sectional analysis, or single cell analysis of disassociated organoids, and light sheet microscopy allow relative high throughput whole organoid analysis via ICC (Lancaster et al., 2013; Tanaka et al., 2020). These
approaches would permit molecular, cellular, spatial, and architectural characterisation on intact samples (Albanese et al., 2020).

7.4 Tau expression and splicing in organoids

Through PCR and qPCR, enCORs were shown to express multiple MAPT isoforms, but some isoforms were expressed at variable levels, especially transcripts that included both exon 2 and 3 (chapter 4). This issue of the larger MAPT transcripts being difficult to detect in iPSC derived neurons has also been shown by others (Iovino et al., 2015; Miguel et al., 2019).

Fetal tau, 0N3R, was the predominant tau isoform in WT enCORs analysed at 50 and 100 DIV, recapitulating what is seen in early neural development in the analysis of fetal brain samples (Hefti et al., 2018). Further, this also recapitulates what was previously observed by our group and others both in vitro 2D iPSC derived neuronal models (Sposito et al., 2015). The 10+16 MAPT mutation was also shown to drive early 0N4R tau, again as previous groups have reported (Iovino et al., 2015; Sposito et al., 2015; Verheyen et al., 2018). This excess 4R tau being expressed within a 3D iPSC derived culture could have potential for 4R tauopathy modelling, as extended culture time might not be needed if 0N4R tau or tau ratio imbalance was the subject of study.

The discovery of multiple tau isoforms at earlier time points in enCORs than in the 2D cultures correlates with suggestions of early maturation within 3D cultures when compared to 2D cultures (Chandrasekaran et al., 2017). This result suggests 3D cultures, or the developmental processes and architecture they recapitulate, are important in neuronal maturation and the switch between fetal and adult tau isoforms. As the analysis performed in this thesis solely focussed on bulk analysis of whole organoids, we cannot say conclusively whether the changes in tau splicing we observed are due to maturation or being driven by the presence of a particular cell type/subset of cells that express these isoforms. This could be resolved by analysis at single cell resolution as described above, although this was beyond the scope of this project.

Other research championing this involves human cortical neuronal cell (HNC) cultures generated from human fetal brain tissue obtained at 6 to 21 week gestation. Despite dissociation and plating into 2D cultures, the neurons mature in culture and multiple tau isoforms are detectable after 20 DIV (Deshpande, Win and Busciglio, 2008). This suggests that the intrinsic regulators that drive the switch of MAPT to adult isoforms are present within these cell cultures. These could include expression of splicing factors, for example the CELF and MBNL proteins which coordinate a post-natal splicing switch in heart tissue, and have both been suggested to regulate tau splicing (Kalsotra et al., 2008; Dhaenens
et al., 2011). However, the differences between 2D and 3D suggest the presence of signals could be party recapitulated within organoids but not in 2D iPSC-neurons. These potential signals are currently unknown, however work from Sood et al, 2019, shows increased functional maturation of neural stem cells when within a 3D model enriched with fetal ECM proteins. Unfortunately, tau isoform expression is not examined within Sood et al, 2019, however it would be fascinating to see if fetal ECM proteins, along with the 3D architecture of enCORs, would allow for MAPT isoform switch, in a similar manner to HCNs. Another example involves ECM compounds, HAPLN1, lumican, and collagen I, showing regulation of cortical folding (Long et al., 2018).

MAPT splicing work form Miguel et al, 2019, is currently the most promising iPSC derived multi-isoform tau model, with mRNA from all 6 isoforms being detected at 25 weeks in culture when neurons were cultured in 3D suspension with Brainphys media (Bardy et al., 2015; Miguel et al., 2019). BrainPhys was developed to be a more in vivo like media which also promotes electrically active neurons and synapses, with reports that it also increased APP processing and Aβ secretion (Satir et al., 2020). However as discussed in section 1.14, almost 80% of the tau detected at the RNA level is ON3R fetal tau, which was also shown at the protein level, where mostly fetal tau was observed by immunoblot with 3 other isoforms visible at low levels. This alginate encased 3D model uses dual SMAD inhibition to drive a cortical fate, which arguably loses some of the architecture and cell heterogeneity observed in enCORs that appears to support neuronal maturation. This work demonstrates that the morphology of 3D cultures can drive adult MAPT splicing, something we have shown in our enCORs. Using Brainphys media promoted increase isoform expression when compare to N2B27 media, something that would be interesting to explore when culturing enCORs and could further enhance maturity. Especially as 2D cultures grown in Brainphys have been shown to have increased deep layer neuronal marker expression as well as more mature APP processing / Aβ ratios (Satir et al., 2020).

Other groups have used organoid models to investigate AD, for example by Raja et al, 2016, and Gonzalez et al, 2018, but with a focus on the aggregation of both Aβ and tau aggregation (Raja et al., 2016; Gonzalez et al., 2018). However tau splicing was not investigated in these papers. Therefore, our data showing that iPSC derived enCORs more rapidly acquire adult tau expression and splicing when compared to 2D iPSC derived neurons is an important advancement in this area. Importantly, this is the first description of a human in vitro model with tau expression that is relatively comparable with human adult brain. This will enable investigation of the mechanisms regulating both MAPT splicing, the function of specific tau isoforms in development and disease, and perhaps most importantly, the incorporation of specific tau isoforms into tau fibrils and aggregates (Scheres et al., 2020).
Despite enCORs showing enhanced expression of adult tau isoforms compared with other models, and reduced culture time required for this to be attained, the long culture time still remains a practical issue that could be a barrier to widespread adoption of this technique in the field. Long-term cultures are becoming more widely used, with a 3D assembloid model showing that astrocyte maturity required 590 days of culture, and an iPSC model of PD showing the temporal emergence of cellular phentoyes at 70 vs. 150 DIV (Burbulla et al., 2017; Sloan et al., 2017). However, the requirement to culture enCORs to 200 DIV or longer to allow multiple tau isoforms to be expressed at the protein level is still unfeasible for most laboratories. A deeper understanding of the regulation of this tau isoform switch will enable the development of strategies to accelerate this process in vitro.

7.5 Protein aggregation in organoid models of AD

As mentioned above and described in the introduction (1), aggregates of the two main AD associated proteins, Aβ and tau has been described in organoid models (Raja et al., 2016; Gonzalez et al., 2018). APP and PSEN1 mutation over expression 3D models utilising Ren cells have shown Aβ plaques as well as presumed NFTs (Choi et al., 2014). Organoid models harbouring single PSEN1 mutation, A246E, have also reported Aβ plaques with DS organoid models showing similar Aβ aggregates (Gonzalez et al., 2018; Hernández-Sapiéns et al., 2020)

However, this does not correlate with findings in this thesis. Staining using anti-Aβ did not reveal any aggregates in 2D cortical neurons or COs, compared to the widespread Aβ deposition observed in post-mortem tissue from the same individual. This could be due in part to the fact that accumulation of Aβ in disease is occurring over decades, compared to the relatively short time frames examined in cell culture (Jack, 2013). Further, although Aβ deposition in 2D cultures is rare, it has been reported in models of Down Syndrome, which carry an extra copy of the APP gene on chromosome 21 (Shi, Kirwan, Smith, MacLean, et al., 2012). This also suggests that Aβ dosage could be important for aggregation, and a critical concentration of Aβ required to initiate the aggregation process may not be reached in our model. Diffusion gradient limits for nutrients and oxygen within organoid models lead to cell death and any staining of protein aggregates should be carefully considered (Lancaster et al., 2013; Lancaster and Knoblich, 2014). For example robust pathology has been seen in APOE4 models of sAD using organoids, however the APOE3 organoids grown in parallel also showed apparent pathology, just to a lesser extent (Lin et al., 2018). There is also the possibility of groups failing to report that no pathology was seen, leading to publication bias. With results of this project, and differing results within the literature, it becomes ever more important for whole organoid analysis to be used, rather than sections.
When considering the development of tau pathology, one has to consider the different ways this can be defined. Tau hyperphosphorylation is the most common readout used, this leads to the formation of detergent insoluble tau aggregates and Gallyas positive NFTs. Only NFT can be considered bona-fide tau pathology, the others are likely precursors to the development of tangles. Further, with tau being shown to spread through neurons in a prion like manner (Clavaguera et al., 2015), it is more likely that smaller tau seeds are the propagation factor, with tau filaments/tangles being the pathology after polymerisation has occurred (Wu et al., 2013). This can been seen within sAD brain tissue that exhibits no tau pathology, but will seed tau pathology when applied to seed competent HEK cells (Holmes et al., 2014; DeVos et al., 2018). It is also likely that these pre-tangle species are more neurotoxic. In an inducible tau transgenic mouse, neurodegeneration was halted when expression of the tau transgene was suppressed, although remarkably NFT persisted (Atwood et al., 2005). This strongly suggests that pre-tangle tau species are the toxic entity.

We therefore decided to focus our analysis on whether seed-competent tau species could be detected within our enCORs using a well-established in vitro assay. Extracts from enCORs of all genotypes at 100 and 300 DIV were analysed, however only lysates at 300 DIV derived from the double 10+16/P301S biallelic mutation enCORs were able to seed aggregation in the HEK biosensor cells. It is interesting that seeding of pathological tau was only observed at 300 DIV and not at 100 DIV. This is likely to be related to the amount of mutant tau present within the enCORs. P301S is only present in 4R tau isoforms, and we shows that the levels of 4R expression increase between 100 and 300 DIV. Therefore the levels of P301S mutant tau are likely to be higher at 300DIV. It is also not known whether the expression of N-terminal splice variants contributes to this seeding activity.

Additionally, if aggregation was entirely due to the coding P301S mutation, it would be reasonable to expect aggregation at D100, due to 4R tau being present harbouring the coding mutation, but this is not the case. Only 300 DIV 10+16bi/P301Sbi enCORs displaying any aggregation. This could possibly suggests other tau isoforms are required to push the aggregation of tau, something the 300 DIV enCORs express. As another major difference in the 300 DIV enCORs is the larger range of tau isoforms present.

This exciting preliminary data is currently being validated by FACs, which allows quantification of seeding levels but was unfortunately delayed due to the COVID-19 shutdown (Furman and Diamond, 2017). It is also important to note that recent work has questioned whether the structure of the inclusions in this model is reflective of disease aggregates (Kaniyappan et al., 2020), however it still provides a sensitive assay for the detected of early pathology in organoids. Impressive work by Dujardin et al, 2018, has shown the isoform composition of tau alters the rate of tau fibrillisation and
spread (Dujardin et al., 2018). It will be interesting to see if the same mechanism could be at work here when using aged 3D neuronal in vitro samples.

In summary, our preliminary data suggests that enCORs with MAPT mutations offer a unique way to investigate the formation of tau pathology. Future work would aim to identify and characterise the seed competent tau species, and further develop this system in order to model tau propagation.

### 7.6 Phosphorylation of tau within enCORs

Tau phosphorylation is important for neuronal development, in the regulation of the microtubule binding function of tau, as well as in the development of tau pathology (Kenessey and Yen, 1993; Jovanov-Milošević et al., 2012). We examined 5 phospho-sites (S199, T181, S202 and T205, S396, and S396 and S404) using phospho-specific antibodies on western blot, at 100 and 300 DIV (chapter 4).

Our analysis did not reveal any significant differences in tau phosphorylation between genotypes at any of the epitopes examined. However, many of the same phospho-sites on tau are utilised during development as well as during disease stages, and so it is difficult to assign changes as pathological rather than developmental (Kenessey and Yen, 1993). Our data is in contrast to many studies on iPSC neurons showing increased tau phosphorylation both in 2D and 3D neuronal cultures (Iovino et al., 2015; Ochalek et al., 2017; Paonessa et al., 2019; Readhead et al., 2019). It is important to consider how this data is normalised, for example normalisation of a pTau signal to a loading control such as actin doesn’t take into account the neuronal content of the cultures being compared, and expressing pTau relative to total tau gives a better indication of the stoichiometry of tau phosphorylation.

Due to this early phosphorylation, changes in phospho-sites of tau could be hard to study within this model. However analysis of sites that have been shown to be more pathological in nature may be interesting to investigate, for example serine 356 situated at the end of the MTBD (Yoshida and Goedert, 2012; Šimić et al., 2016).

Recently, quantitative mass spectrometry assays for analysis the levels of specific phospho sites on tau have been developed and applied to iPSC models (Sato et al., 2018). We have prepared 300 DIV enCORs from all genotypes which are being analysed in collaboration with Chihiro Sato and Nicolas Barthelemy at Washington University, St Louis using this approach. This highly sensitive and quantitative assay will allow the analysis of multiple sites of phosphorylation in parallel, and even small changes in phosphorylation levels will be accurately detected.
7.7 **Future directions**

The fact that tau exists as multiple isoforms as well as being a highly phosphorylated protein means it is difficult to visualise on classic immunoblots due to the multiple species present (Šimić et al., 2016). Studies have looked at tau isoforms in 3D neuronal model, but failed to dephosphorylate the samples, meaning definite isoform bands are hard to distinguish (Choi et al., 2014; Kim et al., 2015). Despite dephosphorylation of tau within this project, it is still difficult to conclusively say how many tau isoforms are present in samples run on immunoblot due to slight smearing of bands, possibly because of other PTMs (Gong et al., 2005).

New immunoblot techniques utilising capillary electrophoresis, like the Peggy Sue™ system, have shown increased definition of bands with options for protein quantification (Harris, 2015). Future work looking at tau protein isoforms present within enCORs of different time points would be enhanced by using these systems, allowing more clarity and certainty around the isoforms present, and their ratios within enCORs. Analysis of enCOR lysates using this system together with 3R and 4R specific antibodies is currently in progress in collaboration with Dale Starkie at UCB.

In the adult brain higher molecular weight tau proteins, containing two N-terminus repeats, account for only 9.5% of total tau (Hong et al., 1998; Liu and Gong, 2008). Immunoblot analysis can be difficult when 90% of the total tau overpowers the signal from the less abundant higher molecular weight tau. This could be combated by probing with N-terminus specific tau antibodies that have been recently described (Verelst et al., 2020). These antibodies could also be combined with the Peggy Sue™ and other high resolution immunoblot systems for quantitative data regarding the tau isoforms more elusive and with lower abundance in cell culture (Sposito et al., 2015).

To further investigate MAPT splicing, investigations into the influence of different ECMs would be interesting. Contemplating the work showing HCNs exhibit multiple tau isoforms even in 2D culture, the culture of enCORs using fetal derived ECM would be fascinating (Deshpande, Win and Busciglio, 2008). For example, there could be growth factors within the fetal ECM that promote maturation of neurons and accelerate mature MAPT splicing within enCORs.

Few PTMs, apart from phosphorylation, have been looked at in iPSC derived models. With PTMs of tau showing correlation with oligomerisation and disease progression (Ercan-Herbst et al., 2019). Investigation into acetylation of tau would be enlightening, especially with reports of tau auto acetylation and K80 acetylation promoting auto-cleavage of tau (Cohen et al., 2013, 2016).

The seeding assays presented in this thesis were highly reproducible (n=6), however the efficiency of the detectable aggregates was extremely low. With more time, the more efficient and quantitative...
method of fluorescence activated cell sorting (FACS) could be implemented (Bonner et al., 1972; Shapiro, 2018). This is currently being performed on our samples in collaboration with Dr Stephanie Fowler and Prof Karen Duff at UCL. This would give quantitative data on the seeding activity of enCOR lysates and avoid bias from immunofluorescence.

Finally, extending the culture times further, potentially in combination with tau seeding to induce pathology, could result in the development of NFTs which could be analysed by cryo-EM. As discussed in 1.6, ground-breaking work by Scheres, Zhang, Falcon, Goedert et al, 2020, has shown different tau filament structures within different tauopathies (Scheres et al., 2020). Recapitulating tau pathology is a key aim of in vitro models, and the structure of tau filaments generated from recombinant tau has been shown to differ from all other tauopathies (Zhang et al., 2019). Long term culture of 10+16bi/P301Sbi enCORs could eventually develop insoluble tau aggregates, as shown in other 3D models. Comparing the structure via cryo-EM to known tau filament structures would be fascinating and allow us to see if tau true disease filaments could be created in vivo. Further to this, seeding within enCORs using recombinant tau and AD tau, with comparison via cryo-EM would also be interesting, potentially observing templating of the endogenous tau into specific filament structures depending on the seed used.

### 7.8 Conclusions

In conclusion, I developed and optimised a novel organoid model for the study of MAPT mutations. For the first time in an iPSC-derived model, I was able to demonstrate tau splicing similar to what is observed in human brain, highlighting the physiological relevance of this system. Importantly, the maturation of tau splicing was accelerated in comparison to 2D cultures. Preliminary data suggests the presence of pre-tangle, seed competent tau species in enCORs. Thus, this model could be suitable platform to understand the development of tau pathology and elucidate the mechanisms linking this to neurodegeneration.
8 References


Alessandri, K., Feyeux, M., Gurchenkov, B., Delgado, C., Trushko, A., Krause, K. H., Vignjević, D.,


Eiraku, M. and Sasai, Y. (2012). ‘Self-formation of layered neural structures in three-dimensional...


Forster, J. I., Köglberger, S., Trefois, C., Boyd, O., Baumuratov, A. S., Buck, L., Balling, R. and Antony,


Mastroberardino, P. G., Vega, I. E., Moreno, S., Castillo-Carranza, D. L., Guerrero-Muñoz, M. J. and


mature neurons from human pluripotent stem cells in a three-dimensional suspension culture system’. Stem Cell Reports, 6 (6), pp. 993–1008. doi: 10.1016/j.stemcr.2016.05.010.


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