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Investigation of the cell- and non-cell autonomous impact of the C9orf72 mutation on human induced pluripotent stem cell-derived astrocytes

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赵 晨

Doctor of Philosophy
The University of Edinburgh
2016
Declaration

The work contained within this thesis has been conducted by me unless otherwise stated. No part of this thesis has been submitted for any other degree or professional qualification.

In Chapter 3, generation of iPSC lines was collaborative work between our group, Sir Ian Wilmut’s group at The University of Edinburgh, Prof. Christopher E. Shaw’s group at King’s College of London and Prof. John Hardy’s group University College London. Generation of the isogenic control iPSC line was conducted by Dr. Bhuvaneish T. Selvaraj, and Figure 3.3 was adapted from his manuscript. Validation of the pluripotency of iPSCs was performed by Dr. Karen Burr. Samples for karyotyping were prepared by Dr. Karen Burr, Dr. Bhuvaneish T. Selvaraj, Dr. Navneet A.Vasistha and Dr. Dario Magnani, and Figure 3.5 & Figure 3.6 were adapted from results provided by TDL and PerkinElmer, respectively. Repeat-primed polymerase chain reaction was performed by Elaine M. Cleary. Maintenance of all iPSC lines and derivation of motor neurons from iPSCs were conducted by Dr. Karen Burr, Nicola Miller, Dr. Bhuvaneish T. Selvaraj, Dr. Navneet A.Vasistha and Dr. Dario Magnani.

Chen Zhao

May 2016
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Entering scientific research with hardly any backgrounds, it would not have been possible for me to complete the project without enormous help and guidance of my supervisors and colleagues in the group. I would like to thank Dr. Andrea Serio for teaching me all the basics and explaining every detail in experiments when I first joined the lab, even some had to be repeated for many times. I am especially grateful to Dr. Bhuvaneish Selvaraj who has guided me through my PhD work. He is always patient and has helped me with everything, experiments, presentations, reports and even providing counselling while I am in blues. I would also like to thank Dr.
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Last, but by no means least, I would like to thank the China Scholarship Council for providing me funding (scholarship No. 2011601061) which allowed me to come to Edinburgh and work on the project that I am really interested in.
Abstract

Amyotrophic lateral sclerosis (ALS) is a late onset neurodegenerative disorder characterised by selective loss of upper and lower motor neurons (MNs). Recently, the GGGGCC (G₄C₂) hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (C9orf72) has been identified as the most common genetic cause of ALS, highlighting the importance of studying the pathogenic mechanisms underlying this mutation. Accumulating evidence implicates that ALS is a multisystem and multifactor disease. Specifically, non-neuronal cells, astrocytes in particular, are also affected by toxicity mediated by ALS-related mutations, and they can contribute to neurodegeneration, suggesting astrocytes as a key player in ALS pathogenesis. Here, a human induced pluripotent stem cells (iPSCs)-based in vitro model of ALS was established to investigate the impact of the C9orf72 mutation on astrocyte behaviour—both cell- and non-cell autonomous. Work in this study shows that patient iPSC-derived astrocytes recapitulate key pathological features associated with C9orf72-mediated ALS, such as formation of G₄C₂ repeat RNA foci, production of dipeptide repeat (DPR) proteins and reduced viability under basal conditions compared to controls. Moreover, C9orf72 mutant astrocytes in co-culture result in reduced viability and structural defects of human MNs. Importantly, correction of the G₄C₂ repeat expansion in mutant astrocytes through targeted gene editing reverses these phenotypes, strongly confirming that the C9orf72 mutation is responsible for the observed findings. Altogether, this iPSC-based in vitro model provides a valuable platform to gain better understandings of ALS pathophysiology and can be used for future exploration of potential therapeutic drugs.
Lay Summary

“I am dying. And fast.” This quote is from a patient diagnosed with amyotrophic lateral sclerosis or ALS at age 29. Unlike Professor Hawking who has had this disease for decades, 80% of ALS patients die within 5 years after diagnosis. If a person had ALS, he or she could feel and think, but would gradually lose the ability to button a shirt, move legs and even speak. Eventually, the person would not be able to breathe. These happen due to the degeneration of a group of nerve cells in the brain and spinal cord, called motor neurons (MNs), which control muscle contraction. Since the first description of ALS in 1869, no cure has been found. Why? One reason is because the neighbouring cells surrounding MNs have long been neglected. Among these neighbouring cells, there is one cell type with a star-like shape, called astrocytes. If a MN was a flower in a garden, astrocytes would be the soil. In a healthy brain, astrocytes produce nutrients and facilitate neuronal functions. Whereas in patients, astrocytes may lose these abilities and even become detrimental to MNs. In my research, I wanted to ask what would happen to MNs, if astrocytes carry the gene mutation that is the most common genetic cause of ALS. To find answers, I used a human stem cell technique that allowed me to make astrocytes from both healthy individuals and ALS patients who have this mutation. I found mutant astrocytes died much faster than normal ones, suggesting the mutant astrocytes are less healthy. What does this mean to MNs? To answer this question, I divided healthy MNs into two groups, one cultured with normal astrocytes and the other cultured with mutant astrocytes. I found MNs in the second group were less viable and much smaller. This finding indicates that these mutant astrocytes can adversely affect MN viability, and death of MNs is responsible for patients’
symptoms. Therefore, work in this study has established a platform to investigate the cause of this adverse effect and whether it can be stopped. If the soil that MNs are living in can be modified, we may be able to rescue the dying MNs in patients, to slow, stop or even reverse this cruel disease.
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List of Abbreviations

- βIII-tubulin: tubulin beta-3 chain
- AAV: adeno-associated virus
- ADARB2: adenosine deaminase RNA-specific B2
- ALS: amyotrophic lateral sclerosis
- AMOs: antisense morpholino oligonucleotides
- AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- APC: astrocyte progenitor cell
- ASO: antisense oligonucleotide
- ATP: adenosine triphosphate
- BAC: bacterial artificial chromosome
- BBB: blood brain barrier
- BDNF: brain-derived neurotrophic factor
- BMP: bone morphogenetic protein
- C. elegans: Caenorhabditis elegans
- C9orf72: chromosome 9 open reading frame 72
- Cas9: CRISPR-associated protein 9
- CDM: chemically defined medium
- cDNA: complementary DNA
- c-Myc: proto-oncogene c-Myc
- cNeuron: cortical neuron
- CNS: central nervous system
- CNTF: ciliary neurotrophic factor
- CRISPR: clustered regularly interspaced short palindromic repeats
- DENN: differentially expressed in normal and neoplasia
- DM2: myotonic dystrophy type 2
- DMEM: Dulbecco's Modified Eagle Medium
- DMSO: dimethyl sulfoxide
- DNA: deoxyribonucleic acid
- Dnase: deoxyribonuclease
- DPR: dipeptide repeat
- DSB: double-stranded break
- EAAT: excitatory amino acid transporter
- EDTA: ethylenediaminetetraacetic acid
- EGF: epidermal growth factor
- eGFP: enhanced green fluorescent protein
- EOMES: eomesodermin homolog
- ER: endoplasmic reticulum
- ESCs: embryonic stem cells
- fALS: familial amyotrophic lateral sclerosis
- FDA: Food and Drug Administration
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>FGF2</td>
<td>fibroblast growth factor 2</td>
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<td>FISH</td>
<td>fluorescent in situ hybridization</td>
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<td>fluo-4 AM</td>
<td>fluo-4 acetoxyethyl ester</td>
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<td>FOXA2</td>
<td>forkhead box protein A2</td>
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<td>FTD</td>
<td>frontotemporal dementia</td>
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<td>FUS</td>
<td>fused in sarcoma</td>
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<td>G4C2</td>
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<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GATA binding protein 4</td>
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<td>G-banding</td>
<td>Giemsa-banding</td>
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<td>glial cell line-derived neurotrophic factor</td>
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<td>guanosine diphosphate</td>
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<td>guide RNA</td>
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<td>guanosine triphosphate</td>
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<td>GWAS</td>
<td>genome-wide association study</td>
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<tr>
<td>HBSS</td>
<td>Hanks' Balanced Salt Solution</td>
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<td>HDR</td>
<td>homology-directed repair</td>
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<td>hnRNP</td>
<td>heterogeneous nuclear ribonucleoprotein</td>
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<td>HR</td>
<td>hazard ratio</td>
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<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
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<td>iPSCs</td>
<td>induced pluripotent stem cells</td>
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<td>krueppel-like factor 4</td>
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<td>median lethal dose</td>
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<td>lactate dehydrogenase</td>
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<td>leukaemia inhibitory factor</td>
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<td>MN</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>NEAA</td>
<td>non-essential amino acids</td>
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<td>NFIA</td>
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<td>nonhomologous end-joining</td>
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<td>NPCs</td>
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<td>OCT3/4</td>
<td>octamer-binding protein 3/4</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
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<td>RAN</td>
<td>repeat-associated non-ATG</td>
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<td>RBPs</td>
<td>RNA binding proteins</td>
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<td>rfu</td>
<td>relative fluorescence units</td>
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<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
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<td>RNA</td>
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<td>RNA interference</td>
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<td>ribonuclease</td>
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<td>regions of interests</td>
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<td>RP-PCR</td>
<td>repeat-primed PCR</td>
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<td>S100B</td>
<td>S100 calcium-binding protein B</td>
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<td>sALS</td>
<td>sporadic amyotrophic lateral sclerosis</td>
</tr>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>sonic hedgehog</td>
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<td>single nucleotide polymorphisms</td>
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<td>SSC</td>
<td>Saline Sodium Citrate</td>
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<td>TALEN</td>
<td>transcription activator–like effector nuclease</td>
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<td>transactive response</td>
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<td>TAR DNA binding protein</td>
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<td>transmembrane protein 106B</td>
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<td>tetracycline response element</td>
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<td>transfer ribonucleic acid</td>
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<td>UBQLN2</td>
<td>ubiquilin 2</td>
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<td>UPIs</td>
<td>ubiquitin-positive inclusions</td>
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<td>ubiquitin proteasome pathway</td>
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<td>unfolded protein response</td>
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<td>vesicle-associated membrane protein</td>
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<td>VAPB</td>
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<td>zinc finger nuclease</td>
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Chapter 1 Introduction
1.1 Background of amyotrophic lateral sclerosis (ALS)

1.1.1 Clinical features

ALS, also known as Lou Gehrig's disease in the US, is a progressive, fatal, adult-onset neurodegenerative disorder characterised by selective loss of upper and lower motor neurons (MNs) in the motor cortex, brainstem and spinal cord\(^1\). It was first described by a French neurologist, Jean-Martin Charcot, in 1869\(^2\), and is the most common form of adult motor neurone disease (MND), with a peak age of onset between 45 and 65 years\(^1\). The prevalence of ALS is approximately 6/100,000 people\(^3\), and its incidence is about 2/100,000 people per year in Europe\(^4\). Typical ALS symptoms include muscle stiffness, muscle wasting and progressive paralysis, and respiratory failure caused by diaphragmatic weakness is the primary reason for death, with an average prognosis of 3 to 5 years after the first symptom onset\(^1\). Approximately 10% of patients have clear family histories, classified as familial ALS (fALS), whereas the vast majority (~90%) are sporadic (sALS) and appear to occur randomly\(^1\). The pathogenic mechanisms of ALS are still unknown, thereby no effective cures are available. To date, Riluzole is the sole US Food and Drug Administration (FDA)-approved medication for ALS\(^5\). It reduces glutamate-mediated excitotoxicity but can merely extend patients’ survival for 2 to 3 months\(^5\).
1.1.2 Genetics

Pathogenic mutations in multiple genes have been identified as genetic causes of ALS, implicating several molecular mechanisms underlying the pathogenesis. The first mutated gene causative of fALS was superoxide dismutase 1 (SOD1) discovered in 1993, followed by identification of over 100 pathogenic variants, in total accounting for ~12% of fALS and ~1% of sALS (~2% of all ALS). The SOD1 protein is widely expressed in various tissues and responsible for clearing highly toxic superoxide radicals. The cellular mechanisms accounting for SOD1-mediated neurodegeneration is proposed to result from gain of toxic properties of the mutant protein form.

After a long hiatus, the genetics of ALS progressed substantially with identification of mutations in several genes encoding proteins that interact with ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), or RNA/DNA binding proteins. These include transactive response (TAR) DNA binding protein (TARDBP), fused in sarcoma (FUS) and matrin 3 (MATR3). Mutation in these genes primarily fall in the protein region responsible for their interactions with other RNA binding proteins (RBPs). This was followed by a landmark discovery of a GGGGCC (G_{4}C_{2}) hexanucleotide repeat expansion in the non-coding region of chromosome 9 open reading frame 72 (C9orf72), which account for ~40% of fALS cases and ~7% of sALS cases, making it the most common genetic cause of ALS. Research in other repeat expansion-mediated neurodegenerative diseases has implicated that the cytotoxicity of these mutations is mediated through disrupted RNA metabolism. Therefore, discoveries of mutated genes encoding RBPs and the G_{4}C_{2} repeat
expansion in C9orf72 together reinforce the involvement of abnormal RNA metabolism in ALS pathogenesis.

Another group of causative mutations is featured by genes involved in protein clearance pathways, such as ubiquilin 2 (UBQLN2)\textsuperscript{17}, sequestosome 1 (SQSTM1)\textsuperscript{18} and vesicle-associated membrane protein (VAMP)-associated protein B (VAPB)\textsuperscript{19}. Proteins encoded by these genes play important roles in transporting undesired cellular proteins to targeted degradation pathways, such as autophagy, ubiquitin proteasome pathway (UPP) and unfolded protein response (UPR)\textsuperscript{20}. These findings indicate that abnormalities in protein homeostasis may play a role in ALS pathogenesis, although such mutations are relatively rare in ALS\textsuperscript{8,20}.

To date, the genetic aetiology of approximately 68% of fALS and 11% of sALS has been uncovered\textsuperscript{8} (Figure 1.1). Although less is known about the factors involved in sporadic cases, clinically and pathologically sALS are indistinguishable from fALS, raising the hope that studying fALS may shed light on common pathogenic pathways underlying different subgroups of ALS.
Figure 1.1 Pathogenic genes in ALS
Proportions of main causal genes of fALS and sALS, respectively (adapted from Renton et al.\textsuperscript{8}, with modifications).

1.1.3 Pathology

ALS pathology is featured by selective degeneration and loss of upper and lower MNs, accompanied by astro- and micro-gliosis\textsuperscript{21}. Cytoplasmic and/or nuclear ubiquitin-positive inclusions (UPIs) are the characteristic pathology in degenerating MNs, and they appear either as filamentous skeins or as compact rounded bodies\textsuperscript{22,23}. In addition to the motor cortex and the spinal cord, UPIs are often found in other regions outside the motor system, such as the frontotemporal cortex, hippocampus, neostriatum and substantia nigra\textsuperscript{22,24}. Although UPIs are predominantly present in affected MNs, detection of UPIs in other types of neurons and glial cells, such as cortical neurons, astrocytes and oligodendrocytes, is not rare\textsuperscript{21,24-27}. Together, these findings indicate ALS as a multisystem degenerative disease, and that both neurons and glial cells are pathologically affected.

Although UPIs had long been described as characteristic pathology in ALS since early 1990s\textsuperscript{22,23}, the major protein component of UPIs was not identified until 2006.
as misfolded TAR DNA-binding protein 43 (TDP-43)\textsuperscript{25,28}. TDP-43 is a highly conserved and ubiquitously expressed DNA-RNA binding protein, encoded by \textit{TARDBP}\textsuperscript{29,30}. It was first discovered as a transcriptional repressor binding to the TAR DNA element of the human immunodeficiency virus type 1\textsuperscript{31}, and later revealed as a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family, playing crucial roles in RNA metabolism\textsuperscript{29,30} (Figure 1.2). Under normal conditions, TDP-43 is predominantly located in the nucleus and constantly shuttles between the nucleus and the cytoplasm\textsuperscript{32}.

\textbf{Figure 1.2 Normal functions of TDP-43}

Roles of TDP-43 in gene expression and RNA metabolism in mammalian cells, including (a) transcriptional regulation, (b) pre-messenger RNA (mRNA) splicing, (c) RNA trafficking and (d) modulation of mRNA stability. (adapted from Lee et al.\textsuperscript{29}, with modifications)

In the context of ALS, several pathological changes occur to TDP-43. Specifically, TDP-43 translocates from the nucleus into the cytoplasm, and is misfolded as
insoluble protein inclusions that are strongly positive for ubiquitin, indicative of ubiquitination of TDP-43\textsuperscript{25,28}. This is often accompanied by depletion of nuclear TDP-43\textsuperscript{28}. Abnormal phosphorylation is another pathological modification of TDP-43, primarily at S409/410 sites, leading to a smear of high molecular weight bands of TDP-43 detected by immunoblot in patient tissues\textsuperscript{25,28,33,34}. In addition, TDP-43 is often cleaved into carboxyl-terminal fragments, which are identified as ~25kD bands in immunoblot\textsuperscript{28}. Altogether, these pathological forms of TDP-43, including ubiquitination, hyper-phosphorylation, truncation, mislocalisation and increased insolubility, are collectively termed as TDP-43 proteinopathies\textsuperscript{29,35}. Of note, nearly all UPIs (~97%) found in ALS patients are composed of TDP-43\textsuperscript{20}, apart from those in SOD1-mediated ALS\textsuperscript{26,27}. Hence, TDP-43 proteinopathies are regarded as the pathological signature of ALS.

1.1.4 SOD1 rodent models of ALS

Following the discovery of mutations in \textit{SOD1}\textsuperscript{6}, a transgenic mouse model was generated (SOD1\textsuperscript{G93A}), which overexpressed human SOD1 protein with a G93A mutation under control of the human SOD1 promoter\textsuperscript{36}. Since then, over thirty other rodent models overexpressing various mutant forms of human SOD1 have been developed\textsuperscript{37}. They recapitulate most pathological and clinical phenotypes of ALS, including progressive motor deficits, premature death, MN loss, gliosis and presence of ubiquitinated protein inclusions, and manifestation of these phenotypes is highly reproducible\textsuperscript{37-40}. In contrast to SOD1 models, transgenic rodents carrying other ALS causing mutations often show variable phenotypes across different research groups.
and cannot fully recapitulate pathological and clinical features of ALS. For example, multiple lines of transgenic rodents overexpressing mutant TDP-43 or C9orf72 do not manifest any TDP-43 proteinopathies or develop motor deficits, the pathological and clinical signatures of ALS. Therefore, the SOD1 rodent models have been commonly used as the primary ALS model to investigate molecular pathomechanisms and explore therapeutic treatments.

1.1.5 Frontal temporal dementia (FTD) and ALS

FTD is a progressive neurodegenerative disorder with atrophy of prefrontal and temporal cortices, and is clinically characterised by behavioural and personality changes together with impairment of language skills. It is the second most common dementia in the population under age 65. Multiple lines of evidence have suggested that FTD and ALS belong to a spectrum disorder, termed as FTD-ALS, as many features are shared between these two diseases. Clinically, about 20-50% of ALS patients develop cognitive impairments accompanied by atrophy of frontotemporal cortices, reminiscent of FTD, and ALS-like mobility deficits can be observed in ~15% FTD patients. Pathologically, TDP-43 protein inclusions are frequently observed in both FTD (~45%) and ALS (~97%). Even in patients with typical ALS symptoms, TDP-43 proteinopathies are often found in prefrontal and temporal cortices, the brain regions most affected by FTD. In terms of genetics, identification of mutations in \textit{TARDBP} and \textit{C9orf72} in both diseases provide the most convincing link between FTD and ALS. Overall, these findings indicate that FTD and ALS are closely related diseases with considerably clinical,
pathological and genetic overlaps, thereby strongly supporting the idea of the FTD-ALS continuum.
1.2 \textit{C9orf72-mediated} ALS

1.2.1 The \textit{C9orf72} mutation

Genome wide association studies (GWAS) first discovered several single nucleotide polymorphisms (SNPs) in the chromosome 9p21 locus which were highly associated with both sALS and fALS in the Finnish population as well as several other European populations\textsuperscript{51-53}. The mutation was later identified as a G\textsubscript{4}C\textsubscript{2} hexanucleotide repeat expansion in \textit{C9orf72}, located at the non-coding region between exon 1a and exon 1b\textsuperscript{13,14}. This mutation is inherited in an autosomal dominant manner, and is the most common genetic abnormality in ALS\textsuperscript{8,15}. Frequency of the \textit{C9orf72} mutation shows regional variation, with the highest occurrence in the Finnish ALS population and low relevance in the Asian populations\textsuperscript{15,54}. It also shows incomplete penetrance, with healthy carriers over age 80\textsuperscript{15,55}. Furthermore, the \textit{C9orf72} mutation has been found in small proportions of other neurodegenerative disorders, such as Alzheimer’s disease and Parkinson’s disease\textsuperscript{56-58}.

In the normal populations, the G\textsubscript{4}C\textsubscript{2} repeat size varies between 2 and 25\textsuperscript{59}, whereas in ALS patients the expansion size ranges from 600-800 to 1700-5000 repeats\textsuperscript{14,60,61}. Of note, repeat number as low as 60-70 has been reported in occasional ALS cases\textsuperscript{62}. Although 30 repeats are commonly used as a diagnostic criterion, no clear cut off has been confirmed as the threshold of minimum repeat numbers required for ALS manifestation\textsuperscript{13,14,63}. The G\textsubscript{4}C\textsubscript{2} repeat expansion size shows somatic instability, as its number varies across different tissues from the same patient\textsuperscript{60,61,64}. Additionally, it is still unclear whether the repeat length has a positive correlation with disease...
Compared to other ALS causing mutations, the G₄C₂ repeat expansion carriers are associated with earlier disease onset and higher percentage of familial cases⁶⁵,⁶⁶.

1.2.2 The C9orf72 protein

The gene *C9orf72* is transcribed into three variants through alternative splicing. The G₄C₂ repeat expansion is either located at the first intron of variant 1 and 3 or at the promoter region of variant 2⁶³ (Figure 1.3). Variant 2 and 3 are translated into the same protein isoform with an estimated molecular weight of ~54kD, and variant 1 is translated into a shorter protein isoform with an estimated molecular weights of ~24kD⁶⁷.
Figure 1.3 The C9orf72 transcripts and C9orf72 proteins

(A) A schematic diagram of the three transcript variants of C9orf72. Location of the G₄C₂ repeat expansion is indicated as yellow dots. Variant 1 is a short transcript with 1a as the 5’-untranslated noncoding exon (black), exon 2 to exon 5 (green) and partial retention of intron 5 (red) consisting of the coding sequence for the short C9orf72 protein isoform—C9-S. Variant 2 and Variant 3 differ in their inclusion of noncoding exon 1a or 1b at the 5’-untranslated region, respectively, and share exons 2 to 11 (green) as coding sequence. Both variants are translated into the long C9orf72 protein isoform—C9-L.

(B) A schematic diagram of the two protein isoforms of C9orf72. Partial retention of intron 5 in Variant 1 adds a unique lysine residue at the C-terminus of C9-S.

(adapted from Xiao et al.⁶⁷, with modifications)
Since the discovery of the G\textsubscript{4}C\textsubscript{2} repeat expansion, accumulating studies start to shed light on the normal cellular functions and regulation of the C9orf72 protein. The first hint came from bioinformatics studies, where sequence and structure analysis predicted the C9orf72 protein as a homolog of the differentially expressed in normal and neoplastic cells (DENN) protein family\textsuperscript{68,69}. Proteins in this family are known to function as guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange factors (GEFs), which catalyse the GDP to GTP switch of Rab-guanosine triphosphatases (GTPases)\textsuperscript{70}. Given that Rab-GTPases are master regulators of almost all vesicle traffic in eukaryotic cells\textsuperscript{71}, the C9orf72 protein was therefore proposed to play a role in Rab-mediated membrane trafficking\textsuperscript{68,69}. In support of this hypothesis, a recent study found co-localisation and co-immunoprecipitation of the C9orf72 protein with several Rab proteins that are involved in endocytosis and autophagy in primary mouse neurons and human spinal cord tissues\textsuperscript{72}. Moreover, knockdown of the C9orf72 protein in neuronal cell lines led to impaired endocytic transport and accumulation of autophagosomes\textsuperscript{72}, further supporting the involvement of the C9orf72 protein in intracellular membrane trafficking.

Recently, one group generated two isoform-specific antibodies for the C9orf72 protein, and observed distinct expression patterns and pathological changes of each isoforms in human tissues\textsuperscript{67}. Under normal conditions, the long isoform showed diffuse cytoplasmic expression, whereas the short isoform was specifically localised at the nuclear membrane. In addition, the short isoform co-localised and co-immunoprecipitated with Importin-b1 and RanGTPase, two proteins involved in the nuclear transport. In post-mortem tissues of ALS patients, regardless of the presence
or absence of the G\textsubscript{4}C\textsubscript{2} repeat expansion, expression of the short isoform was reduced or absent, accompanied by lost nuclear staining of Importin-b1 and RanGTPase. Interestingly, loss of the short C9orf72 isoform correlated with clearance of nuclear TDP-43 and mislocalisation of TDP-43 into cytoplasmic inclusions. In contrast, the long protein isoform showed highly variable intensities in ALS patients\textsuperscript{67}. Altogether, these findings indicate distinctive normal functions of the two C9orf72 protein isoforms and the involvement of the short isoform in nucleus-cytoplasm shuttling.

In the attempt to understand the spatial-temporal expression pattern of C9orf72, one study utilised the beta-galactosidase reporter system to investigate transcription of the mouse C9orf72 orthologue, which is highly homologous to human C9orf72 (>90%)\textsuperscript{73}. Expression of mouse C9orf72 was primarily detected in cortical neurons and spinal MNs, the cell types most vulnerable to degeneration in FTD-ALS. In contrast, C9orf72 was rarely transcribed in non-neuronal cells, such as microglia, astrocytes and tissues outside the nervous system. Developmental characterisation showed C9orf72 expression was not detectable until postnatal week 2\textsuperscript{73}. In line with this study, a recent report also observed high neuronal enrichment of C9orf72 expression by using in situ hybridisation on wild type (WT) mice\textsuperscript{74}. However, C9orf72 transcripts were also detectable in embryonic and early postnatal neurons\textsuperscript{74}. Although conditional knockout of C9orf72 in neuronal and glial precursors from E10.5 did not lead to overt neurodevelopmental abnormalities\textsuperscript{74}, the necessity of C9orf72 in early embryonic development remains to be addressed. Nevertheless, these two studies strongly suggest that the C9orf72 expression is differentially
regulated in various cell types in the central nervous system (CNS). This expression pattern is distinctive from other ALS-associated proteins, such as TDP-43, FUS and SOD1, which are ubiquitously expressed in all cell types.

1.2.3 Pathology of C9orf72-mediated ALS

Similar to other subgroups of ALS, TDP-43 proteinopathies have been found in various brain regions in patients with the C9orf72 mutation. However, TDP-43 negative but p62 positive protein inclusions are also commonly observed in the cerebral cortex, hippocampus and cerebellum. This pathology is specific to patients carrying the G₄C₂ repeat expansion, thereby has been proposed as a defining feature of C9orf72-mediated ALS.

As the G₄C₂ repeat expansion is located in the non-coding regulatory region, the expression of C9orf72 is proposed to be affected by the mutation. Although one study did not find any change in C9orf72 expression, down-regulation of total C9orf72 transcript levels has been widely described in various brain regions as well as lymphoblast cells from patients. Such reduction is, at least partially, caused by epigenetic modifications, including hypermethylation of C9orf72 and trimethylation of histone. Further, reduced C9orf72 protein levels have also been observed in frontal cortex of patients tissues.

Transcription of C9orf72 is bidirectional, and the antisense transcripts are produced as a result of naturally occurring antisense transcription. Bidirectional transcription
of the mutant allele of *C9orf72* leads to production of sense and antisense pre-mRNAs containing the G₄C₂ and C₄G₂ repeat expansion, respectively, which form complex secondary structures, such as G-quadruplex. These repeat expansion containing pre-mRNAs aggregate and cluster in nuclei, which can be detected as RNA foci using RNA fluorescent in situ hybridization (FISH). Intranuclear and occasionally cytoplasmic RNA foci have been observed in various cell types from patients, including neurons, astrocytes, white blood cells and fibroblasts, making it another pathological feature of *C9orf72*-mediated ALS.

Furthermore, presence of repeat expansions can also initiate an unconventional translation independent of the traditional starting codon ATG, termed as repeat associated non-ATG (RAN) translation. Both sense and antisense transcripts of the G₄C₂ repeat expansion are amenable to RAN translation, resulting in five dipeptide repeat (DPR) products of all six possible reading frames. They are poly-glycine:alanine (poly-GA), poly-glycine:proline (poly-GP) and poly-glycine:arginine (GR) translated from the sense transcripts, and poly-proline:alanine (poly-PA), poly-proline:glycine (poly-PG) and proline:arginine (poly-PR) translated from the antisense transcripts. Among these, poly-GP can be translated from both sense and antisense transcripts. Such DPR products are prone to aggregation and have been detected as inclusions in various brain regions of patient tissues. Additionally, immunohistochemistry shows co-localisation of DPR proteins with p62, suggesting that DPR proteins are the component of p62 positive but TDP-43 negative protein inclusions found in patients.
Collectively, in addition to TDP-43 proteinopathies, C9orf72-mediated ALS is pathologically characterised by down-regulation of C9orf72 expression, RNA foci, DPR products through RAN translation and p62 positive but TDP-43 negative protein inclusions.

**1.2.4 Hypotheses of C9orf72-mediated pathogenesis**

The pathological features found in patient tissues indicate three possible pathogenic mechanisms mediated by the G_{4}C_{2} repeat expansion in C9orf72: 1) loss of C9orf72 functions, 2) gain of RNA toxicity (RNA foci) and 3) gain of protein toxicity (DPR proteins). In light of these, multiple *in vitro* and *in vivo* models have been developed to address whether and how these pathological changes are neurotoxic.

**Haploinsufficiency of C9orf72**

Reduced C9orf72 expression in various brain regions of patients indicates that haploinsufficiency of C9orf72 may contribute to neurodegeneration. To test this hypothesis, a zebrafish model was first generated by injecting antisense morpholino oligonucleotides (AMO) to block C9orf72 expression^{81}. These zebrafish developed loco-motor deficits accompanied by axonopathy, which could be rescued by overexpression of human C9orf72 transcripts^{81}. Similarly, a null mutation of C9orf72 orthologue led to progressive paralysis and MN degeneration in *Caenorhabditis elegans* (*C. elegans*)^{95}. Together, these findings support the notion that haploinsufficiency of C9orf72 is pathogenic.
However, several studies did not find evidence of cytotoxicity associated or caused by C9orf72 down-regulation. For example, reduced C9orf72 expression was not found in patient induced pluripotent stem cell (iPSC)-derived neurons, even though these cells showed increased vulnerability to stressors compared to control neurons\textsuperscript{96}. Similarly, RNA interference (RNAi) mediated knockdown of C9orf72 in primary mouse neurons did not lead to any change in neuronal vulnerability\textsuperscript{97}. In addition to \textit{in vitro} evidence, rodent models of C9orf72 loss of function have shown contradicting findings to zebrafish and \textit{C. elegans} models. Conditional Nestin-Cre knockout mice did not manifest any ALS-like behavioural phenotypes or pathological hallmarks, apart from moderate weight loss\textsuperscript{74}. In line with this finding, antisense oligonucleotide (ASO)-mediated knockdown of C9orf72 in the CNS of mice was very well tolerated, even though a 40\% reduction of C9orf72 was achieved\textsuperscript{90}. The discrepancy between rodent models and non-mammal models may be accounted by the low homology of \textit{C. elegans} (58\%) and zebrafish (76\%) orthologues compared to 98\% similarity shared between mouse orthologue and human C9orf72\textsuperscript{74}. It is worth noting, however, that both rodent models had knockdown or knockout of mouse C9orf72 orthologue only in the CNS since postnatal stages, which does not fully mimic the condition in ALS patients, where the mutation is present in all tissues and throughout their entire lifespan. Nevertheless, these \textit{in vitro} and \textit{in vivo} findings oppose the pathogenic roles of C9orf72 haploinsufficiency.
**RNA- and protein-mediated gain of toxicity**

Another pathogenic mechanism is proposed to be gain of toxicity caused by the repeat expansion. Overexpressing G\textsubscript{4}C\textsubscript{2} repeats led to increased cell death in neuronal like cells and primary mouse neurons\textsuperscript{97,98}, and it also initiated development of rough eye morphology and motor deficits in transgenic Drosophila lines, indicative of neurodegeneration\textsuperscript{99,100}. In addition, one study utilised adeno-associated virus (AAV)-mediated expression of 66 x G\textsubscript{4}C\textsubscript{2} repeats in the CNS of mice. These mice developed motor deficits and antisocial behaviours, and multiple pathological features of C9orf72-mediated ALS were also observed, including TDP-43 proteinopathies, G\textsubscript{4}C\textsubscript{2} repeat RNA foci and DPR proteins\textsuperscript{101}. Altogether, these findings support the gain of toxicity hypothesis, although whether the neurotoxicity is mediated through RNA foci and/ or DPR proteins could not be distinguished.

In the attempt to address this question, one study specifically developed an *in vivo* Drosophila model engineered to overexpress RNA-only repeats or protein-only repeats\textsuperscript{102}. Stop codons were introduced to the G\textsubscript{4}C\textsubscript{2} repeats in the RNA-only repeat construct to prevent RAN translation, without affecting the formation of RNA foci. As for the protein-only repeat construct, alternative codons encoding the same DPR products were designed. Using these two constructs, transgenic Drosophila lines were generated, and degeneration of photoreceptors and MNs was only observed in Drosophila expressing the DPR proteins but not in those expressing the G\textsubscript{4}C\textsubscript{2} repeat-containing RNAs\textsuperscript{102}. Using the same design strategy of the protein-only construct, another study also found increased vulnerability in primary mouse neurons overexpressing the DPR proteins\textsuperscript{97}. Although these studies provide convincing
evidence to support that DPR proteins are responsible for the neurotoxicity, RNA-mediated toxicity still could not be completely ruled out. One argument is that introduction of stop codons may alter the interaction between the repeat containing RNAs with other molecules, hence leading to a lack of toxic effects.

Nevertheless, using these models, several studies start to gain insights into the molecular mechanisms underlying the neurotoxic effects caused by the G₄C₂ repeats. Given that the G₄C₂ repeats form stable secondary structures, such as G-quadruplets, it is proposed that RBPs can be sequestered into RNA foci. Indeed, several RBPs have been identified that specifically bind to the G₄C₂ repeats both in vitro and in vivo, including hnRNP A3, Pur-alpha, adenosine deaminase RNA-specific B2 (ADARB2) and hnRNP H. Neurodegenerative phenotypes could be rescued either by overexpressing these RBPs or by ASOs targeted degradation of RNA foci in order to release the sequestered RBPs. These findings further support the notion that impaired normal functions of sequestered RBPs lead to RNA foci-mediated toxicity.

Nucleolar stress is the second molecular mechanism proposed to explain repeat containing RNA-mediated toxicity. Nucleolin, a primary component of the nucleoli, was found binding to G₄C₂ repeat containing transcripts in a conformation dependent way, i.e. nucleolin specifically binds to G-quadruplex formed by repeat containing transcripts. This interaction caused mislocalisation of nucleolin from its normal nucleus sites, and resulted in impaired nucleolin functions, including maturation of
ribosomal RNA (rRNA). Such changes initiated abnormal molecular cascades and led to dysregulated RNA processing and subsequent cell vulnerability to stress\textsuperscript{104}.

Most recently, a third mechanism has been proposed to cause G\textsubscript{4}C\textsubscript{2} RNA-mediated toxicity. Genetic screening of modifiers of G\textsubscript{4}C\textsubscript{2} repeat mediated neurodegeneration in Drosophila led to the discovery of proteins involved in nucleocytoplasmic transport that directly interacted with the G-quadruplex formed by G\textsubscript{4}C\textsubscript{2} repeats\textsuperscript{100,105}. As a consequence, nuclear RNA export and protein import were impaired, leading to RNA retention in the nucleus and mislocalisation of nuclear proteins in the cytoplasm, such as the GTP-binding nuclear protein Ran\textsuperscript{105}. These molecular abnormalities resulted in increased cellular susceptibility to stressors, which could be rescued by small molecules and ASOs that could destabilise the G-quadruplex\textsuperscript{105}. Hence, the impaired nucleocytoplasmic transport might account for gain of toxicity mediated by the G\textsubscript{4}C\textsubscript{2} repeat-containing RNAs.

As for the DPR protein-mediated toxicity, four pathogenic mechanisms have been hypothesised. Firstly, given that expressing the DPR proteins causes neurodegeneration both in vitro and in vivo, the RAN translation products themselves are suggested to be toxic\textsuperscript{97,102}. Secondly, the DPR proteins are prone to aggregation, and thereby may overload the protein clearance pathway and result in ER stress\textsuperscript{106}. Additionally, DPR proteins have been shown interacting with nucleolin and proteins involved in nucleocytoplasmic transport\textsuperscript{107,108}, similar to G\textsubscript{4}C\textsubscript{2} containing RNAs. Therefore, nucleolar stress and defect in nucleocytoplasmic transport are suggested as another two possible pathogenic mechanisms underlying DPR-mediated toxicity.
In contrast to these reports, which support a gain of toxicity model in \( C9orf72 \)-mediated ALS, recent studies pose contradicting findings towards this hypothesis. One transgenic mouse model utilised the tetracycline response element (TRE)-induced expression of 80 x \( G_4C_2 \) RNA repeats, where no pathological changes or behavioural abnormalities were observed in 12-week old mice, apart from intranuclear and cytoplasmic ubiquitin-positive inclusions\(^{109} \). Follow-up studies have not yet been published on this model to address whether there are late onset pathological and motor phenotypes. In addition, two most recent transgenic mouse models carrying a bacterial artificial chromosome (BAC) containing the full length or the first 6 exons of patient \( C9orf72 \) gene with hundreds of \( G_4C_2 \) repeats did not manifest any motor dysfunctions or signs of neurodegeneration, despite the presence of RNA foci and DPR proteins\(^{43,44} \). These findings indicate that the repeat containing RNAs or RAN translation products are not sufficient to initiate neurodegeneration in the lifespan of mice. Notably, the regulatory region of human \( C9orf72 \) was incorporated in the BAC vectors, and the expression levels of \( C9orf72 \) were much lower in these mice compared to animal models discussed above, which may account for the differences in behavioural and neurodegenerative phenotypes between these models. Hence, further studies are still required to determine whether the \( G_4C_2 \) repeat expansion in \( C9orf72 \) is responsible for neurodegeneration.
1.3 Astrocytes in ALS

Considerable efforts have been put into unravelling the pathogenic mechanisms of ALS, and a long-standing idea is that toxicity accumulated within MNs drives the neurodegeneration. However, increasing evidence has challenged this neuron-centric viewpoint and brought the surrounding glial cells, astrocytes in particular, to light as potential non-cell autonomous contributors to ALS pathogenesis. Indeed, most ALS-relevant proteins are ubiquitously expressed in all cell types in the CNS, and astroglial pathology is commonly observed in both fALS and sALS\textsuperscript{21}. Additionally, astrocytes are not merely supporting cells but actively participate in various housekeeping functions of the CNS. Hence, it is hard to imagine that astrocytes do not contribute to the pathogenesis of ALS.

1.3.1 Cellular functions of astrocytes in the healthy CNS

Astrocytes are a group of specialised glial cells (the word “glia” means “glue” in Greek). They are the most abundant cell type in the CNS, and used to be regarded as purely structural cells, filling the gaps between neuronal networks\textsuperscript{110,111}. However, it has become increasingly evident that astrocytes play crucial roles in the maintenance of health and functions of the CNS, and actively engage with neurons to fine-tune neuronal activities\textsuperscript{110,111}.

**Maintenance of glutamate homeostasis**

Glutamate is the major excitatory neurotransmitter in the CNS, and its homeostasis is precisely regulated by neurons and glial cells through glutamate transporters.
expressed on their membranes, named as excitatory amino acid transporters (EAATs) in human. There are five EAATs: EAAT1 and EAAT2 are predominantly expressed on astrocytes; EAAT3 and EAAT4 are neuron specific; EAAT5 is only found in retina\textsuperscript{112}. The clearance of excessive glutamate in the extracellular space is primarily achieved by transporters expressed on astrocytes, where glutamate is converted to glutamine by glutamine synthetase\textsuperscript{113,114}. Glutamine is subsequently recycled back to pre-synaptic terminals, where it is utilised for the synthesis of glutamate\textsuperscript{113,114}. This astrocytic glutamate-glutamine cycle is crucial to the maintenance of glutamate homeostasis, which prevents neurons from glutamate-mediated excitotoxicity\textsuperscript{115,116}.

Control of synapse number and regulation of synaptic transmission

There is a growing body of evidence that astrocytes are essential to synaptogenesis in the CNS\textsuperscript{117}. They not only promote the formation of developing synapses\textsuperscript{118,119}, but also contribute to selective elimination of redundant synapses through the classical complement pathway in coordination with microglia\textsuperscript{120}.

In addition to regulation of synaptic structures, there is an emerging view that astrocytes are an active partner in synaptic functions, termed as tripartite synapses\textsuperscript{121,122}. A single astrocyte may have contact with \textasciitilde 100,000 synapses\textsuperscript{123,124}, and astrocytes are believed to process information from the neuronal elements, respond to neuronal activities and regulate synaptic transmission. Several metabotropic neurotransmitter receptors are expressed on astrocytes. Pre-synaptic release of neurotransmitters bind to these G-protein coupled astrocytic receptors and subsequently activate the inositol 1,4,5-trisphosphate (IP\textsubscript{3}) pathway, which results in
calcium release from the intracellular calcium store endoplasmic reticulum (ER)\textsuperscript{125,126}. This calcium signal can be transmitted to adjacent astrocytes, producing calcium waves, which mediate intercellular communication\textsuperscript{125,126}. The propagation of calcium waves is achieved through two pathways, either by transfer of calcium via intercellular connexin 43 gap junctions\textsuperscript{127} or by the calcium-dependent paracrine release of adenosine triphosphate (ATP) which activates IP\textsubscript{3}-mediated calcium rise in neighbouring astrocytes in a feed-forward manner\textsuperscript{128}. Elevations of cytosolic calcium in astrocytes can trigger exocytosis of several neuroactive molecules, such as glutamate\textsuperscript{129}, ATP\textsuperscript{130} and d-serine\textsuperscript{131} (together termed as gliotransmitters), which can bind to receptors expressed on neurons and modulate synaptic transmission\textsuperscript{122,125,126}. This calcium-dependent astrocytic regulation of synaptic functions is termed as gliotransmission\textsuperscript{122}.

**Blood brain barrier (BBB)**

Astrocytes make extensive contact with cerebral blood vessels in the CNS via numerous endfeet, as one cellular constituent of the BBB\textsuperscript{132}. They modulate the barrier properties of BBB, i.e. molecular exchange between the blood and the brain\textsuperscript{132}. Moreover, astrocytes can couple the neuronal activities with cerebral blood flow. Synaptic release of glutamate can trigger IP\textsubscript{3}-dependent calcium rise at astrocytic endfeet, which results in vasodilation of nearby arterioles\textsuperscript{133,134}. Hence, astrocytes play an important role in maintaining the microenvironment for neuronal activities.
1.3.2 Non-cell autonomous roles of astrocytes in ALS

The first piece of evidence supporting the non-cell autonomous mechanism was the discovery of focal loss of EAAT2, an astrocyte specific glutamate transporter, in pathologically affected brain regions of post-mortem tissues\textsuperscript{135}, which explained the initial observation of glutamate transport defect in ALS patients\textsuperscript{136}. Although whether the loss of EAAT2 was a primary pathological change or secondary response to neuronal loss was not clear, this finding highlighted the importance of non-neuronal cells in ALS.

**Non-cell autonomous toxicity of astrocytes in SOD1-mediated ALS**

As previously introduced in Section 1.1.4 (p.7), overexpressing mutant human SOD1 in rodents leads to MN degeneration and motor deficits\textsuperscript{36,38-40}. However, solely expressing mutant SOD1 in MNs did not initiate these ALS-like phenotypes\textsuperscript{137}, indicating that other cell types are also involved in ALS development. In line with this idea, a chimeric mouse model, which was composed of mixtures of cells expressing either WT or mutant human SOD1, exhibited delayed disease onset and extended survival\textsuperscript{138}. Further investigation showed that non-neuronal cells expressing mutant SOD1 triggered degeneration of nearby WT MNs, whereas non-neuronal cells expressing WT SOD1 extended the survival of nearby mutant MNs\textsuperscript{138}. This study strongly supports the pathogenic role of non-neuronal environment in SOD1-mediated ALS, even though which type of glial cells responsible for this non-cell autonomous effect could not be identified in the chimeric mice.
Following this report, several *in vitro* studies provided direct evidence that primary mouse or human astrocytes expressing mutant human SOD1 could adversely affect viability of primary WT mouse MNs or human embryonic stem cell (ESC)-derived MNs through co-culture experiments\textsuperscript{139-142}. Such astrocytic toxicity was specific to MNs, and could be exacerbated by expression of mutant SOD1 in MNs\textsuperscript{140}. Moreover, the deleterious effects could be recapitulated by culturing MNs in medium conditioned by mutant astrocytes, indicating that the non-cell autonomous toxicity is, at least partially, mediated by soluble factors released from mutant astrocytes\textsuperscript{139}.

*In vivo* studies further supported the negative role of astrocytes in *SOD1*-mediated ALS. Using the Cre-loxP system to selective excise the mutant *SOD1* transgene in an astrocyte-specific manner revealed that astrocytes played a role in disease onset and/or progression in rodent models\textsuperscript{143,144}. In addition, transplantation of precursors of mutant SOD1 expressing astrocytes into the cervical spinal cord of WT rats was sufficient to trigger development of ALS-like phenotypes\textsuperscript{145}, whereas WT astrocyte precursor grafts could attenuate neurodegeneration and slow down disease progression in the SOD1 rat model\textsuperscript{146}.

Collectively, these studies strongly support that ALS pathogenesis is dependent on multicellular interactions, and that astrocytes are an important non-cell autonomous driver of MN degeneration in *SOD1*-related ALS.
Non-cell autonomy in sALS

Mutations in \textit{SOD1} merely account for 2\% of total ALS cases\(^8\). An important question is whether the non-cell autonomous toxicity of astrocytes is a common pathogenic mechanism in all subgroups of ALS, particularly in sporadic cases, which are the majority of ALS patients. Using primary astrocytes generated from post-mortem tissues of sALS patients, survival of primary rodent MNs\(^{147}\) or human ESC-derived MNs\(^{148}\) was decreased when co-cultured with these astrocytes \textit{in vitro}. Together, these studies suggest a shared pathomechanism between sALS and \textit{SOD1}-mediated fALS, by which astrocytes induce MN toxicity.

Non-cell autonomy in other subgroups of fALS

In the context of a seeming consensus in non-cell autonomous toxicity in ALS, our group investigated the role of \textit{TARDBP} mutant astrocytes generated from patient iPSCs\(^{149}\). To our surprise, co-culture of control human iPSC-derived MNs o mutant astrocytes did not alter MN vulnerability, suggesting a distinct pathogenic mechanism in \textit{TARDBP}-mediated ALS from \textit{SOD1}-related fALS or sALS. This hypothesis was supported by an independent \textit{in vivo} study, where transplantation of mutant TDP-43 expressing astrocyte precursors into WT rat spinal cords did not induce MN degeneration\(^{150}\). However, findings in these two studies were contradicted by a transgenic rat model, where selectively overexpressing human mutant TDP-43 in astrocytes led to MN degeneration and motor deficits\(^{151}\). Hence, there is still controversy about the non-cell autonomous toxicity of astrocytes in \textit{TARDBP}-mediated ALS, and further investigations are required. It also highlights the need to investigate the role of astrocytes in different subgroups of ALS.
Hypotheses of molecular mechanisms underlying the non-cell autonomous influence of astrocytes in ALS

A long-standing question is whether the involvement of astrocytes in ALS is mediated through loss of normal functions or gain of toxic functions. Studies of both patient post-mortem tissues and SOD1 rodent models have shown focal loss of EAAT2, known as glutamate type I transporter (GLT-1) in rodents, on astrocytes in ALS affected regions\(^{39,40,135}\). Overexpression or knockdown of GLT-1 could alter the disease onset and early phase of progression in SOD1 rodent models, despite no influence on disease outcome\(^{152,153}\). These led to the hypothesis that loss of astrocytic EAAT2 results in chronic accumulation of extracellular glutamate, which causes excitotoxic MN death. In addition, astrocytes have been shown to possess the capacity to up-regulate the glutamate receptor 2 (GluR2) subunit of the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor on MNs, which reduces the calcium permeability and protects MNs from AMPA receptor-mediated excitotoxicity\(^{154}\). However, presence of the mutant SOD1 in astrocytes abolished this neuroprotective function, and led to increased vulnerability of MNs to AMPA in co-culture\(^{154}\).

Another popular hypothesis is that astrocytes contribute to ALS pathogenesis through neuroinflammatory responses in concert with microglia. Indeed, focal transplantation of mutant SOD1 expressing astrocyte precursors led to microgliosis and MN loss in WT rats\(^{145}\), whereas reactivation of microglia was attenuated following selective silencing the mutant $SOD1$ transgene in astrocytes, resulting in extended survival of transgenic mice\(^{143}\). Upregulation of the transforming growth
factor β (TGF-β) in astrocytes is suggested to mediate this gain of toxic function, which renders reduced production of neuroprotective factors from microglia\textsuperscript{155}. Instead, blocking the astrocytic TGF-β pathway in the mutant SOD1 mouse model at post-symptomatic stage could slow down the disease progression and extend the survival time\textsuperscript{155}.

Taken together, the non-cell autonomous role of astrocytes in ALS pathogenesis is likely mediated by a combination of both loss of normal functions and gain of toxic functions. It is worth noting, however, that most, if not all, of these pathogenic hypotheses are based on studies on rodent models of $SOD1$-related ALS, which accounts for a small proportion of patients and appears to have distinct pathophysiology from other subgroups of ALS. Hence, elucidation of molecular mechanisms responsible for astrocytic involvement in other subgroups of ALS is highly important.

Collectively, studies reviewed in this section highlight the important contributions of astrocytes to both normal CNS functions and ALS pathogenesis. Understanding the non-cell autonomous roles of astrocytes in ALS are beneficial to developing potential treatments via enhancing a supportive environment around MNs.
1.4 Human iPSCs and disease modelling

1.4.1 Challenges of modelling human neurodegenerative disorders

Identification of ALS causing mutations and characterisation of disease-relevant pathology on post-mortem patient tissues are informative, and have provided hints on potential pathogenic pathways. In order to gain a better understanding of ALS pathophysiology and ultimately to develop preventive or disease-modifying strategies, good in vitro and in vivo models of ALS are required. This is particularly challenging because of the inability to readily and reproducibly study living primary human CNS cells. Although cultures of primary human neurons and glial cells are achievable in vitro, materials obtained from biopsy or post-mortem tissues are very limited, and it faces regulation and ethical issues. In addition, the resulting cultures are often heterogeneous and composed of various cell types, and complex tissue processing procedures and stringent culture conditions are required to ensure good cell viability. These limitations together restrict the wide use of primary human neurons and glia to study ALS in vitro.

Transgenic animals, rodents in particular, are frequently used for modelling human diseases in vivo. However, it is highly challenging to model neurodegenerative disorders, such as ALS, in rodents for the following reasons. Firstly, compared to human, rodents have evolutionarily much less advanced CNS and significantly shorter lifespan, which make them difficult to fully replicate phenotypes of late-onset neurodegenerative disorders of human beings. For example, two BAC transgenic mouse models expressing human C9orf72 with hundreds of G4C2 repeats do not
manifest any motor deficits, cognitive abnormalities or develop neurodegeneration, which are the defining characteristics of ALS-FTD\textsuperscript{43,44}.

Secondly, causative mutations of neurodegenerative disorders are mostly inherited in an autosomal dominant manner, meaning that one copy of the mutant allele is adequate to initiate neurodegeneration. Most transgenic rodent models, however, rely on non-physiological overexpression of disease relevant proteins, which can be as high as over 20 copies of mutant human SOD\textsubscript{1}\textsuperscript{36} or about 8-fold increase of mutant human TDP-43\textsuperscript{42} in transgenic rodent models of ALS. In contrast, mice expressing much lower levels of mutant proteins manifest slow onset of moderate, if not at all, ALS-like phenotypes\textsuperscript{42,160}.

Thirdly, neurodegenerative disorders often manifest clinical heterogeneity, suggesting the existence of disease modifiers. Indeed, many genetic, epigenetic and environmental factors can significantly affect the course and severity of these diseases. For example, SNPs in transmembrane protein 106B (TMEM106B) and hypermethylation of \textit{C9orf72} are associated with prolonged disease duration and lower risk of FTD symptom manifestation\textsuperscript{161-163}, thereby suggested as protective modifying factors. In addition, smoking, physical exercise, head trauma and several other environmental factors are proposed as potential non-genetic contributors to ALS development\textsuperscript{164}. Such genetic, epigenetic and environmental diversity in patients cannot be easily mimicked in transgenic animal models that are generated by successive rounds of inbreeding and maintained in highly controlled environment.
Due to these limitations, additional models are required to complement transgenic animals in order to mimic diverse aspects in neurodegenerative disorders, which will facilitate advancing our understanding of pathogenesis and ultimately translating research findings into clinical therapy.

1.4.2 Modelling neurodegenerative diseases using iPSCs

Disease modelling has been revolutionised by the recently discovered human iPSC technique, which reprograms adult somatic cells to pluripotent stem cells by delivering four transcription factors, including octamer-binding protein 3/4 (OCT3/4), sex determining region Y (SRY)-box 2 (SOX2), proto-oncogene c-Myc (c-Myc) and krueppel-like factor 4 (KLF4). This technique allows generation of patient specific iPSCs, which can be subsequently differentiated into disease relevant cell types in an unlimited supply. In the context of neurodegenerative disorders, various types of neurons and glial cells have been successfully derived from iPSCs, with significantly shortened generation time and highly homogenous differentiated cells established over the past few years. Of note, patient iPSC-derived neurons and glial cells have been shown to recapitulate clinical and pathological features of neurodegenerative diseases in vitro. For example, ALS patient iPSC-derived MNs harbouring an M337V mutation in TARDBP manifested TDP-43 proteinopathies, progressive hypoexcitability and increased vulnerability compared to controls. Importantly, the physiological expression levels of mutant proteins allow the investigation of pathophysiology with strong disease
relevance. Moreover, using different disease relevant cell types, it enables the examination of selective cell vulnerability and non-cell autonomous neurotoxicity\textsuperscript{149}.

Another advantage of using iPSCs for modelling neurodegenerative diseases is that there is no species difference, and diverse genetic backgrounds of individual patients are retained. Although studies of patient iPSC-derived cells carrying a disease causing mutation have provided informative evidence about pathogenesis, establishing iPSCs from sporadic patients is also invaluable to elucidate pathogenic mechanisms that may be distinct from familial cases, which is impossible to be modelled by transgenic animals. In addition, cells derived from iPSCs enables the discovery of cellular and molecular events that may occur prior to disease onset\textsuperscript{170}. This is in contrast to end-stage observations in patient post-mortem tissues which cannot address such pathologies as causes or results of a certain disease. Moreover, iPSC-derived \textit{in vitro} models provide valuable platforms for drug screening and pre-clinical validations of candidate molecules\textsuperscript{175}. Such extensive therapeutic screening is highly time-consuming in small transgenic animal models, such as worms and zebrafish, and is not achievable in transgenic rodents\textsuperscript{176}. Last but not least, iPSC-derived cells also provide an opportunity for cell replacement therapies in regenerative medicine\textsuperscript{177}.

Overall, neurons and glial cells derived from patient iPSCs have opened a new, and complementary to existing experimental systems, avenue to model neurodegenerative diseases \textit{in vitro}. 

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1.4.3 Variability of iPSCs and targeted human genome editing

A major challenge of using patient iPSCs for disease modelling is the significant variability among different cell lines at genetic, epigenetic, transcriptional and functional levels\textsuperscript{178}. Genetic backgrounds and epigenetic memory of donor cells, derivation methods, passage number, environmental and technical factors can all contribute to such variability\textsuperscript{178}, although genetic background diversity of donors has been suggested as the predominant driving factor\textsuperscript{179}. Using whole genome sequencing, one study has found that the difference between any given two iPSC lines is much more pronounced than the difference between human iPSCs and human ESCs\textsuperscript{180}. Significant clonal variation has also been widely reported\textsuperscript{181}, possibly as a result of mutations derived from clonal selection\textsuperscript{178}. This remarkable variation can significantly affect phenotypic differences revealed through comparisons between control and patient lines by exaggerating, masking or even reversing the true effects caused by a disease relevant mutation\textsuperscript{182}. It is therefore difficult to interpret findings, and the identified molecular pathways may not be pathologically relevant to diseases.

To overcome this problem, three methods have been applied by researchers. Firstly, the power of findings increases by recruiting a large number of control and patient lines\textsuperscript{172}, but such strategy requires substantial money and time input. Another possibility to limit the background noise is to use healthy relatives of patients. Nevertheless, there clearly is a risk that these “healthy” relatives may develop the same disease in the near future. Of note, these two methods can only associate the pathological phenotypes and proposed molecular mechanisms with certain diseases and mutations, without providing evidence of causative relationship.
The third option to reduce variability is to generate isogenic iPSC lines, either by introducing disease-relevant mutations to control iPSCs or by correcting the mutations in patient iPSCs. By directly comparing cellular and molecular phenotypes between the isogenic pair lines, it enables studies of the causal relationship between mutations and phenotypes\textsuperscript{183}. This strategy has been empowered by the recently developed human genome editing system—clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 (Cas9)\textsuperscript{184}. Adapted from the bacterial immune system\textsuperscript{185}, the CRISPR/Cas9 technique utilises guide RNAs (gRNAs) to direct the Cas9 nuclease to cleave targeted DNA sequences and induce double-stranded break (DSB), followed by homology-directed repair (HDR)- or nonhomologous end-joining (NHEJ)-mediated repair to edit human genome at a desired locus\textsuperscript{186,187}. This technique is fast, simple and highly efficient compared to previous protein-based genome editing techniques, such as zinc finger nucleases (ZFNs) and transcription activator–like effector nucleases (TALENs)\textsuperscript{184}. Recently, correction of mutations using the CRISPR/Cas9 mediated genome editing has been shown to reverse disease relevant phenotypes in iPSC-based \textit{in vitro} models\textsuperscript{188}. Therefore, targeted gene editing techniques have greatly enhanced the opportunities to use human iPSCs for \textit{in vitro} disease modelling, such as neurodegenerative disorders.
**Aims**

Against the background described in Chapter 1, my hypothesis was that the *C9orf72* mutation affected human astrocyte behaviour—both cell- and non-cell autonomous. This was addressed in the following aims.

1) To establish an *in vitro* model of ALS using patient iPSC-derived astrocytes (Chapter 3)

2) To examine the pathological features associated with *C9orf72*-mediated ALS in iPSC-derived astrocytes (Chapter 4)

3) To evaluate the cell-autonomous impact of the *C9orf72* mutation on astrocyte viability (Chapter 5)

4) To elucidate the non-cell autonomous contribution of *C9orf72* mutant astrocytes to control human MNs (Chapter 6)
Chapter 2  Materials and Methods
2.1 Generation and maintenance of iPSC lines

Skin fibroblasts were obtained from two healthy individuals (CTRL1 and CTRL2) and three ALS patients carrying the G₄C₂ repeat expansion in C9orf72 (Carrier1, Carrier2 and Carrier3). These were conducted under full Ethical/Institutional Review Board approval at the University of Edinburgh (Prof Ian Wilmut’s group, for CTRL1 and CTRL2), at University College London (Prof John Hardy’s group, for Carrier1) and at King’s College of London (Prof Christopher Shaw’s group, for Carrier2 and Carrier3). Generation of iPSC lines was conducted by Judy Flecther at the University of Edinburgh (for CTRL1 and CTRL2), Dr Selina Wray at University College London (for Carrier1) and Dr Shyamanga Booroah at University of Cambridge (for Carrier2 and Carrier3) using the Yamanaka transcription factors (OCT3/4, SOX2, c-Myc, and KLF4) as previously described. The resulting iPSC lines were maintained and characterised by Dr Karen Burr, Nicola Miller, Dr Bhuvaneish T. Selvaraj, Dr Navneet A.Vasistha and Dr Dario Magnani in our group at the University of Edinburgh using previously described methods. Demographic information of iPSC lines used in this study is listed in Chapter 3 Table 3.1 (p.62).

2.2 Generation of isogenic control iPSCs using CRISPR/Cas9-mediated genome editing

An isogenic control line of Carrier3 iPSCs was generated by Dr Bhuvaneish T. Selvaraj in the group by using CRISPR/Cas9-mediated genome editing to selectively
remove the G₄C₂ repeat expansion as previously described\textsuperscript{187}. Two gRNAs (1 & 2) flanking the G₄C₂ repeats were used, and their sequences are AACTCAGGAGTCGC GCGCTAGGG (gRNA-1) and GGCCCGCCCCGGACCACGCCCCGG (gRNA-2), respectively. 164 individual clones were picked and screened for deletion of the G₄C₂ repeat expansion using repeat-primed polymerase chain reaction (RP-PCR) as described below in Section 2.9. One positive clone was identified and expanded to establish an iPSC line, named as Carrier3-ΔG₄C₂.

### 2.3 Differentiation of iPSCs into three germ layers

Three germ layers differentiation of iPSCs was conducted by Dr Karen Burr in the group using the following method. Confluent iPSC cultures were dissociated into small colonies using Dispase and Collagenase and split from 1 well of a 6-well plate onto 3 wells of a 12-well plate in presence of endoderm, mesoderm and neuroectoderm medium, respectively, to induce three germ layer differentiation. Medium was changed every day for endoderm and mesoderm differentiation until day 3, whereas medium was changed every other day for neuroectoderm differentiation until day 7. At the end of differentiation, cells were fixed for immunocytochemistry as described below in Section 2.15.

### 2.4 Generation of MNs from iPSCs

MNs were generated from iPSCs by Dr Karen Burr, Nicola Miller, Dr Bhuvaneish T. Selvaraj, Dr Navneet A. Vasistha and Dr Dario Magnani in the group using a
previously described protocol with a minor modification of the dual-SMAD inhibition step, where a small molecule LDN (100μM) was used to replace the dorsomorphin (2.5μM) as a bone morphogenetic protein (BMP) inhibitor to enhance the efficiency of neurolisation.

2.5 Generation of astrocytes from iPSCs

Astrocytes were generated from iPSCs using a previously established protocol with minor modifications. iPSCs were first converted to MN spheres as described above in Section 2.4. After 2-4 weeks of culture in MN maturation medium, MN spheres were mechanically chopped into small spheres and then cultured in EL20 medium for 4-6 weeks to induce astrogliogenesis. At the end of this conversion phase, medium was switched to EF20 medium to maintain the proliferation of astrocyte progenitor cells (APCs) in spheres. These astrospheres were mechanically chopped every 4 weeks to ensure good viability and maintained long-term in EF20 medium. Astrospheres were dissociated into single cells using the Papain Dissociation System and plated onto 6-well Matrigel (1:80 diluted) coated plates at a density of 7.5x10^5. The resulting monolayers of APCs were either propagated as progenitors upon confluence in the presence of EF20 medium with a split ratio of 1:2 or 1:3, or differentiated into astrocytes by switching the medium to ciliary neurotrophic factor (CNTF) medium for 14 days. All media were changed every 2–3 days during the astrocyte generation process. Components of media are described below in Section 2.8.
2.6 Cryopreservation and recovery of APCs

Upon confluence APCs were dissociated into single cells using Accutase and cryopreserved in EF20 medium supplemented with 10% dimethyl sulfoxide (DMSO, Sigma, D2438), which were stored within a CoolCell® Cell Freezing Containers (biocision, BCS-405) at -80°C overnight before being transferred to liquid nitrogen for long-term storage. Cryopreserved APCs were recovered by being defrosted at 37°C and washed once in EF20 medium, after which APCs were plated onto 6-well Matrigel (1:80 diluted) coated plates with a split ratio of 1 cryogenic vial of APCs onto 2 wells of a 6-well plate.

2.7 MNs and astrocytes co-culture

2-week old differentiated astrocytes were dissociated into single cells using Accutase and plated onto 96-well plates at a density of 4x10⁴ cells per well in CNTF medium. 4 days later, MN spheres were dissociated into single cells using the Papain Dissociation System and 1x10⁴ cells per well were plated on monolayers of astrocytes for co-culture experiments in presence of MN maturation medium. All media were changed every 2–3 days. Components of media are described below in Section 2.8.
2.8 Cell culture media

All base medium and cell culture supplements were purchased from Life Technologies unless otherwise specified.

<table>
<thead>
<tr>
<th>Name</th>
<th>Component</th>
<th>Final Concentration</th>
<th>Catalogue</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL20</td>
<td>Advanced DMEM/F12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antibiotic-Antimycotic</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamax</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B27</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>20ng/mL</td>
<td>R&amp;D systems, 236-EG</td>
</tr>
<tr>
<td></td>
<td>LIF</td>
<td>20ng/mL</td>
<td>Sigma, L5283</td>
</tr>
<tr>
<td>EF20</td>
<td>Advanced DMEM/F12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antibiotic-Antimycotic</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamax</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B27</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>20ng/mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FGF2/Heparin</td>
<td>20ng/mL</td>
<td>Peprotech, 450-33</td>
</tr>
<tr>
<td>CNTF</td>
<td>Neurobasal</td>
<td></td>
<td>21103-049</td>
</tr>
<tr>
<td></td>
<td>Antibiotic-Antimycotic</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamax</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B27</td>
<td>1%</td>
<td></td>
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<tr>
<td></td>
<td>NEAA</td>
<td>1%</td>
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</tr>
<tr>
<td></td>
<td>CNTF</td>
<td>10ng/mL</td>
<td>R&amp;D systems, 257-NT-010</td>
</tr>
</tbody>
</table>

Abbreviations: ciliary neurotrophic factor (CNTF), Dulbecco's Modified Eagle Medium (DMEM), epidermal growth factor (EGF), leukaemia inhibitory factor (LIF), non-essential amino acids (NEAA).
2.9 RP-PCR

RP-PCR was conducted by Elaine M. Cleary to confirm the presence or absence of the G4C2 repeat expansion in carrier or control lines at both iPSCs and astrosphere stages using the following method. Genomic DNA was isolated using a Wizard® Genomic DNA Purification Kit (Promega, A1120) following the manufacturer’s instructions. PCR reaction setup is shown in Table 2.2, and primer sequences are shown in Table 2.3. Cycling was performed using a Veriti® thermal cycler (Life Technologies) with conditions shown in Table 2.4. PCR products were separated on an ABI 3130 x 1 analyser (Life Technologies), and data were analysed using the GeneMarker software (Soft Genetics).
### Table 2.2 RP-PCR reaction setup

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Catalogue</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimized DyNAzyme EXT buffer 10x</td>
<td>ThermoFisher Scientific, F-511L</td>
<td>1.6</td>
</tr>
<tr>
<td>Betaine, 5M</td>
<td>Sigma, B0300</td>
<td>7.2</td>
</tr>
<tr>
<td>HPE dNTP mix</td>
<td>Scientific Lab Supplies, 28406551</td>
<td>0.3</td>
</tr>
<tr>
<td>F2 primer, 5µM</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>Repeat R primer, 5µM</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Tail R</td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>DyNAzyme EXT DNA Polymerase 1U/µl</td>
<td>ThermoFisher Scientific, F505-L</td>
<td>2.4</td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>DNA, ~200ng/µl</td>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

### Table 2.3 Primer sequences for RP-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td>F2</td>
<td>CTGTAGCAAGCTCTGGAACTCAGGAGTCG</td>
</tr>
<tr>
<td>Repeat R</td>
<td>TACGCATCCCAGTTTGAGACGGCCGGCCCCGGCCGGCC</td>
</tr>
<tr>
<td>Tail R</td>
<td>TACGCA TCCAGTTTGAGACG</td>
</tr>
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</table>

### Table 2.4 Cycling conditions for RP-PCR

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Cycles</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>45 seconds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>10 seconds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>30 seconds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>5 seconds (slow ramp, 0.6°C/second)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>2 seconds</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>86</td>
<td>2 seconds</td>
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<td></td>
</tr>
<tr>
<td>78</td>
<td>2 seconds</td>
<td>7</td>
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<tr>
<td>90</td>
<td>2 seconds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>10 minutes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.10 Karyotyping

Giemsa-banding karyotyping was conducted by TDL (The Doctors Laboratory), and iPSCs samples were prepared as follows. BrdU (33.3µg/mL) /Colcemid (5ng/mL) solution was added onto ~75% confluent iPSC cultures. 15 hours later, iPSCs were harvested in 1:1 0.56% KCl:0.4% sodium citrate solution and fixed with 3:1 Methanol:Acetic acid. The KaryoLite BoBs™ assay was conducted by PerkinElmer with genomic DNA isolated from iPSCs using a Wizard® Genomic DNA Purification Kit.

2.11 Glutamate uptake assay

2-week old differentiated astrocytes were dissociated into single cells using Accutase and plated on Matrigel (1:80 diluted) coated 96-well plates at a density of 2.5x10^4 cells per well in CNTF medium. 5 days after plating, astrocytes were exposed to 100µM of L-glutamic acid (ATT Bioquest, 10054) diluted in Hanks’ Balanced Salt Solution (HBSS, Sigma, H8264). Supernatants were collected at 30, 60 and 120 minutes. In addition, 100µM L-glutamic acid in HBSS supplemented with 2mM L-trans-Pyrrolidine-2,4-dicarboxylic acid (PDC, Sigma, P7575) was applied for 120 minutes as a negative control. The residual concentrations of L-glutamic acid in supernatants were determined using an Amplite™ Fluorimetric Glutamic Acid Assay Kit (ATT Bioquest, 10054) following the manufacturer’s instructions. Fluorescence intensity was measured on a FLUOstar OPTIMA microplate reader (BMG LABTECH). Raw data was processed first by subtracting the average background signal of empty wells, which was then used to determine the residual glutamate
concentration based on a standard curve generated by a serial dilution of glutamate. Next, the glutamate uptake was calculated by subtracting the remaining concentration from the starting concentration, 100μM.

To control for the cell number, the amount of DNA in each well was first determined by using a CyQUANT® NF Cell Proliferation Assay Kit (Life Technologies, C35006) following the manufacturer’s instructions. Fluorescence intensity was measured on a FLUOstar OPTIMA microplate reader. Raw data was processed first by subtracting the average background signal of empty wells, which was then converted to cell numbers based on a standard curve generated by a serial dilution of cells. Next, the glutamate uptake was normalised to the cell number and presented as uptake concentration per 1000 cells.

### 2.12 Calcium imaging

2-week old differentiated astrocytes were dissociated into single cells using Accutase and plated on Matrigel (1:80 diluted) coated μ-Slide 8 Well Glass Bottom (ibidi, 80827) chambers at a density of 1.5x10^5 cells per well. 4 days later, astrocytes were loaded with fluo-4 acetoxyethyl ester (Fluor-4 AM) (Life Technologies, F-14201) in Neurobasal® Medium (Life Technologies, 21103-049) for 1 hour at 37°C. After three washes with Neurobasal® Medium, astrocytes were left in Neurobasal® Medium for 30 minutes at 37°C to allow fully de-esterification. As a negative control, 50μM 2-APB (Calbiochem, 100065) was applied at this stage to block IP3-dependent calcium release. The medium was then switched to Dulbecco's Phosphate-Buffered
Salines (DPBS, Life Technologies, 14190-094) prior to imaging. To trigger calcium waves, astrocytes were stimulated by dropping glass beads (200µm diameter) suspended in HBSS on top of the cultures. Time-lapse imaging was performed using an Axio Observer.Z1 (Carl Zeiss) epifluorescence microscope at 10x magnification with a 488nm excitation filter at 37°C and 5% CO₂.

2.13 Quantitative real-time PCR (qRT-PCR)

2-week old differentiated astrocytes were harvested using Accutase, and total RNA was isolated using an RNeasy Mini Kit (Qiagen, 74106) following the manufacturer’s instructions, with a post-isolation deoxyribonuclease (DNase) digestion using a TURBO DNA-free™ Kit (Life Technologies, AM1907). 500ng RNA was reverse transcribed to complementary DNA (cDNA) using a DyNAmo cDNA Synthesis Kit (Thermo Scientific, F-470) following manufacturers’ instructions. RT-PCR reactions were performed in triplicate using a DyNAmo™ ColorFlash SYBR® Green qPCR Kit (Thermo Scientific, F-416), and cycling was conducted using a C1000™ Thermal Cycler with a CFX96 Real-time System (Bio-Rad). The RT-PCR cycling conditions are shown in Table 2.5. Sequences of primers used in this study are listed in Table 2.6. Primers for beta-actin were designed using the NCBI/Primer-BLAST tool. Primers for total C9orf72 transcripts were published by Sareen et al.⁹⁶. Primers for 3 individual C9orf72 transcript variants were published by Fratta et al.¹⁸⁹. Of note, C9orf72 T1, T2 and T3 primers detect C9orf72 variant 2, 1 and 3, respectively, in the current study. Locations of the C9orf72
primers are shown in Figure 4.4 on page 101. Relative gene expression levels were calculated using the CFX Manager™ Software (Bio-Rad) with the 2^{-ΔΔCt} method.

### Table 2.5 Cycling conditions for qRT-PCR

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>7 minutes</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>15 seconds</td>
<td>40</td>
</tr>
<tr>
<td>59</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>0.5°C/5 seconds</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>(melt curve)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.6 Primer sequences for qRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
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</thead>
<tbody>
<tr>
<td>Beta-Actin F</td>
<td>GTTACAGGAAGTCCCTTGCCATCC</td>
</tr>
<tr>
<td>Beta-Actin R</td>
<td>CACCTCCCCCTGTGGACTTGGG</td>
</tr>
<tr>
<td>C9orf72 total F</td>
<td>TGTGACAGTTGGAATGCAGTGA</td>
</tr>
<tr>
<td>C9orf72 total R</td>
<td>GCCACTTAAAGCAATCTCTGTCTTG</td>
</tr>
<tr>
<td>C9orf72 T1 F</td>
<td>GCGGTGGCGAGTGATAT</td>
</tr>
<tr>
<td>C9orf72 T2 F</td>
<td>TCATCTATGAAATCACAAGCTGTTC</td>
</tr>
<tr>
<td>C9orf72 T2 R</td>
<td>GGTATCTGCTTCACTCAGCTT</td>
</tr>
<tr>
<td>C9orf72 T3 F</td>
<td>GAGCAGGTGTGGGTAGAGGAGA</td>
</tr>
<tr>
<td>C9orf72 T1+T3 R</td>
<td>TGGGCAAAGAGTGACATCA</td>
</tr>
</tbody>
</table>
2.14 RNA FISH

Cells were fixed with 4% paraformaldehyde (Agar Scientific, AGR1026) for 15 minutes at room temperature and then permeabilised in 70% ethanol at 4°C overnight. Prior to hybridisation, cells were re-hydrated in 50% formamide (Sigma, 47680)/2x Saline Sodium Citrate (SSC, Sigma, S6639) for 10 minutes at room temperature and blocked in hybridisation buffer for 30 minutes at 45°C. An Alexa Fluor® 546-conjugated (GGCCCC)_4 probe (Integrated DNA Technologies) was diluted in the hybridisation buffer (Table 2.7) and 50ng was applied on cells for 2 hours at 45°C in a humidified chamber. Next, cells were washed twice with 50% formamide/2x SSC for 30 minutes at 45°C and then once with 2x SSC for 30 minutes at room temperature. After another three washes with phosphate-buffered saline (PBS, Life Technologies, 10010023), immunocytochemistry was performed as described below in Section 2.15.

As controls, cells were treated with 3U/mL DNase (Life Technologies, AM1907) or 100µg/mL ribonuclease (RNase, Sigma, R4642) diluted in 2x SSC prior to hybridisation for 1 hour at 37°C. In addition, an anti-sense RNA probe against the CCTG repeat expansion, the mutation associated with myotonic dystrophy type 2 (DM2), was also applied on astrocytes to assess the specificity of the (GGCCCC)_4 probe.
Table 2.7 Hybridisation buffer for RNA FISH

<table>
<thead>
<tr>
<th>Component</th>
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<th>mL</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>Sigma, 47680</td>
<td>5</td>
<td>50%</td>
</tr>
<tr>
<td>SSC</td>
<td>Sigma, S6639</td>
<td>1</td>
<td>2×</td>
</tr>
<tr>
<td>Dextran Sulfate</td>
<td>Millipore, S4030</td>
<td>1</td>
<td>10%</td>
</tr>
<tr>
<td>Yeast transfer RNA (tRNA)</td>
<td>Invitrogen, 15401-029</td>
<td>1</td>
<td>1mg/mL</td>
</tr>
<tr>
<td>Salmon sperm DNA</td>
<td>Invitrogen, 15632-011</td>
<td>1</td>
<td>1mg/mL</td>
</tr>
</tbody>
</table>

2.15 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 minutes. After three washes with PBS, cells were permeabilised with 0.2% Triton X-100 (Sigma, T8787) for 5 minutes and then blocked in 3% goat serum (Dako, X090710-8) or donkey serum (Sigma, D9663) for 45 minutes. Next, cells were incubated with primary antibodies diluted in blocking buffer for 1 hour, after which cells were washed three times with PBS and then incubated with Alexa Fluor®-conjugated secondary antibodies (Life technologies) for 30 minutes. After another three washes with PBS, nuclei were visualised by counterstaining with 0.2μg/mL of 4',6-diamidino-2-phenylindole (DAPI, Sigma, D8417) for 5 minutes. Coverslips were mounted on slides using FluorSave™ Reagent (Merck Millipore, 34578921). All procedures were performed at room temperature. Primary antibodies used in this study are listed in Table 2.8.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Isotype</th>
<th>Catalogue</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NANOG</td>
<td>Goat</td>
<td>IgG</td>
<td>R&amp;D Systems, AF-1997</td>
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</tr>
<tr>
<td>SOX2</td>
<td>Rabbit</td>
<td>IgG</td>
<td>Millipore, AB5603</td>
<td>1:250</td>
</tr>
<tr>
<td>TRA-1-60</td>
<td>Mouse</td>
<td>IgM</td>
<td>Santa Cruz, SC-21705</td>
<td>1:250</td>
</tr>
<tr>
<td>OCT3/4</td>
<td>Mouse</td>
<td>IgG2b</td>
<td>Santa Cruz, SC-5279</td>
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<tr>
<td>SOX1</td>
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<td>IgG</td>
<td>R&amp;D Systems, AF3369</td>
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</tr>
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<td>Mouse</td>
<td>IgG1</td>
<td>Millipore, MAB5326</td>
<td>1:1000</td>
</tr>
<tr>
<td>BRACHYURY</td>
<td>Goat</td>
<td>IgG</td>
<td>R&amp;D Systems, AF2085</td>
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<tr>
<td>EOMES</td>
<td>Rabbit</td>
<td>IgG</td>
<td>abcam, ab23345</td>
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<tr>
<td>FOXA2</td>
<td>Goat</td>
<td>IgG</td>
<td>R&amp;D Systems, AF2400</td>
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<td>GATA-4</td>
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<td>IgG2a</td>
<td>Santa Cruz, sc-25310</td>
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<tr>
<td>NFIA</td>
<td>Rabbit</td>
<td>IgG</td>
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<td>Mouse</td>
<td>IgM</td>
<td>Millipore, MAB1681</td>
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<tr>
<td>GFAP</td>
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<td>IgG</td>
<td>Dako, Z0334</td>
<td>1:500</td>
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<tr>
<td>GFAP</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Sigma, C9205</td>
<td>1:500</td>
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<tr>
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<td>Rabbit</td>
<td>IgG</td>
<td>Dako, Z0311</td>
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<tr>
<td>βIII-tubulin</td>
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<td>IgG2b</td>
<td>Sigma, T8660</td>
<td>1:1000</td>
</tr>
<tr>
<td>TDP-43</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Abnova, H00023435-M01</td>
<td>1:250</td>
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<tr>
<td>p62</td>
<td>Mouse</td>
<td>IgG1</td>
<td>BD Biosciences, 610832</td>
<td>1:250</td>
</tr>
</tbody>
</table>

Abbreviations: SRY box-1 (SOX1), eomesodermin homolog (EOMES), forkhead box protein A2 (FOXA2), GATA binding protein 4 (GATA-4), nuclear factor I/A (NFIA), S100 calcium-binding protein B (S100B), glial fibrillar acidic protein (GFAP), tubulin beta-3 chain (βIII-tubulin),
2.16 Imaging and image analysis

Fluorescence imaging was performed either by using an Axio Observer.Z1 (Carl Zeiss) epifluorescence microscope or by using a LSM710 confocal microscope (Carl Zeiss). At least six randomly selected fields from two coverslips were imaged for each condition. All images were blindly analysed using the ImageJ64 (v 1.47) software.

For the densitometric analysis of TDP-43 in subcellular compartments, confocal images were acquired at 63X magnification, and acquisition parameters were set using the range indicator to avoid saturation or clipping, which ensured the linearity of the signal. Subcellular TDP-43 signal intensity was analysed as follows. The DAPI channel was first converted to a thresholded binary mask to define the regions of interests (ROIs), which were used to measure nuclear TDP-43 intensity. Cytoplasmic TDP-43 signal intensity was measured by subtracting the nuclear TDP-43 signal from the TDP-43 channel, and the average signal intensity was normalised to the number of astrocytes in the field. Nuclear and cytoplasmic background signal was evaluated of a secondary only staining using the same method, and was subtracted from nuclear and cytoplasmic TDP-43 signal intensity readouts, respectively. At least 3 independent imaging of each cell line was conducted for the densitometric analysis, and for each imaging 6 randomly selected fields with similar cell density (~10 cells/field) were analysed to ensure comparability.

For the morphological assessment of MNs, images acquired at 20x magnification were used. Number of neurites projected from each MN was counted, and individual
neurites were traced manually using the segmented tracing tool in ImageJ to measure the length, which was used to calculate the longest neurite length and the total neurite length.

### 2.17 Western blotting

2-week old differentiated astrocytes were harvested using Accutase, and cell pellets were lysed in cold radioimmunoprecipitation assay (RIPA) buffer supplied with 1x protease inhibitor (Roche, 04693132001) and 1x phosphatase inhibitor (Roche, 04906837001). After incubation for 30 minutes on ice, lysate was centrifuged at 13000 rpm for 30 minutes at 4°C, and the supernatant was collected as the RIPA-soluble fraction. After a wash with RIPA buffer to minimize the carryover contamination from the soluble fraction, RIPA-insoluble pellets were dissolved in Urea buffer with 1x protease inhibitor and 1x phosphatase inhibitor of a volume in proportion to the soluble fraction. This was followed by sonication for further dissolving the protein. After centrifugation at 13000 rpm for 30 minutes at 4°C, the supernatant was collected as the RIPA-insoluble fraction.

Protein concentrations in the RIPA-soluble fraction were determined by a Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23227), and 10μg of protein was loaded in an 8-20% Precise™ Protein Gel (Thermo Scientific, 25200-25244) for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The amount of insoluble protein was adjusted based on Coomassie Brilliant Blue staining on a duplicate gel to ensure equal loading across samples. Separated proteins were then
transferred to an Immobilon-FL PVDF membrane (Millipore, IPFL00010) and blocked in Odyssey Blocking Buffer (LI-COR, 927-40000) for 1 hour at room temperature. Primary antibody was applied at 4°C overnight. After three washes with PBS supplemented with 0.1% TWEEN® 20 (Sigma, P9461), IRDye® Secondary Antibodies (LI-COR) were applied for 1 hour at room temperature followed by another 3 washes with PBS-Tween. Primary and secondary antibodies were diluted in Odyssey Blocking Buffer. Membranes were imaged using an Odyssey® Fc Imager (LI-COR), and images were processed and analysed using the Image Studio™ software (LI-COR). Semi-quantitative densitometric analysis was performed with β-Actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control for the RIPA-soluble fraction and the Coomassie Brilliant Blue staining as a loading control for the RIPA-insoluble fraction. Components of buffers are shown in Table 2.9 and Table 2.10. Primary antibodies used for Western blotting are shown in Table 2.11.
### Table 2.9 RIPA Buffer

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<tr>
<td>NaCl</td>
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<td>150mM</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma, T8787</td>
<td>1%</td>
</tr>
<tr>
<td>sodium deoxycholate</td>
<td>Sigma, D6750</td>
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</tr>
<tr>
<td>SDS</td>
<td>Sigma, L3771</td>
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</tr>
<tr>
<td>ethylenediaminetetraacetic acid (EDTA)</td>
<td>Sigma, E9884</td>
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### Table 2.10 Urea Buffer

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<td>Thiourea</td>
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<td>Trizma® base</td>
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<td>30mM</td>
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### Table 2.11 Primary antibodies for immunocytochemistry

<table>
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<th>Antibody</th>
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<th>Catalogue</th>
<th>Dilution</th>
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</tr>
<tr>
<td>p62</td>
<td>Mouse</td>
<td>BD Biosciences, 610832</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Mouse</td>
<td>Sigma, A2228</td>
<td>1:5000</td>
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<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>Calbiochem, CB1001</td>
<td>1:15000</td>
</tr>
</tbody>
</table>
2.18 Single cell longitudinal survival analysis

2-week old differentiated astrocytes were dissociated into single cells using Accutase and plated on Matrigel (1:80 diluted) coated 96-well plates at a density of $2.5 \times 10^4$ cells per well in CNTF medium. 4 days later, astrocytes were transfected with a construct encoding enhanced green fluorescent protein (eGFP), pGW1-eGFP, using the Lipofectamine® 2000 Transfection Reagent (Invitrogen, 11668019). This was performed using 0.5µL of Lipofectamine® 2000 Transfection Reagent with 0.5µg plasmid DNA per well following the manufacturer's instructions. 24 hours after transfection, live imaging was performed every 24 hours for 10 days using an Axio Observer.Z1 epifluorescence microscope at 37°C and 5% CO₂.

Images were analysed using the ImageJ software. Images were stacked together for each well, and individual astrocyte was blindly traced to determine the time of cell death, which is defined as disappearance of fluorescence or dissolution of cells. Kaplan-Meier survival analysis was performed using the R software with the cumulative hazard function, and hazard ratio (HR) was calculated using the Cox-proportional regression function. At least 3 independent experiments were performed for survival analysis, and for each experiment at least 6 wells of each cell line were imaged and analysed with an average number of 15 cells per well.

2.19 Cytotoxicity assay

2-week old differentiated astrocytes were dissociated into single cells using Accutase and plated on 96-well plates at a density of $2.5 \times 10^4$ cells per well. Cells were washed
once with Neurobasal® Medium prior to replacement with fresh CNTF medium with or without an autophagy inhibitor, chloroquine. 24 hours later, conditioned medium was collected to measure the concentration of lactate dehydrogenase (LDH) using a CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, G7891) following the manufacturer’s instructions. LDH release from untreated cells served as a negative control, whereas cells lysed by 0.1% Triton X (Promega, G7891) were used as positive control. Percentage of cytotoxicity was calculated by subtracting the negative control first and then normalised to the positive control. The resulting data was independent of cell number, and therefore could be compared across different conditions.

### 2.20 Data analysis

At least three independent derivations of astrocytes and MNs were used in each assay, and at least three independent experiments were conducted. All data are presented as mean ± SEM. For comparisons between control and C9orf72 mutant astrocytes, data of 2 control iPSC lines (CTRL1 & 2) and data of 3 patient iPSC lines (Carrier1, 2 & 3) were pooled together, respectively. Differences between means of two groups were analysed by two-sided Student’s t-test, whereas differences between means of three or more groups were analysed by one-way ANOVA with Bonferroni correction. Two-way ANOVA was performed where two independent factors were involved. Cox-proportional regression was performed for Kaplan-Meier survival analysis. Kolmogorov-Smirnov test was performed for morphology assessment of MNs. For all analyses, the null hypothesis was rejected at 0.05.
Chapter 3

Generation of astrocytes from iPSCs
3.1 Introduction

Modelling neurodegenerative disorders such as ALS has been a long-term challenge because of the difficulty in obtaining cellular materials from the human CNS. Although transgenic rodent models have been extensively generated, they typically only recapitulate certain aspects of ALS with significant variability in the behavioural and pathological phenotypes, as a result of difference in transgene expression methods, lack of crucial disease modifiers and species differences (reviewed in Chapter 1 Section 1.4.1, p.31).

The discovery of human iPSCs has opened a new avenue to model ALS in vitro, which allows generation of patient specific iPSCs and subsequent differentiation into disease relevant cell types with indefinite cell numbers. Recently, several studies have shown that patient iPSC-derived neurons recapitulate core pathological features of C9orf72-mediated ALS, such as C9orf72 haploinsufficiency, RNA foci and DPR proteins, and manifest vulnerability to glutamate mediated excitotoxicity and autophagy inhibition. However, the impact of carrying the C9orf72 mutation on human astrocytes has not yet been investigated. To address this question, iPSC lines were first established in the group. Skin fibroblasts were obtained from two healthy individuals (CTRL1 and CTRL2) and three ALS patients carrying the G4C2 repeat expansion in C9orf72 (Carrier1, Carrier2 and Carrier3) through collaborative work with Prof Ian Wilmut’s group at the University of Edinburgh, with Prof John Hardy’s group at University College London and with Prof Christopher Shaw’s group at King’s College of London. These fibroblasts were expanded and then reprogrammed by Judy Fletcher at the University of Edinburgh, Dr Selina Wray at
University College London and Dr Shyamanga Booroah at University of Cambridge using the conventional cocktail of four transcription factors (OCT3/4, SOX2, c-Myc, and KLF4) through Sendai virus- or retrovirus-mediated transduction. Colonies with pluripotent stem cell like morphology were manually selected and expanded to establish iPSC lines as shown in Figure 3.1, which was followed by detailed validation as described below. Demographic information of iPSC lines used in this study is listed in Table 3.1.

![Figure 3.1 Phase contrast images of iPSC colonies](image)

**Figure 3.1 Phase contrast images of iPSC colonies**

Representative phase contrast images of iPSC colonies of all iPSC lines used in this study. Typical pluripotent stem cell-like morphology could be observed and was used as a criterion for manual selection of iPSC colonies following reprogramming. (Scale bars, 200µm)
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Gender</th>
<th>Age at Biopsy / ALS Diagnosis</th>
<th>Reprogramming Method</th>
<th>Gene Editing Method</th>
<th>Reprogrammed Cell Line</th>
<th>Diagnosed Disease</th>
<th>Gender</th>
<th>Reprogrammed Cell Line</th>
<th>Diagnosed Disease</th>
<th>Diagnosis</th>
<th>Reprogrammed Cell Line</th>
<th>Diagnosed Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>BECKERS6</td>
<td>Female</td>
<td>39 / ~750 ALS / ALS ALS</td>
<td>Retrovirus</td>
<td>Sendai virus</td>
<td>Camb</td>
<td>Female</td>
<td>39</td>
<td>BECKERS6 Camb</td>
<td>ALS ALS</td>
<td>ALS</td>
<td>BECKERS6 Camb</td>
<td>ALS ALS</td>
</tr>
<tr>
<td>M2112</td>
<td>Male</td>
<td>67 / TBD / TBD / TBD</td>
<td>Retrovirus</td>
<td>Sendai virus</td>
<td>Camb</td>
<td>Female</td>
<td>39</td>
<td>M2112 Camb</td>
<td>ALS ALS</td>
<td>ALS</td>
<td>M2112 Camb</td>
<td>ALS ALS</td>
</tr>
<tr>
<td>DN194</td>
<td>Male</td>
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<td>Camb</td>
<td>Female</td>
<td>39</td>
<td>DN194 Camb</td>
<td>ALS ALS</td>
<td>ALS</td>
<td>DN194 Camb</td>
<td>ALS ALS</td>
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<tr>
<td>34DC</td>
<td>Female</td>
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<td>Sendai virus</td>
<td>Camb</td>
<td>Female</td>
<td>39</td>
<td>34DC Camb</td>
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<td>Healthy / Healthy / Healthy</td>
<td>Healthy</td>
<td>33D9 Camb</td>
<td>Healthy / Healthy / Healthy</td>
</tr>
</tbody>
</table>

Table 3.1: Demographical Information of iPSC Lines used in this study.
Following the establishment of iPSC lines, RP-PCR was conducted by Elaine M. Cleary in the group to examine the presence or absence of the G₄C₂ repeat expansion in iPSCs. RP-PCR involves a specifically designed reverse primer composed of several C₄G₂ repeats that can bind at different loci within the repeat expansion, thereby creating multiple amplicons of incrementally larger sizes. This technique has been widely used for diagnosing the C9orf72 mutation, thus was utilised as a genotyping method for established iPSC lines. As expected, the G₄C₂ repeat expansion (>30 repeats) was detected in all patient iPSC lines but absent (<30 repeats) in control iPSC lines (Figure 3.2). Hence, these data confirm that the genotypes are preserved in established iPSC lines after reprogramming.
Elaine M. Cleary conducted the RP-PCR and provided these representative results of all iPSC lines used in this study. Each peak represents amplicons of a particular size, and the interval between two peaks is always 6 nucleotides, corresponding to one G₄C₂ repeat. Thus, the longer the product is, the more G₄C₂ repeats it contains. The relative fluorescence unit (rfu) value represents the relative amount of each product. 30 repeats is the diagnostic cut off value (Normal, <30 repeats; Affected, >30 repeats). Both control and the gene edited iPSC lines only showed short products with less than 30 repeats, whereas mutant iPSC lines showed multiple peaks falling into the affected region corresponding to over 30 repeats. Hence, these data confirm the presence of G₄C₂ repeat expansion in patient iPSC lines and its absence in control and gene edited iPSC lines.
Heterogeneity of genetic backgrounds can have significant impacts on phenotypic differences between control and patient iPSC lines. In order to overcome this problem and to determine the causal effects of carrying the \textit{C9orf72} mutation, an isogenic control of a patient iPSC line was generated by Dr Bhuvaneish T. Selvaraj in the group using the CRISPR/Cas9-mediated genome editing. The Carrier3 iPSC line was selected, which has a heterozygous condition with 3 x G\textsubscript{4}C\textsubscript{2} repeats in the WT allele (analysed by Sanger sequencing) and approximately 750 x G\textsubscript{4}C\textsubscript{2} repeats in the mutant allele (analysed by Southern blotting) of \textit{C9orf72}. Two gRNAs flanking the G\textsubscript{4}C\textsubscript{2} repeat expansion were designed (Figure 3.3A), and one positive clone was identified by using RP-PCR to screen 164 individual iPSC clones for deletion of G\textsubscript{4}C\textsubscript{2} repeats (Figure 3.3B). Sanger sequencing of the G\textsubscript{4}C\textsubscript{2} locus in \textit{C9orf72} further confirmed this positive clone with complete deletion of the G\textsubscript{4}C\textsubscript{2} repeat expansion in the mutant allele and one remaining G\textsubscript{4}C\textsubscript{2} repeat in the WT allele (Figure 3.3B). This iPSC clone, named as Carrier3-ΔG\textsubscript{4}C\textsubscript{2} (Table 3.1), was expanded and subjected to detailed validation in the following section.
Figure 3.3 Generation of isogenic control iPSCs

Dr Bhuvaneish T. Selvaraj conducted the CRISPR/Cas9-mediated genome editing and provided this figure.

(A) The targeting strategy for excising the G₄C₂ repeat expansion from C9orf72 using the CRISPR/Cas9-mediated genome editing. Two gRNAs were designed flanking the G₄C₂ repeats to generate DSB (indicated by //).

(B) Sanger sequencing of the G₄C₂ locus confirmed excision of the G₄C₂ repeats in the mutant allele and 1 repeat remained in the WT allele.
To characterise the established iPSC lines and the isogenic control iPSC line, immunocytochemistry was performed by Dr Karen Burr to examine whether the established iPSCs possessed pluripotency, the fundamental characteristic of iPSCs. As shown in Figure 3.4A, iPSCs of all six lines displayed high expression levels of pluripotency markers, including NANOG, SOX2, podocalyxin (recognised by the TRA-1-60 antibody) and OCT3/4.

In addition to expression of various pluripotency makers, these iPSCs were functionally tested for pluripotency by *in vitro* three germ layer differentiation conducted by Dr Karen Burr. iPSCs were cultured in defined media to induce neuroectoderm, mesoderm and endoderm, respectively. Expression of germ layer specific markers was noted in all iPSC lines following differentiation (Figure 3.4B). These markers included SRY-box 1 (SOX1) and Nestin for the neuroectoderm, Brachyury and eomesodermin homolog (EOMES) for the mesoderm, and forkhead box protein A2 (FOXA2) and GATA binding protein 4 (GATA-4) for the endoderm. Altogether, these data confirm that the established iPSC lines are pluripotent, and that CRISPR/Cas9-mediated gene editing does not affect the pluripotency of iPSCs.
Figure 3.4 Pluripotency of iPSC lines

Dr Karen Burr conducted the immunocytochemistry and provided these images.

(A) Representative immunofluorescent images showed high expression of pluripotency markers (NANOG, SOX2, TRA-1-60 and OCT3/4) in all iPSC lines.

(B) Representative immunofluorescent images showed high expression of three germ layer specific markers in all iPSC lines upon differentiation. These markers included SOX1 and Nestin for the neuroectoderm, Brachyury and EOMES for the mesoderm, and FOXA2 and GATA-4 for the endoderm. (Scale bars, 50µm)
Next, karyotypes of all iPSC lines were examined using either the conventional Giemsa-banding (G-banding) or a molecular karyotyping assay (KaryoLite BoBs™ assay), conducted by TDL and PerkinElmer, respectively. The latter assay is based on the BACs-on-Beads™ technology which utilises BAC probes bound with fluorescently coded beads that can be distinguishable by the Luminex® instrument. The amount of targets bound to each bead type in each sample is compared with the amount of target bound in reference samples generating ratio plots, which indicate where a gain or a loss of genomic material has occurred. Compared to the conventional G-banding karyotyping, this method is less time-consuming and only requires small amounts of genomic DNA material. Figure 3.5 and Figure 3.6 show karyotype results using these two methods, which confirm normal karyotypes of all iPSC lines used in this study.

Figure 3.5 Karyotypes of iPSC lines (G-banding)
G-banding of four iPSC lines used in this study confirmed their normal karyotypes.
**Figure 3.6 Karyotypes of iPSC lines (KaryoLite BoBs™ assay)**

The KaryoLite BoBs™ assay conducted by PerkinElmer confirmed normal karyotypes of the rest two iPSC lines used in this study. Red bars are the normalised ratios compared to a female reference DNA sample. Blue bars are the normalised ratios compared to a male reference DNA sample.
Our lab has previously established a chemically defined protocol to generate highly pure (>90%) astrocytes from iPSCs by following the developmental principle that neurogenesis precedes gliogenesis\textsuperscript{149}. These iPSC-derived astrocytes also display abilities to uptake extracellular glutamate and to propagate calcium waves, the two fundamental functions of astrocytes in the normal CNS\textsuperscript{110,112,126}. Hence, I decided to apply this protocol to iPSC lines established in the group in order to obtain human astrocytes for investigating the impact of carrying the \textit{C9orf72} mutation on human astrocytes.

Hence, the aims of this chapter were to derive and characterise astrocytes from iPSC lines in order to establish an \textit{in vitro} platform for understanding the role of astrocytes in \textit{C9orf72}-mediated ALS.
3.2 Results

3.2.1 Derivation of astrocytes from iPSCs

Having characterised iPSCs, astrocytes were generated from all iPSC lines following a previously developed protocol\textsuperscript{149}, which contains two major stages—generation of MNs and astrocyte conversion (Figure 3.7A). iPSCs were first neuralised by dual-SMAD inhibition\textsuperscript{192}. The resulting early neural progenitor cells (NPCs) were cultured in suspension as neurospheres, and sequentially patterned through RA-mediated caudalisation and sonic hedgehog (SHH)-mediated centralization to generate MN precursors. Next, differentiation of MNs was initiated by removal of the mitogen FGF2 and addition of forskolin and neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF).

Astroglial conversion was achieved by application of LIF together with mechanical chopping to promote astrogliogenesis and eliminate post-mitotic neurons. This led to enriched populations of proliferative APCs in spheres, termed as astrospheres. At the end of conversion, astrospheres were maintained in EGF and FGF2 containing medium, and dissociated to obtain monolayers of APCs. APCs could be further expanded and differentiated into astrocytes by application of CNTF. Homogenous cell cultures with astrocyte like morphology were generated from all six iPSC lines (Figure 3.7B), and RP-PCR was used to ensure genotypic status of each line at the end of derivation (data not shown). In the following section, differentiated cells were further characterised to determine whether they were indeed astrocytes.
Figure 3.7 Derivation of astrocytes from iPSCs

(A) A schematic diagram of the protocol used in this study to derive astrocytes from iPSCs (adapted from Serio et al.\textsuperscript{149}, with modifications).

(B) Representative phase contrast images of differentiated cells generated from all six iPSC lines. They displayed homogenous astrocyte-like morphology without overt morphological alterations across different lines. (Scale bars, 50µm)
3.2.2 Characterisation of iPSC-derived astrocytes

Expression of astrocyte-specific markers

To characterise iPSC-derived astrocytes, immunostainings against well-known astrocyte lineage markers were first performed to examine the cell identities. As shown in Figure 3.8, iPSC-derived APCs showed high expression of NFIA and Vimentin, confirming their APC identities. After two weeks of differentiation from APCs to astrocytes, high expression levels of S100B and GFAP, two well-known astrocyte markers, were observed (Figure 3.9). The vast majority of non-astrocytes in the cultures showed neuron-like morphology and were positive for βIII-tubulin, a pan-neuronal marker (Figure 3.9).
Figure 3.8 Immunocytochemistry of iPSC-derived APCs

Representative images of immunostainings on APCs derived from all six iPSC lines, showing high expression levels of Vimentin and NFIA, two well-known APC markers. (Scale bars, 50µm)
Figure 3.9 Immunocytochemistry of iPSC-derived astrocytes

Representative images of immunostainings on astrocytes derived from all six iPSC lines. These showed high expression of S100B and GFAP, two well-known astrocyte markers, and minimal expression of βIII-tubulin, a pan-neuronal marker. (Scale bars, 50µm)
Quantification of the proportion of differentiated cells positive for each marker revealed that over 90% of differentiated cells were positive for astrocyte markers with less than 10% of cells positive for the neuronal marker (Figure 3.10, Table 3.2). These data confirm that highly pure populations of astrocytes are generated from all six iPSC lines. Importantly, no significant difference in cell identity quantification was found across different cell lines (Figure 3.10), indicating that the G_{4}C_{2} repeat expansion does not interfere with the astrocyte differentiation efficiency from iPSCs.
Figure 3.10 Quantification of immunocytochemistry of iPSCs-derived astrocytes

A bar graph shows the quantification of proportions of cells positive for S100B, GFAP and βIII-tubulin, confirming the high purity of astrocytes derived from all six iPSC lines. In addition, the astrocyte differentiation efficiency was comparable across all lines ($n \geq 3$, one-way ANOVA with Bonferroni correction, $p>0.05$).

Table 3.2 Quantification of immunocytochemistry of iPSCs-derived astrocytes

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<th>CTRL1</th>
<th>CTRL2</th>
<th>Carrier1</th>
<th>Carrier2</th>
<th>Carrier3</th>
<th>Carrier3-ΔG+C2</th>
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<td>S100B</td>
<td>96.8±0.9</td>
<td>90.2±1.2</td>
<td>90.6±2.7</td>
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<tr>
<td>GFAP</td>
<td>94.5±0.9</td>
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<td>90.2±1.4</td>
<td>92.3±1.4</td>
<td>90.6±1.7</td>
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<tr>
<td>βIII-tubulin</td>
<td>2.5±0.8</td>
<td>6.2±0.9</td>
<td>6.9±1.6</td>
<td>6.3±1.5</td>
<td>8.6±1.8</td>
<td>1.4±0.5</td>
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Functional characterisation—glutamate uptake assay

To determine function of iPSC-derived astrocytes, a glutamate uptake assay was next undertaken. 2-week old differentiated astrocytes were exposed to HBSS containing 100µM glutamate. At 30min, 60min and 120min post-application, the residual concentrations of glutamate in the supernatant were measured, and the uptake of glutamate was calculated by subtracting the remaining concentration of glutamate from the starting concentration—100µM. The resulting glutamate uptake was normalised to cell numbers. To validate the glutamate uptake assay, PDC, a pan-EAAT inhibitor\(^{193}\), was simultaneously applied to astrocytes for 120min as a negative control. As expected, iPSC-derived astrocytes were able to take up extracellular glutamate in a time dependent manner, and the clearance of glutamate was significantly blocked in the presence of PDC as compared to glutamate uptake at 120min (Figure 3.11). These data support the capability of iPSC-derived astrocytes to take up extracellular glutamate.
Figure 3.11 Glutamate uptake assays of iPSC-derived astrocytes

A glutamate uptake assay showed that astrocytes derived from all six iPSC lines could take up extracellular glutamate in a time-dependent manner (n≥3 for each cell line). In the presence of PDC, a pan-EAAT inhibitor, the glutamate uptake was significantly blocked in comparison to the 120’ data without PDC application.
In addition to the importance to normal functions of the CNS, the glutamate clearance by astrocytes is also pathologically relevant to ALS. Impaired glutamate transport and focal loss of EAAT2 has been found in ALS patient samples\textsuperscript{135,136} as well as in SOD1 rodent models\textsuperscript{39,40}, indicating that glutamate dependant excitotoxicity may play a role in the pathogenic mechanisms of ALS. Hence, data of glutamate uptake of iPSC-derived astrocytes were pooled together for comparisons. As shown in Figure 3.12, no difference was found between control and mutant lines, or between the isogenic pair, Carrier3 and Carrier3-ΔG\textsubscript{4}C\textsubscript{2}. These data demonstrate that carrying the G\textsubscript{4}C\textsubscript{2} repeat expansion in \textit{C9orf72} does not lead to loss of glutamate uptake ability in iPSC-derived astrocytes.
Figure 3.12 Comparison of the glutamate uptake ability in iPSC-derived astrocytes

(A) Pooled data of control and C9orf72 mutant astrocytes did not show significant difference in glutamate uptake. (two-way ANOVA with Bonferroni correction, p>0.05)

(B) Direct comparison between Carrier3 and Carrier3-ΔG4C2 astrocytes did not show significant difference in glutamate uptake. (two-way ANOVA with Bonferroni correction, p>0.05)
Functional characterisation—calcium imaging

Another important function of astrocytes is their ability to propagate calcium waves in response to neuronal activities\(^\text{126}\). Taking advantage that the astrocytic calcium waves can be triggered by stimulation \textit{in vitro}, calcium imaging was performed. 2-week old differentiated astrocytes were loaded with a fluorescent calcium indicator, fluor-4 AM\(^\text{194}\), and stimulated by dropping glass beads on top of the culture. Live imaging was performed using an epifluorescence microscope to record the change of intracellular calcium over time. In addition, simultaneous application of 2-APB, an inhibitor of IP\(_3\)-dependent calcium release\(^\text{195}\), was used as a negative control. As shown in Figure 3.13, in the absence of 2-APB, intracellular calcium levels were increased upon stimulation and propagated from the centre of the stimulus to the periphery over time. However, in the presence of 2-APB, the calcium waves could not be triggered by stimulations. No overt differences were observed either between control and mutant astrocytes, or between the isogenic pair. These data confirm the capability of iPSC-derived astrocytes to propagate calcium waves under stimulation, and that carrying the G\(_4\)C\(_2\) repeat expansion does not lead to impaired calcium wave propagation in iPSC-derived astrocytes.
Figure 3.13 Calcium imaging of iPSC-derived astrocytes

Representative serial images of calcium imaging. In the upper panel, iPSC-derived astrocytes displayed the ability to propagate calcium waves over time under mechanical stimulation (white arrow). Increase of intracellular calcium levels was propagated from the centre of stimulus to the periphery. This phenotype was blocked in the presence of 2-APB (lower panel), where no calcium waves were observed. (Scale bars, 50µm)
Collectively, using various characterisation assays, findings in this section demonstrate that highly pure astrocytes are derived from all six iPSC lines, and exhibit functions to take up extracellular glutamate as well as to propagate calcium waves under stimulation. Moreover, these data also indicate that harbouring the G_4C_2 repeat expansion in C9orf72 does not affect the differentiation efficiency or functional capacities of iPSC-derived astrocytes.
3.3 Discussion

This chapter demonstrate that highly enriched astrocytes are derived from healthy control, C9orf72 patient and isogenic control iPSC lines, and manifest functional properties as evidenced by clearance of extracellular glutamate and propagation of calcium waves upon stimulation, without overt alterations caused by the C9orf72 mutation.

The presence or absence of the G4C2 repeat expansion in iPSCs was assessed by RP-PCR, which is rapid and simple, and thereby suitable for routine genotyping. It is worth noting that this technique can detect a maximum of ~60 repeats, as longer products are highly difficult to be amplified through PCR reactions\(^\text{13}\). Southern blotting, instead, can be utilised for estimating the approximate number of G4C2 repeats in the mutant allele of C9orf72\(^\text{14}\). As the expansion size is a potential disease modifier\(^\text{60,63}\), which may affect the severity and duration of ALS, it would be interesting to evaluate the repeat number in each iPSC lines using Southern blotting. This will allow a correlation study between severity of *in vitro* phenotypes and the G4C2 repeat number. Additionally, genome instability is a well-known feature of repeat expansion-relevant mutations, and iPSC-derived cells provide a powerful tool to examine such characteristic *in vitro*. Various cell types of high purity can be derived from the same iPSC line, such as NPCs, MNs, APCs and astrocytes in the current study. Examination of the repeat size in these cell types can address whether the G4C2 repeats are unstable *in vitro*. Hence, defining the G4C2 repeat size via Southern blotting can be explored in the future.
Here, correction of the G\textsubscript{4}C\textsubscript{2} repeat expansion was achieved through targeted gene editing using the CRISPR/Cas9 system, which did not affect pluripotency of the resulting iPSCs or subsequent astrocyte differentiation efficiency. This recently established technique has provided a powerful tool to conduct targeted human genome editing with high specificity and efficiency, and yet been widely utilised in iPSC-based disease modelling. One challenge of using this technique is off-target events, leading to undesired mutagenesis. This may result in alteration in cellular functions, thereby impairing the examination of disease relevant phenotypes. Recent studies have suggested that such events are relatively rare\textsuperscript{196}. Nevertheless, it is still worth to examine potential off-target events in the isogenic control iPSC line, which can be explored in the future studies.

Using the protocol previously established in the group, highly pure functional astrocytes were generated from all six iPSC lines, strongly supporting the wide applicability of this protocol. Since the discovery of human iPSCs, several groups have developed protocols for astrocyte derivation, which share many similarities in terms of neuronalisation methods, enrichment of astrocytes following neurogenesis and total generation time\textsuperscript{169,197-200}. Additionally, the purity and functional capacities of resulting cultures are comparable, as similar developmental principles and molecular pathways are targeted in these protocols. Recently, an alternative pathway to generate astrocytes has been developed, which is direct conversion from adult somatic cells with\textsuperscript{201} or without\textsuperscript{202} an intermediate state of NPCs. These protocols significantly shorten the time required for generation of astrocytes to 3-4 weeks. However, the efficiency of generating astrocytes is as low as ~2% through direct
conversion from human fibroblasts\textsuperscript{202}. Although astrocytes derived from induced NPCs appeared to be much more enriched, the efficiency was not clearly stated in the report and direct conversion from human fibroblast into NPCs showed variability across different iPSC lines\textsuperscript{201}. Moreover, serum was applied for inducing astrocyte differentiation\textsuperscript{201}, in contrast to chemically defined media utilised in the current study. Serum is known to have high variation across batches, and it contains multiple unknown factors that can potentially affect astrocyte behaviours, which are hard to be specified. Additionally, serum-containing medium restricts the potential use of derived astrocytes for cell replacement therapies due to the risk of transmitting animal pathogens. Furthermore, these protocols are lack of patterning procedures, leading to undefined regional identities of the resulting astrocytes. Given that ALS has regional specificity, using these astrocytes may not fully mimic the clinical features of ALS. Therefore, despite the long generation time of the current protocol, it has been chosen to derive astrocytes from iPSCs \textit{in vitro}.

Notably, the purity of iPSC-derived astrocytes was comparable across all lines, indicating that the \textit{C9orf72} mutation does not affect the generation efficiency of astrocytes. Moreover, the glutamate uptake function is not impaired by carrying the \textit{C9orf72} mutation in iPSC-derived astrocytes. It is worth noting that presence of GlutaMax and NEAA in the astrocyte culture medium, which contains high concentrations of glutamate, may saturate its transporters and consequently affect the ability of astrocytes to clear up glutamate. Hence, culturing astrocytes in the minimum medium prior to the glutamate uptake assay may allow identification of subtle but disease relevant difference in their glutamate uptake function. In addition,
examination of the expression levels and alternative splicing of EAAT2 still remains to be addressed, as such features have been proposed as pathogenic contributors to ALS\textsuperscript{203}.

Propagation of calcium waves was observed in astrocytes derived from all six iPSC lines, providing another convincing evidence for functional capacities of astrocytes. The importance of astrocytic calcium waves not only is because of its involvement in normal astrocyte communications, but also, and more importantly, because of its potential pathogenic contributions to neurodegenerative disorders. Specifically, increased activity of astrocytes has been proposed to play a role in dysfunction of synaptic activities in the Alzheimer’s disease\textsuperscript{204}. Such impairment has yet to be observed in ALS, but remains as an important target, particularly given that FTD is associated with ALS through the \textit{C9orf72} mutation\textsuperscript{35}. Therefore, it would be interesting to compare the activity of astrocytes as a consequence of carrying the G\textsubscript{4}C\textsubscript{2} repeat expansion. This question cannot be addressed using the current setting of calcium imaging in this chapter, which is a qualitative assessment. Controlled mechanical stimulation by micromanipulator or chemical stimulation applied by a computer regulated perfusion system is required to perform quantitative calcium imaging, and these settings were not available in the lab. Nevertheless, future studies can pursue achieving these to measure the speed of calcium propagation and magnitude of intracellular calcium rise, which will enable to address the role of calcium signalling in \textit{C9orf72}-mediated ALS/FTD.
Altogether, findings in this chapter show that an *in vitro* cellular platform has been established to model *C9orf72*-mediated ALS with a primary focus on astrocytes. This allows further investigation of astrocyte pathology (Chapter 4) and the impacts of the *C9orf72* mutation on both cell autonomy (Chapter 5) and non-cell autonomy (Chapter 6) of astrocytes derived from human iPSCs.
Chapter 4

*In vitro* human astrocyte pathology of C9orf72-mediated ALS
4.1 Introduction

Pathologically, ALS is characterised by intracellular and/or intranuclear UPIs, and the primary component of these inclusions is TDP-43\(^{25,28}\). Of note, protein inclusions that are negative for TDP-43 but positive for p62 are commonly found in the cerebral cortex, hippocampus and cerebellum of patients carrying the \(C9orf72\) mutation\(^{76,77}\). In addition, there are three other pathological changes that are specific to \(C9orf72\)-mediated ALS, including down-regulation of \(C9orf72\), repeat RNA foci and DPR products\(^{63}\), which have been widely described in patient tissues, \textit{in vitro} and \textit{in vivo} models (as previously reviewed in Chapter 1 Section 1.2.3, p.15).

Although these pathological changes are predominantly observed in MNs, detection of these pathologies in glial cells, such as astrocytes, is not rare. For example, both TDP-43 protein inclusions and the \(G_{4}C_{2}\) repeat RNA foci have been reported in astrocytes in affected brain regions of ALS patients\(^{25,90}\), suggesting that not only MNs but also astrocytes are affected by the mutation. Several studies have shown that patient iPSC-derived neurons carrying the \(G_{4}C_{2}\) repeat expansion recapitulate pathological features of \(C9orf72\)-mediated ALS\(^{87,96,190}\). However, no studies have performed detailed pathological characterisation of astrocytes derived from \(C9orf72\) patient iPSCs. Following the work presented in Chapter 3, I sought to determine whether patient iPSC-derived astrocytes displayed any of the pathological features associated with the \(C9orf72\) mutation. Addressing this question would enable the determination of whether the patient iPSC-derived astrocytes were a valid \textit{in vitro} model of \(C9orf72\)-mediated ALS.
Hence, the aim of this chapter was to investigate whether patient iPSC-derived astrocytes recapitulated key pathological changes associated with $C9orf72$-mediated ALS, including TDP-43 proteinopathies, $C9orf72$ haploinsufficiency, repeat RNA foci, DPR proteins and p62 inclusions.
4.2 Results

4.2.1 Examination of TDP-43 proteinopathies in C9orf72 mutant astrocytes

Our group previously have shown that iPSC-derived astrocytes carrying an M337V mutation in TARDBP displayed increased cellular TDP-43\textsuperscript{149}. To address whether patient iPSC-derived astrocytes carrying the C9orf72 mutation recapitulated features of TDP-43 proteinopathies \textit{in vitro}, immunostaining against TDP-43 using a carboxyl-terminal antibody was first performed on 2-week old astrocytes. As shown in Figure 4.1, TDP-43 was predominantly located in nuclei of astrocytes with minimal expression in the cytoplasm, resembling normal localisation of TDP-43\textsuperscript{29}. No apparent aggregates or inclusions of TDP-43 were observed in C9orf72 mutant astrocytes.
Figure 4.1 Immunocytochemistry of TDP-43 in iPSC-derived astrocytes

Representative confocal images of TDP-43 immunostaining in iPSC-derived astrocytes. TDP-43 showed predominant nuclear staining with moderate signals in the cytoplasm of astrocytes derived from all lines. No apparent loss of nuclear TDP-43 or mislocalisation of TDP-43 into the cytoplasm was observed in mutant astrocytes, nor did I detect aggregates or inclusions of TDP-43 in astrocytes derived from patient iPSC lines. (Scale bars, 10µm)
Next, to further characterise the subcellular localisation of TDP-43 in astrocytes, densitometric analysis of TDP-43 immunostaining was performed using confocal images. To exclude the influence of background fluorescent signals, a negative control of immunocytochemistry was performed by applying the Alexa Fluor® 555-conjugated secondary antibody only, without application of the primary TDP-43 antibody. Cytoplasmic and nuclear density of the 555 channel was measured both in the negative control and in the TDP-43 immunostaining.

As shown in Figure 4.2A & B, both cytoplasmic and nuclear background signals were detectable and were also found to be variable across different cell lines and imaging experiments. Hence, the density of backgrounds was subtracted from corresponding nuclear or cytoplasmic densities of TDP-43 staining in order to accurately assess the subcellular distribution. As shown in Figure 4.2C & D, no significant differences of cytoplasmic or nuclear TDP-43 levels were observed in C9orf72 mutant astrocytes compared to controls using pooled data, suggesting a lack of mislocalisation of TDP-43 in mutant astrocytes. Similarly, the densities of cytoplasmic and nuclear TDP-43 were comparable between Carrier3 astrocytes and Carrier3-ΔG4C2 astrocytes (Figure 4.2C & D), further supporting that the subcellular localisation of TDP-43 is not affected by harbouring the C9orf72 mutation in astrocytes.
Figure 4.2 Densitometric analysis of subcellular TDP-43 in iPSC-derived astrocytes

(A-B) Densitometric analysis was first performed on confocal images of astrocytes stained only with the Alexa Fluor® 555 conjugated secondary antibody to determine the fluorescence background. Cytoplasmic (A) and nuclear (B) fluorescence intensities were plotted for astrocytes derived from individual iPSC lines, which showed variation across different cell lines and experiments (error bars).

(C-D) Densitometric analysis was performed on confocal images of astrocytes stained with TDP-43. Background signals measured in (A) and (B) were subtracted first in each experiment. The cytoplasmic intensity of TDP-43 (C) was much lower than its nuclear intensity (D), supporting predominant nuclear localisation of TDP-43. No significant difference of cytoplasmic or nuclear TDP-43 was detected between control and mutant astrocytes using pooled data (n≥3, Student’s t-test, ns p>0.05). Similarly, the comparison between Carrier3 astrocytes and Carrier3-ΔG4C2 astrocytes did not show a significant difference in subcellular TDP-43 intensity either (n≥3, Student’s t-test, ns p>0.05).
To complement the immunocytochemistry findings of TDP-43 localisation, biochemical analysis was performed to further assess TDP-43 proteinopathies in iPSC-derived astrocytes. Western blotting of TDP-43 was performed using both RIPA soluble and RIPA insoluble but urea soluble protein fractions isolated from 2-week old astrocytes (Figure 4.3A & C). In both soluble and insoluble fractions, no significant changes of TDP-43 protein levels were detected between control and mutant astrocytes, and the comparison between Carrier3 astrocytes and Carrier3-ΔG4C2 astrocytes did not reveal any differences either (Figure 4.3B & D). Moreover, protein bands corresponding to hyper-phosphorylated or truncated products of TDP-43 (~25kD) were not detected in the mutant astrocytes in the insoluble fraction (Figure 4.3C).

Together with the findings of TDP-43 immunostaining, these data indicate that harbouring the C9orf72 mutation does not lead to changes in subcellular distribution, protein levels, solubility or post-translational modifications of TDP-43 in astrocytes.
Figure 4.3 Western blotting of TDP-43 in iPSC-derived astrocytes

(A) A representative Western blot of TDP-43 in the RIPA soluble protein fraction isolated from 2-week old astrocytes. GAPDH was used as a loading control.

(B) Quantification of soluble TDP-43 levels normalised to GAPDH levels showed no significant difference between control and mutant astrocytes using pooled data, or between Carrier3 and Carrier3-ΔG4C2 astrocytes (n≥3, Student’s t-test, ns p>0.05).

(C) A representative Western blot of TDP-43 in the insoluble protein fraction isolated from 2-week old astrocytes. Protein bands corresponding to hyper-phosphorylated or truncated products of TDP-43 were not observed. Coomassie staining was used as a loading control.

(D) Quantification of insoluble TDP-43 levels showed no significant difference between control and mutant astrocytes using pooled data, or between Carrier3 and Carrier3-ΔG4C2 astrocytes (n≥3, Student’s t-test, ns p>0.05).
4.2.2 Examination of \textit{C9orf72} expression in iPSC-derived astrocytes

Having found a lack of TDP-43 proteinopathies in patient iPSC-derived astrocytes, other pathological features specific to \textit{C9orf72}-mediated ALS were next investigated. Given that the \textit{C9orf72} expression is differentially regulated in the CNS, with predominant expression in neurons and moderate expression in astrocytes\textsuperscript{73,74}, it is possible that the down regulation of \textit{C9orf72} found in patient tissues is a result of neuron loss combined with astrogliosis. In addition, although down-regulation of total \textit{C9orf72} transcripts was found in patient iPSC-derived neurons\textsuperscript{87}, another study did not observe such change using a similar iPSC-based neuronal model\textsuperscript{96}. Thus, it is important to examine the \textit{C9orf72} expression in individual cell types separately in order to determine whether harbouring the G\textsubscript{4}C\textsubscript{2} repeat expansion leads to haploinsufficiency of \textit{C9orf72}.

To achieve this, qRT-PCR was conducted to measure the transcript levels of \textit{C9orf72} in iPSC-derived astrocytes. One primer set, located at the mRNA region shared with all three variants, was utilised in order to evaluate the total transcript levels of \textit{C9orf72}. Additionally, three variant specific primer sets were utilised to assess each individual transcript variant levels. The locations of the primer sets were illustrated in Figure 4.4.
As noted previously, \( C9orf72 \) has much higher expression in neurons compared to astrocytes\(^{73,74} \). Therefore, cortical neurons (~95% purity) derived from a control human ESC line were used as a positive control for the \( C9orf72 \) transcript levels. QRT-PCR was performed on 2-week old differentiated astrocytes and 5-week old differentiated cortical neurons (n≥3 for each cell line).

As for the total \( C9orf72 \) transcripts, ESC-derived cortical neurons showed significantly higher levels compared to iPSC-derived astrocytes (Figure 4.5). Next, comparison of total transcript levels of \( C9orf72 \) between 2 control lines and 3 mutant lines was undertaken, and total \( C9orf72 \) transcript levels remained unchanged (Figure 4.5). However, by directly comparing Carrier3 astrocytes with Carrier3-\( \Delta G_4C_2 \) astrocytes, a significant 2-fold increase of total \( C9orf72 \) transcripts was detected after removal of the \( G_4C_2 \) repeat expansion (Figure 4.5). With moderate expression of \( C9orf72 \) in astrocytes, such change can easily be masked by genetic variation.

**Figure 4.4 C9orf72 transcript variants and locations of qRT-PCR primers**
A schematic diagram of three transcript variants of \( C9orf72 \). Locations of the primer set for total \( C9orf72 \) transcripts are illustrated by red arrows, and black arrows indicate the locations of variant specific primer sets.
background variation across different iPSC lines, and therefore may explain why reduction of the total C9orf72 transcript levels was not observed in mutant astrocytes using pooled data.

Figure 4.5 Total transcript levels of C9orf72 in iPSC-derived astrocytes and in ESC-derived cortical neurons

Total transcript levels of C9orf72 in cortical neurons (cNeuron) derived from a control human ESC line and in astrocytes derived from control, C9orf72 mutant and the gene edited iPSC lines. Cortical neurons had much higher levels of total C9orf72 transcripts compared to astrocytes derived from all six iPSC lines. No significant change was detected between control and mutant astrocytes (n≥3, one-way ANOVA with Bonferroni correction, ns p>0.05). However, a significant reduction was found in Carrier3 astrocytes compared to its isogenic control, Carrier3-ΔG4C2 astrocytes (n≥3, Student’s t-test, # p<0.05).
As expected, ESC-derived cortical neurons again showed significantly higher levels of all three transcript variants compared to iPSC-derived astrocytes (Figure 4.6A, B & C). Similar to the total C9orf72 transcript levels, two control lines and three mutant lines were compared individually using one-way ANOVA. As shown in Figure 4.6, although significant changes of each variant were detected in some mutant lines, these changes were not consistent across all mutant lines. These data suggest that the three transcript variants are differentially regulated in astrocytes carrying the G₄C₂ repeat expansion.

In addition, individual variant levels were compared between the isogenic pair, Carrier3 and Carrier3-ΔG₄C₂ (Figure 4.6A, B & C). Notably, significant reductions of variant 1 and variant 2 were detected in Carrier3 mutant astrocytes compared to its isogenic control line, but no change in variant 3 expression was found. Given that expression levels of each individual variant was very low in astrocytes (Ct value was over 32), it is possible that these differences may be due to clonal variation instead of carrying the G₄C₂ repeat expansion.

Collectively, data in this section suggest that expression of the C9orf72 is low in astrocytes compared to cortical neurons, and that the effect of the G₄C₂ repeat expansion on C9orf72 expression is inconclusive.
Levels of C9orf72 transcript variant 1 (A), variant 2 (B) and variant 3 (C) in cortical neurons derived from a control human ESC line and in astrocytes derived from control, C9orf72 mutant and the gene edited iPSC lines. Cortical neurons had much higher levels of all three transcript variants compared to astrocytes derived from all six iPSC lines. One-way ANOVA was used to compare 3 mutant lines with 2 control lines individually (Compared to CTRL1, ** p<0.01, *** p<0.001, with Bonferroni correction; Compared to CTRL2, ++ p<0.01, with Bonferroni correction). Student’s t-test was used to compare Carrier3 with Carrier3-ΔG4C2 (ns, p>0.05, # p<0.05, ## p<0.01)
4.2.3 Formation of G₄C₂ repeat RNA foci in C9orf72 mutant astrocytes

The second pathological change related to C9orf72-mediated ALS is the formation of G₄C₂ repeat RNA foci, which can sequester RBPs potentially leading to their dysfunction in cells. In recent studies using iPSC-derived neurons as in vitro models of ALS, G₄C₂ repeat RNA foci have been reported in C9orf72 mutant neurons. No study has yet reported the presence of RNA foci in patient iPSC-derived astrocytes. Therefore, I decided to ask whether harbouring the C9orf72 mutation leads to formation of G₄C₂ repeat RNA foci in astrocytes.

In order to achieve this, RNA FISH was performed with an anti-sense probe, (GGCCCC)₄, targeting the G₄C₂ repeats to examine the presence of RNA foci in 2-week old differentiated astrocytes. As expected, nuclear RNA foci were detected in mutant astrocytes, but were absent in control astrocytes (Figure 4.7A). Importantly, this pathological change was reversed by removal of the repeat expansion, as nuclear RNA foci disappeared in Carrier3-ΔG₄C₂ astrocytes (Figure 4.7A). In order to validate these findings, DNase and RNase treatments of the samples were also performed prior to application of the (GGCCCC)₄ probe. Detection of nuclear RNA foci in mutant astrocytes was not affected by the DNase treatment, but foci were no longer detectable following the RNase treatment (Figure 4.7B). These findings support that the nuclear foci are indeed RNA foci. Additionally, to assess the specificity of the (GGCCCC)₄ probe, an anti-sense RNA probe against the CCTG repeat expansion, the mutation associated with myotonic DM2, was applied in RNA FISH on astrocytes. In this scenario, no RNA foci could be detected in mutant...
astrocytes (Figure 4.7B), supporting the specificity of the (GGCCCC)$_4$ RNA probe. Altogether, these findings confirm that the nuclear foci are bona fide G$_4$C$_2$ repeat RNA foci.

Quantification of the proportion of astrocytes positive for RNA foci as well as the average number of foci per cell revealed that RNA foci were variable across different patient iPSC lines, ranging from ~20% to ~60% (Figure 4.7C, Carrier1 29.6±7.8, Carrier2 19.5±4.8%, Carrier3 59.8±2.6%). Interestingly, the average number of G$_4$C$_2$ repeat RNA foci was comparable across astrocytes derived from the three patient iPSC lines (Figure 4.7D, Carrier1 2.1±0.3, Carrier2 2.3±0.1, Carrier 3 1.9±0.2). Notably, no foci were detected in control astrocytes, and the nuclear RNA foci completely disappeared in the gene edited line Carreir3-ΔG$_4$C$_2$.

Collectively, findings in this section demonstrate that carrying the C9orf72 mutation leads to formation of nuclear G$_4$C$_2$ repeat RNA foci in iPSC-derived astrocytes.
Figure 4.7 Examination of \( G_4C_2 \) repeat RNA foci in iPSC-derived astrocytes

(A) Using RNA FISH with an antisense probe against \( G_4C_2 \) repeat expansion, nuclear RNA foci (arrow heads) were detected in mutant astrocytes but not in control astrocytes. These foci disappeared after removal of the \( G_4C_2 \) repeat expansion in Carrier3-\( \Delta G_4C_2 \). (Cells were co-stained with an astrocyte marker GFAP; the lower panel shows respective zoom in images of areas indicated by squares in the upper panel; scale bars, 10\( \mu \)m in the upper panel, 2.5 \( \mu \)m in the lower panel)

(B) Representative images of RNA FISH in mutant astrocytes with DNase or RNase treatment prior to application of the antisense probe against \( G_4C_2 \) repeat expansion. Additionally, a probe against the CCTG tetranucleotide repeat expansion, causative
of DM2, was also applied as a negative control. Nuclear RNA foci found in mutant astrocytes could only be detected in DNase treatment, which are indicated by arrow heads. (Scale bars, 5µm)

(C) Quantification of RNA FISH showed variable proportions of astrocytes positive for nuclear RNA foci across different mutant lines (n≥3, Carrier1 29.6±7.8, Carrier2 19.5±4.8%, Carrier3 59.8±2.6%). No nuclear RNA foci were detected in control or gene edited astrocytes.

(D) Quantification of RNA FISH showed approximate 2 foci per cell on average in mutant astrocytes (n≥3, Carrier1 2.1±0.3, Carrier2 2.3±0.1, Carrier 3 1.9±0.2).
4.2.4 RAN translation in C9orf72 mutant astrocytes

In addition to the formation of nuclear RNA foci, another pathological feature observed in C9orf72 patient post-mortem tissues is DPR proteins produced through the RAN translation of all possible reading frames of the G₄C₂ repeat expansion⁸³,⁹². This unconventional translation is believed to be bidirectional, leading to five possible DPR proteins, poly-GA, poly-GP, poly GR, poly-PA and poly-PR⁹³. Although post-mortem studies have suggested that these DPRs are neuron specific⁹², I decided to ask whether iPSC-derived astrocytes produced any DPRs as a result of carrying the C9orf72 mutation.

A commercially available antibody against the poly-GP was utilised to assess the RAN translation in iPSC-derived astrocytes. First, immunocytochemistry was performed on 2-week old differentiated astrocytes. As shown in Figure 4.8, nuclear and cytoplasmic staining of poly-GP was detectable in astrocytes derived from all six iPSC lines, and cytoplasmic aggregates/puncta of poly-GP were observed in both control and mutant astrocytes. Hence, these aggregates are unlikely to be C9orf72-specific poly-GP products. These findings indicate that this antibody unspecifically binds to other proteins, thereby it is difficult to confirm whether harbouring the G₄C₂ repeat expansion leads to production of poly-GP in astrocytes through immunocytochemistry.
Figure 4.8 Immunocytochemistry of poly-GP in iPSC-derived astrocytes

Representative images of immunostaining against poly-GP DPR proteins in 2-week old astrocytes derived from control, C9orf72 mutant and gene edited iPSC lines. Nuclear and cytoplasmic fluorescent signals can be detected in all lines, without apparent difference between control and mutant astrocytes. Potential cytoplasmic aggregates (indicated by arrows) were observed in all lines but not specific to mutant astrocytes. (Scale bars, 25μm)
Next, Western blotting for poly-GP was performed to resolve all proteins detected by this antibody. Urea-soluble protein fractions isolated from 2-week old iPSC-derived astrocytes were used. As shown in Figure 4.9, a band at ~60kD (indicated by red arrowheads) was only detected in mutant astrocytes but not in control astrocytes, and this observed band showed much stronger intensity compared to other unspecific bands. Importantly, this band detected in Carrier3 astrocytes was absent in its isogenic control, Carrier3-ΔG₄C₂ astrocytes, suggesting that it is indeed a poly-GP protein produced as a result of carrying the G₄C₂ repeat expansion. Hence, results in this section provides evidence that the DPR products generated through RAN translation are also present in astrocytes.
Figure 4.9 Western blotting of poly-GP DPR in iPSC-derived astrocytes

A representative Western blot of poly-GP in the urea-soluble fraction isolated from 2-week old astrocytes. Several bands of different molecular weights were detected, and many of them were present in all cell lines, indicative of unspecific binding. A band at ~60kD (indicated by red arrow heads) was only present in mutant astrocytes, likely to be poly-GP DPR. Importantly, this strong band detected in the Carrier3 astrocytes was absent in its isogenic control, Carrier3-ΔG₄C₂.
4.2.5 Investigation of p62 pathology in *C9orf72* mutant astrocytes

In addition to TDP-43 protein inclusions, *C9orf72*-mediated ALS is also characterised by TDP-43 negative but p62 positive inclusions, as evidenced by studies in patient tissues\(^{76,77}\). Using iPSC-derive neurons, one study found increased p62 protein levels in neurons carrying the *C9orf72* mutation\(^{190}\). However, it is not clear whether this pathological feature is relevant to astrocytes. Thus, I utilised the highly pure astrocyte cultures to address by immunocytochemistry whether harbouring the *C9orf72* mutation leads to p62 pathology in human astrocytes. Treatment with Chloroquine, an autophagy inhibitor, for 24 hours robustly increased the expression of p62 in astrocytes with p62 predominantly located in the cytoplasm as puncta (Figure 4.10). This condition was therefore used as a positive control.
Figure 4.10 Immunocytochemistry of p62 in iPSC-derived astrocytes under autophagy inhibition

Representative images of immunostaining against p62 in astrocytes derived from one control, one C9orf72 mutant and the gene edited iPSC lines under Chloroquine-mediated autophagy inhibition. Upon treatment with 100µM Chloroquine for 24 hours, robust p62 expression was detected in the cytoplasm of astrocytes with no nuclear expression. Cytoplasmic p62 was present as numerous puncta. (Scale bars, 25µm)
Under basal culture conditions (Figure 4.11), immunostaining against p62 in iPSC-derived astrocytes showed moderate expression regardless of their genotypes, and sparse p62 puncta were observed in both nucleus and cytoplasm. However, variation of p62 expression was observed across cells, as some cells showed relatively higher p62 levels compared to others in the same field. Additionally, no apparent aggregates or inclusions of p62 were observed in C9orf72 mutant astrocytes. These findings indicate that harbouring the C9orf72 in astrocytes does not lead to formation of p62 protein inclusions.
Figure 4.11 Immunocytochemistry of p62 in iPSC-derived astrocytes under basal culture conditions

Representative images of immunostaining against p62 in astrocytes derived from control, C9orf72 mutant and the gene edited iPSC lines under basal culture conditions. Moderate staining of p62 was detectable in both nucleus and cytoplasm, and sparse p62 puncta were present in both subcellular locations. Notably, the p62 expression was various across cells, as some cells showed relatively higher p62 levels compared to others in the same field. Moreover, no apparent aggregates or inclusions of p62 were observed in C9orf72 mutant astrocytes. (Scale bars, 25µm)
Next, Western blotting was performed to measure the total protein levels of p62 in iPSC-derived astrocytes. As shown in Figure 4.12A, p62 could be detected in RIPA-soluble protein fractions from all six iPSC lines-derived astrocytes, and its levels were measured relative to β-actin, a loading control. Firstly, data of astrocytes derived from two control iPSC lines and data of astrocytes derived from three patient iPSC lines were pooled together, respectively. By comparing the data between these two groups, a moderate but significant increase of p62 protein levels was found in C9orf72 mutant astrocytes (Figure 4.12B). However, when comparing astrocytes derived from the isogenic pair iPSC lines, this increased p62 levels were not reversed after removal of the G₄C₂ repeat expansion (Figure 4.12B), suggesting that the moderate ~0.2-fold increase of p62 in mutant astrocytes may be accounted by variation of p62 expression across different lines.

Collectively, data in this section demonstrate that harbouring the G₄C₂ repeat expansion in astrocytes does not lead to formation of protein inclusions or increased protein levels of p62.
Figure 4.12 Western blotting of p62 in iPSC-derived astrocytes

(A) A representative Western blot of p62 in RIPA-soluble protein fractions isolated from iPSC-derived astrocytes. β-actin was used as a loading control.

(B) Relative protein levels of p62 across different cell lines. A significant increase of p62 was found in mutant astrocytes compared to controls (n≥3, Student’s t-test, ** p<0.01). No difference was found between Carrier3 astrocytes and Carrier3-ΔG4C2 astrocytes controls (n≥3, Student’s t-test, ns p<0.01).
4.3 Discussion

Results in this chapter demonstrate that patient iPSC-derived astrocytes recapitulate key pathological features of C9orf72-mediated ALS. Specifically, harbouring the G_4C_2 repeat expansion leads to down-regulation of variant 1, variant 2 and total transcript levels of C9orf72, formation of nuclear G_4C_2 repeat RNA foci and production of poly-GP DPR proteins in astrocytes. These findings support the use of patient iPSC-derived astrocytes as a valid in vitro model of aspects of C9orf72-mediated ALS.

Although TDP-43 proteinopathies are commonly observed in ALS patients with the C9orf72 mutation, pathological changes of TDP-43 were not observed in patient iPSC-derived astrocytes carrying the G_4C_2 repeat expansion. Previously, our group have reported that iPSC-derived astrocytes carrying an M337V mutation in TARDBP have increased levels of cytoplasmic TDP-43 and higher amount of soluble TDP-43 protein compared to controls^{149}. Given that ALS-relevant mutations in TARDBP can accelerate aggregation of TDP-43^{205}, this may account for the difference in TDP-43 status in astrocytes between these two studies.

The finding that iPSC-derived astrocytes have significantly lower transcript levels of C9orf72 compared to ESC-derived cortical neurons confirms that C9orf72 is differentially regulated in different cell types in the CNS. Importantly, harbouring the G_4C_2 repeat expansion causes down-regulation of variant 1, variant 2 and total transcript levels of C9orf72 in astrocytes, supporting the notion of haploinsufficiency of C9orf72 in ALS. Although many studies have reported reduction of total C9orf72
transcripts, few of them investigated regulation of individual transcripts. Moreover, decreased transcript levels observed in post-mortem tissues could arise due to the loss of MNs and increase of astrocytes as a result of astrogliosis. Here, data not only show that haploinsufficiency is indeed caused by harbouring the C9orf72 mutation in astrocytes, but also demonstrate that the three transcript variants are differentially regulated. Similarly, one study has also found increased transcript variant 3 levels in patient iPSC-derived neurons\textsuperscript{96}, which is consistent with the finding here using the pooled data of all three mutant lines. Although removal of the G\textsubscript{4}C\textsubscript{2} repeat expansion in one mutant line did not reverse this phenotype, it remains interesting to study the variant 3 levels in isogenic controls of the other two mutant lines. This will allow the determination whether this phenotype is indeed not pathologically relevant to ALS but is an artefact due to variation across different cell lines. In addition, further studies are required to compare C9orf72 transcript levels between iPSC-derived MNs and astrocytes, as the astrocytes used in this study have been patterned as spinal astrocytes.

Although astrocytes have low expression of C9orf72, abundant nuclear G\textsubscript{4}C\textsubscript{2} repeat RNA foci were still detectable in mutant astrocytes, the formation of which was reversed by removal of the G\textsubscript{4}C\textsubscript{2} repeat expansion. This finding is in concordance with a study which has found G\textsubscript{4}C\textsubscript{2} repeat RNA foci in various cell types in patient tissues, including astrocytes\textsuperscript{90}. Several reports have identified multiple RBPs that bind to the G\textsubscript{4}C\textsubscript{2} repeat expansion, but it seems that none of these identified RBPs are shared by different model systems. This is very likely a result of different methods utilised in these studies to identify the binding partners of the repeat.
expansion. It also suggests that the RBPs interacting with the repeat expansion may be cell type dependent. Therefore, it is important to further investigate astrocyte specific RBPs sequestered into the G\textsubscript{4}C\textsubscript{2} repeat expansion.

In addition to RNA foci, presence of poly-GP DPR in mutant astrocytes was also observed via Western blotting. Although several studies have implied that these RAN translation products are neuron specific\textsuperscript{92,206}, recent reports have extended such pathology to glial cells\textsuperscript{207}. Data here clearly support the latter finding that DPR proteins can be produced in astrocytes, even though they have low expression levels of \textit{C9orf72}. Importantly, the Western blot result provides more convincing evidence of the presence of DPR products in astrocytes compared to dot blots and immunoassays that are primarily used in published reports. One interesting observation is that bands detected by the poly-GP antibody have similar molecular weights between the two mutant lines. Given that the size of DPR proteins is dependent on the length of the G\textsubscript{4}C\textsubscript{2} repeat expansion, these findings suggest that these two carrier lines may have similar size of the repeat expansion. Alternatively, it is possible that an unknown mechanism restricts the size of DPR products independent of the length of the repeat expansion. Therefore, it would be interesting to measure the size of the repeat expansion in both mutant lines by Southern blotting in order to decipher which possibility accounts for the similar product size of poly-GP DPR.

Although DPR proteins have been widely studied in patient tissues, the antibodies generated by different laboratories are not easily accessible, and the commercially
available antibodies have not been optimised and fully characterised in different model systems. Indeed, unspecific bindings of the antibody used in this study make it difficult to interpret the immunocytochemistry data, as potential aggregates are found in all cell lines but not specific to mutant astrocytes. Although overexpression of poly-GP could be used as a positive control for Western blotting, the size variation of the DPR proteins makes it still challenging to distinguish the true poly-GP bands from the unspecific bindings. Hence, more detailed characterisation of antibodies is required, and it is also interesting to determine whether other DPR proteins are produced in mutant astrocytes.

Collectively, findings in this chapter strongly support the use of patient iPSC-derived astrocytes as a model of C9orf72-mediated ALS, and demonstrate that harbouring the G₄C₂ repeat expansion leads to development of C9orf72-related pathology in astrocytes.
Chapter 5

Cell autonomy of \textit{C9orf72} mutant astrocytes
5.1 Introduction

In addition to examining cell-autonomous ALS-relevant pathologies, another advantage of using iPSC-derived astrocytes to model ALS is the possibility to study the effects of an ALS-causing mutation on astrocyte viability. This is challenging to achieve in animal models, as proliferation of astrocytes due to astrogliosis can easily mask cell loss. Our group have previously found that iPSC-derived astrocytes carrying a mutation in TARDBP manifested increased vulnerability in comparison to control astrocytes\textsuperscript{149}. This discovery was also supported by a study of an ALS rat model, which selectively expresses GFP in astrocytes\textsuperscript{151}. In this model, astrocytic cell death in addition to astrogliosis was observed over the course of disease progression\textsuperscript{151}.

Many cellular stress pathways have been associated with ALS, and dysregulated autophagy has been proposed as a potential pathogenic mechanism in C9orf72-mediated ALS. Firstly, a recent study has shown that the C9orf72 protein specifically interacts with several Rab proteins that are involved in the autophagy pathway\textsuperscript{72}, thereby suggesting the C9orf72 protein as a participant in autophagy. Secondly, accumulated inclusions of p62, a carrier of cargos in the autophagy pathway, also implies the involvement of this pathway in C9orf72-mediated ALS\textsuperscript{208}. In addition, an \textit{in vitro} cellular model has identified selective vulnerability of iPSC-derived neurons to autophagy inhibition through screening of several stressors\textsuperscript{190}. Altogether, these findings highlight the importance of autophagy in C9orf72-mediated ALS as a potential pathogenic pathway. Therefore, I decided to investigate the effects of autophagy stress on astrocyte viability.
Hence, the aim of this chapter was to investigate whether the \textit{C9orf72} mutation induced cell-autonomous vulnerability in astrocytes both under basal culture conditions and upon autophagy inhibition.
5.2 Results

5.2.1 Viability of iPSC-derived astrocytes at basal culture conditions

To assess the viability of astrocytes, a cytotoxicity assay was first conducted to measure the LDH release from astrocyte cultures as a determinant of cell death. LDH is an enzyme produced in normal cells and can be released into the extracellular environment when cell membrane integrity is disrupted, indicative of cell death\(^\text{209}\). One particular advantage of the LDH release assay is that it measures cytotoxicity as a consequence of all possible cell death pathways\(^\text{209}\). Therefore, it has been widely used as a readout of cell death.

To measure the extracellular concentration of LDH, astrocyte culture medium conditioned for 24 hours was collected, and LDH release was measured using an enzymatic fluorescence reaction. As the cell number is a confounding factor for LDH release, the total LDH release by lysing all cells with Triton-X was also measured as a positive control. All measurements of LDH release were normalised to their corresponding positive controls to calculate the percentage of cytotoxicity, which is independent of the cell number and can be compared across different conditions. As shown in Figure 5.1, no significant difference was detected between control and mutant astrocytes using pooled data, or between the isogenic pair. Hence, these data suggest a comparable viability of astrocytes with different genotypes under basal culture conditions.
Figure 5.1 Cytotoxicity of iPSC-derived astrocytes under basal culture conditions

LDH released from astrocytes into medium under basal culture conditions was measured and then normalised to total LDH release in order to calculate the percentage of cytotoxicity. Data of two control iPSC lines and data of three mutant iPSC lines were pooled together, respectively. No significant changes of cell death were revealed between control and mutant astrocytes or between the isogenic pair astrocytes (n≥3, Student’s t-test, ns p>0.05).
The cytotoxicity determined by LDH release reflects cell death in cultures at a population level. Given that the half-life of LDH is only 9 hours\textsuperscript{209}, the LDH release assay has limited sensitivity, and may not be able to reveal subtle differences in cell death. Thus, a single cell longitudinal survival analysis that is highly sensitive was next used to interrogate cell viability\textsuperscript{210}.

To follow the survival of cells individually, iPSC-derived astrocytes were first labelled by transfection with a construct encoding EGFP. 24 hours post-transfection, live imaging was conducted every 24 hours for 10 days. This allowed monitoring of individual astrocytes to determine the time of cell death, which is defined as disappearance of fluorescence or dissolution of cells\textsuperscript{210} (Figure 5.2). Using Kaplan–Meier survival analysis, cumulative hazards, a measurement of risk of cell death, were plotted for astrocytes derived from all six iPSC lines (Figure 5.3A). Firstly, the cumulative hazards were compared between control and mutant astrocytes using pooled data. Cox-proportional regression revealed a hazard ratio (HR) of 1.464 in mutant astrocytes compared to controls (Figure 5.3B). This data indicates that a 1.464-fold increase in risk of cell death is associated with astrocytes carrying the \textit{C9orf72} mutation. Importantly, direct comparison of Carrier3-\Delta G_{4}C_{2} astrocytes to Carrier3 astrocytes showed an improved survival with the HR of Carrier3 over Carrier3-\Delta G_{4}C_{2} as 1.796 (Figure 5.3C). Together, these data demonstrate that carrying the \textit{C9orf72} mutation leads to increased risk of cell death in iPSC-derived astrocytes.
Figure 5.2 Single cell longitudinal survival analysis of iPSC-derived astrocytes

Representative serial images of astrocytes manually tracked for the single cell longitudinal survival analysis. Astrocytes positive for EGFP in the first image (24h) were recruited for analysis (indicated by red arrow heads), and followed until their death or the end of 10 days of observation. (Scale bars, 200µm)
Figure 5.3 Kaplan–Meier survival analysis of iPSC-derived astrocytes under basal culture conditions

(A) Cumulative hazards were plotted for astrocytes derived from all six iPSC lines. The n number for each cell line was the total number of EGFP positive astrocytes analysed, which was collected from 5 independent experiments.

(B) Data of two control lines and data of three mutant lines were pooled together, respectively, and cumulative hazards were plotted for these two groups. Mutant astrocytes showed increased cumulative risk of death compared to controls (HR=1.464; *** p<0.001, Cox proportional regression stratified by experiment dates).

(C) The increased risk of cell death in Carrier3 astrocytes was reversed by removal of the $G_4C_2$ repeat expansion (HR of Carrier3 over Carrier3-$\Delta G_4C_2$ is 1.796; *** p<0.001, Cox proportional regression stratified by experiment dates).
Collectively, findings in this section demonstrate that despite the unchanged astrocyte viability at population levels, harbouring the \textit{C9orf72} mutation causes increased vulnerability in astrocytes at basal culture conditions.

5.2.2 Viability of iPSC-derived astrocytes under autophagy inhibition

Next, to investigate the impacts of harbouring the \textit{C9orf72} mutation on astrocyte viability upon autophagy stress, chloroquine treatment was selected as a stressor. Chloroquine functions as an autophagy inhibitor through blocking the fusion of autophagosome with lysosome\textsuperscript{211}. In order to determine the appropriate concentration of chloroquine, a pilot dose response experiment was first performed to estimate the median lethal dose (LD\textsubscript{50}) of chloroquine on iPSC-derived astrocytes, and cytotoxicity was assessed by the LDH release assay. Untreated astrocytes served as a negative control, whereas total LDH release by lysing all astrocytes in a well was the positive control. LDH release of astrocytes under the stress condition was first controlled for the untreated negative control and then normalised to the corresponding positive control to calculate percentage of cytotoxicity. Using this method, the readout is independent of cell number and reflects the net impact of autophagy inhibition on astrocyte viability. Four concentrations of chloroquine, including 30\textmu M, 100\textmu M, 300\textmu M and 1000 \textmu M, were first selected to estimate its LD\textsubscript{50} on astrocytes derived from one control and one \textit{C9orf72} mutant iPSC lines. Treatment with 300\textmu M of Chloroquine induced approximately 50\% of cell death (Figure 5.4). Therefore, 100\textmu M, 300\textmu M and 600\textmu M of chloroquine were selected as
stress conditions to explore the impacts of autophagy inhibition on astrocyte viability.

Figure 5.4 Dose-response of chloroquine treatment on iPSC-derived astrocytes
Astrocytes derived from CTRL1 and Carrier2 iPSCs were used for a pilot study to estimate the LD$_{50}$ of chloroquine. Astrocytes derived from both lines showed a trend of dose-dependent increase of cell death. The LD$_{50}$ of Chloroquine was approximately around 300µM.
2-week old differentiated astrocytes derived from all six iPSC lines were exposed to 100\(\mu\)M, 300\(\mu\)M and 600\(\mu\)M Chloroquine for 24 hours, and the resulting cell death was measured using the LDH release assay explained above. As shown in Figure 5.5, the cytotoxicity of astrocytes showed a dose-dependent response with increased cell death as the concentration of chloroquine increases. At 300\(\mu\)M and 600\(\mu\)M of chloroquine treatments, there was significantly higher cell death of mutant astrocytes compared to control astrocytes using pooled data. This finding suggests increased vulnerability of mutant astrocytes under autophagy inhibition.

In contrast, cytotoxicity of astrocytes derived from the isogenic pair Carrier3 and Carrier3-\(\Delta G_4 C_2\) was comparable at all three concentrations of chloroquine treatment (Figure 5.5), suggesting that the astrocyte viability was not enhanced by removal of the \(G_4 C_2\) repeat expansion. This finding indicates that the impaired astrocyte viability under stress may not be relevant to ALS pathogenesis. However, it is worth noting that the cytotoxicity of astrocytes under autophagy inhibition is variable across different mutant lines with Carrier1 and Carrier2 showing significantly higher cell death in comparison to Carrier3 astrocytes (Figure 5.5). Hence, the phenotype of reduced viability of mutant astrocytes is predominantly driven by the data from these two cell lines, as pooled data were used to compare control and mutant astrocytes. Therefore, it is not surprising that removing the \(C9orf72\) mutation in the Carrier3 line did not lead to rescue of this phenotype. It would be interesting to generate isogenic control lines of Carrier1 and Carrier2 to investigate whether this phenotype can be reversed. In addition, examination of other autophagy markers, such as the light chain 3 (LC3) protein level, may also provide information on whether the autophagy
pathway is disrupted in astrocytes carrying the C9orf72 mutation. Hence, data in this section demonstrate that although higher cell death seems to be associated with the C9orf72 mutation, carrying the G₄C₂ repeat expansion does not lead to impaired viability of astrocytes under autophagy inhibition.

**Figure 5.5 Cytotoxicity of iPSC-derived astrocytes under autophagy inhibition**

Cytotoxicity of astrocytes populations determined by an LDH release assay revealed higher cell death in mutant astrocytes compared to controls upon exposure to 300µM and 600µM of chloroquine for 24h (n≥3, Student’s t-test between 2 control lines and 3 mutant lines, * p<0.05, ** p<0.01,). Under the same treatments, no difference in cytotoxicity was observed between Carrier3 and Carrier3-ΔG₄C₂ astrocytes (n≥3, Student’s t-test, ns p>0.05,).
5.3 Discussion

Results in this chapter demonstrate that the C9orf72 mutation causes increased vulnerability of astrocytes under basal culture conditions but not upon autophagy inhibition. As our group has previously reported association of increased risk of astrocyte death with a TARDBP mutation\textsuperscript{149}, these findings together highlight that cell-autonomous vulnerability of astrocytes is a common feature in ALS. Taking into account the astrocyte pathology presented in Chapter 4, these findings strongly support the idea that ALS is a multicellular disease rather than purely affecting MNs. Several molecular pathways have been proposed as underlying mechanisms of C9orf72-mediated neurotoxicity, including sequestration of RBPs into G\textsubscript{4}C\textsubscript{2} RNA foci, nucleolar stress and impaired nucleocytoplasmic transport\textsuperscript{63}. Whether these are related to C9orf72-mediated astrocyte vulnerability presented here is an interesting question to address, which can be explored in the future studies.

Of note, results in this chapter and the previous one clearly show that variation across different iPSC lines is unavoidable, and generation of the isogenic control is a powerful tool to tackle this problem. Comparison between the isogenic pair uncovers the true pathological changes as a result of harbouring the C9orf72 mutation, which can be masked or underestimated by using pooled data of various iPSC lines. For example, using the single cell longitudinal survival analysis, the HR of mutant astrocytes over controls was less robust than the comparison of Carrier3 astrocytes over its isogenic control Carrier3-ΔG\textsubscript{4}C\textsubscript{2}. Additionally, the reduction of total C9orf72
transcript levels was not significant between control and mutant astrocytes, but was only unveiled by direct comparison between the isogenic pair. Moreover, identification of the poly-GP DPR was very challenging, as its size is dependent on the length of the G\(_4\)C\(_2\) repeat expansion. Although overexpression of DPR of a particular length could be used to assess the sensitivity of the antibody, it still could not determine whether a band of a different molecular weight is indeed a DPR product. Instead, absence of a particular band in the isogenic control line provides more convincing evidence, which allowed the conclusion that poly-GP was indeed produced in astrocytes. Collectively, these findings emphasise the importance of isogenic controls in iPSC-based disease modelling.

On the other hand, the variation across different iPSC lines also reflects that subgroups of \(C9orf72\)-mediated ALS may be present. Firstly, it is possible that the disease severity is dependent on the length of repeat expansion, although no evidence has shown clear association. Secondly, epigenetic difference can also affect the manifestation of the disease. For example, recent studies have found that a proportion of ALS patients carrying the G\(_4\)C\(_2\) repeat expansion have hypermethylation of the CpG islands located at the upstream of the expansion\(^{66}\). Interestingly, one recent study has reported the hypermethylation of the mutant allele is associated with reduction of \(C9orf72\) transcript levels, fewer pathological RNA foci and DPR products and less vulnerability to autophagy stress, suggesting that the hypermethylation is protective\(^{80}\). Although more studies are required to confirm this association, it still supports the notion that there are subgroups within \(C9orf72\)-mediated ALS. Indeed, by taking a close look of the data in this chapter and the
previous one, Carrier1 and Carrier2 had higher transcript levels of \textit{C9orf72}, higher proportions of cells positive for RNA foci and were more susceptible to autophagy inhibition compared to Carrier3 astrocytes. Therefore, it would be interesting to examine the methylation levels of the mutant allele in these three lines to determine whether the epigenetic difference accounts for the variation across these lines. This is also important to explain why some phenotypes identified using the pooled data, such as the increased levels of transcript variant 3 and increased susceptibility to autophagy inhibition, were not reversed by removal of the repeat expansion in Carrier3. Hence, generation of isogenic controls of Carrier1 and Carrier2 is important to determine whether these phenotypes are indeed not relevant to ALS.
Chapter 6

Non-cell autonomous role of $C9orf72$ mutant astrocytes
6.1 Introduction

MNs had long been thought as the sole cell type being affected in ALS until recently when accumulating studies have indicated glial cells, astrocytes in particular, as important players in the pathogenesis of ALS (reviewed in Chapter 1 Section 1.3.2, p.26). The \textit{C9orf72} mutation is the most common genetic cause of ALS\textsuperscript{8}, thereby deciphering the role of astrocytes in this subgroup of ALS is crucial with a potential to develop novel therapeutic treatments. As previously highlighted, human iPSCs provides a powerful tool to acquire astrocytes for investigating the non-cell autonomous roles of astrocytes \textit{in vitro}. Following the work presented in Chapter 3-5, a platform has been established to use iPSC-derived astrocytes for studying the impacts of the \textit{C9orf72} mutation on the interaction between astrocytes and MNs through co-culture experiments. Survival and morphological analysis of MNs were selected as measurements of the potential astrocyte toxicity, as these aspects of MNs have been shown adversely affected by ALS astrocytes in several \textit{in vitro} studies\textsuperscript{139-142,147,148}.

Hence, the aims of this chapter were to examine the impacts of \textit{C9orf72} mutant astrocytes on 1) viability and 2) morphology of control MNs through \textit{in vitro} co-culture experiments.
6.2 Results

6.2.1 Investigation of the impact of $C9orf72$ mutant astrocytes on MN survival

The central question in understanding the non-cell autonomy in $C9orf72$-mediated ALS is what the impacts of astrocytes are on MNs. To answer this question, survival of control MNs was first assessed upon co-culture with iPSC-derived astrocytes with different genotypes. In order to establish a completely humanised in vitro model, control MNs were derived from a genetically modified human ESC line that selectively expresses green fluorescent protein (GFP) under the MN specific promoter of the MN and pancreas homeobox 1 gene ($MNX1$); more commonly referred as $HB9$ (henceforth referred to as HB9::GFP$^{142}$, which was kindly given by Prof Kevin Eggan. Using a previously established protocol$^{166}$, an enriched neuronal population, primarily composed of MNs (~50%), was generated from the HB9::GFP line$^{166}$. This allowed specific focus on the impacts of astrocytes on MNs, as the toxicity of astrocytes is known to be MN specific.

The HB9::GFP ESC-derived MNs were plated on control, $C9orf72$ mutant and the gene edited iPSC-derived astrocytes, respectively. In addition, MNs were also plated alone as monolayers in order to elucidate if co-cultured with astrocytes could improve their survival in vitro. To determine the MN survival, I took advantage of the GFP expression in these MNs and utilised the single cell longitudinal survival analysis as previously described in Chapter 5 Section 5.2.1 (p.129). 24 hours post-MN plate down, live imaging was performed every 24 hours for 10 days. Serial images of each well were then stacked together and blindly analysed to define the
time of cell death for individual HB9::GFP positive MNs (Figure 6.1A). Images of a representative HB9::GFP positive MN is shown in Figure 6.1B. Over the course of 10 days, continuous local movement of this MN was observed, accompanied by constant alteration in its morphology (Figure 6.1B). These features were commonly observed in MNs used for this experiment. As a result, manual tracking of each individual MNs was necessary in order to clearly define the lifespan of MNs.
Figure 6.1 Single cell longitudinal survival analysis of HB9::GFP positive MNs cultured with or without iPSC-derived astrocytes

(A) A schematic diagram shows the timeline of the single cell longitudinal survival analysis of MNs cultured with or without astrocytes. For co-culture experiments, astrocytes were first plated down 4 days prior to MN plate down. For MN cultured alone, they were directly plated down as monolayers on day 0. In both culture conditions, 24 hours after MN plate down (day 1), live imaging at 10x magnification was performed every 24 hours for 10 days. On day 10, an additional live imaging at higher magnification (20x) was performed to enable detailed morphology analysis of MNs as described below in Section 6.2.2.

(B) Representative serial images of an HB9::GFP positive MN manually tracked for the single cell longitudinal survival analysis. This MN showed GFP expression from Day 1 and survived through to the end of 10 days’ observation. Morphology of this MN constantly changed over the course of 10 days as a result of continuous neurite outgrowth. Local movement of the MN was also apparent. Both of these features were commonly observed in the human ESC-derived MNs used for this experiment.

(Scale bars, 50µm)

The HB9::GFP line was kindly given by Prof Kevin Eggan.
Using the Kaplan-Meier survival analysis, cumulative hazards were plotted for HB9::GFP positive MNs in each culture conditions as a measurement of risk of cell death (Figure 6.2, data collected from 4 independent experiments). Firstly, the cumulative hazard of MNs cultured alone as monolayers was compared with the cumulative hazard of MNs co-cultured with iPSC-derived astrocytes. As shown in Figure 6.2A, MNs in co-culture with iPSC-derived astrocytes, regardless of their genetic background, showed significantly improved survival in comparison to MNs cultured alone. Next, data of MNs co-cultured with astrocytes derived from all six iPSC lines were pooled together, in comparison to which the HR of MN cultured alone was 2.11 (Figure 6.2B). This indicates that HB9::GFP positive MNs are twice as likely to die when cultured alone as compared to when co-cultured with iPSC-derived astrocytes. In addition, regardless of genotype, co-culture with astrocytes increases net MN survival in vitro compared to isolated MN culture.
Figure 6.2 Kaplan-Meier survival analysis of HB9::GFP positive MNs cultured with or without astrocytes

(A) Cumulative hazards were plotted for HB9::GFP positive MNs cultured alone and for their counterparts in co-culture with iPSC-derived astrocytes. Co-culturing MNs with astrocytes, regardless of their genetic backgrounds, significantly improved the survival of MNs in comparison to them cultured alone. The n number for each culture condition was the total number of HB9::GFP positive MNs analysed, which was collected from 4 independent experiments.

(B) Data of MNs co-cultured with astrocytes derived from all six iPSC lines were pooled together and compared to the data of MNs cultured alone. The cumulative hazard of MNs cultured alone was significantly higher than MNs co-cultured with iPSC-derived astrocytes. The HR of MNs alone was 2.11 over MNs in co-culture with astrocytes (Cox proportional regression, stratified by experiment date, **** p<0.0001).
Next, the cumulative hazard of HB9::GFP positive MNs on control astrocytes was compared with those on \textit{C9orf72} mutant astrocytes (Figure 6.3, data collected from 4 independent experiments). Data of MNs on control astrocytes (CTRL1 & 2) and data of MNs on \textit{C9orf72} mutant astrocytes (Carrier1, 2 & 3) were pooled together, respectively. A significantly increased risk of cell death was found in MNs co-cultured with \textit{C9orf72} mutant astrocytes with a HR of 1.36 (Figure 6.3B). This finding indicates an adverse impact of \textit{C9orf72} mutant astrocytes on survival of control MNs.

In order to determine if this negative impact was a consequence of carrying the \textit{G4C2} repeat expansion in astrocytes, I next asked whether this survival phenotype could be reversed by removing the \textit{C9orf72} mutation in astrocytes. Hence, the cumulative hazards of HB9::GFP positive MNs on astrocytes derived from the isogenic pair iPSC lines were directly compared. As shown in Figure 6.3C, the risk of MN death was indeed significantly reduced when co-cultured with Carrier3-\textit{ΔG4C2} astrocytes as compared to co-cultured with the \textit{C9orf72} mutant Carrier3 astrocytes. The HR was 1.92 for MNs on Carrier3 astrocytes in comparison to those on Carrier3-\textit{ΔG4C2} astrocytes (Figure 6.3C). This finding therefore shows that harbouring the \textit{C9orf72} mutation in astrocytes leads to their adverse impact on survival of control MNs.
Figure 6.3 Kaplan-Meier survival analysis of HB9::GFP positive MNs cocultured with iPSC-derived astrocytes of different genotypes

(A) Cumulative hazards were plotted for HB9::GFP positive MNs in co-culture with each individual iPSC line-derived astrocytes, showing variance in risk of MN death across different co-culture conditions. The n number for each culture condition was the total number of HB9::GFP positive MNs analysed, which was collected from 4 independent experiments.

(B) Data of MNs co-cultured with control astrocytes (CTRL1 & 2) and data of MNs co-cultured with C9orf72 mutant astrocytes (Carrier1, 2 & 3) were pooled together, respectively. Cumulative hazards were plotted for these two groups, and MNs co-
cultured with C9orf72 mutant astrocytes showed a significantly increased risk of cell death in comparison to their counterparts co-cultured with control astrocytes with a HR of 1.36 (Cox proportional regression, stratified by experiment dates, ** p<0.01). (C) Cumulative hazards were plotted for MNs co-cultured with Carrier3 astrocytes and for MNs co-cultured with the gene edited Carrier3-ΔG4C2 astrocytes. MNs in the first group showed significantly increased risk of cell death in comparison to their counterparts co-cultured with Carrier3-ΔG4C2 astrocytes with a HR of 1.92 (Cox proportional regression, stratified by experiment dates, *** p<0.001).
Altogether, findings in this section demonstrate that iPSC-derived astrocytes improve the survival of control MNs, which is independent of whether they carry the G₄C₂ repeat expansion in C9orf72. More importantly, astrocytes harbouring the C9orf72 mutation adversely affect survival of control MNs, which can be reversed by excising the G₄C₂ repeat expansion in astrocytes. This demonstrates that the C9orf72 mutation is responsible for the toxicity of astrocytes on MN survival.

6.2.2 Investigation of the impact of C9orf72 mutant astrocytes on MN morphology

Having found that C9orf72 mutant astrocytes were toxic to control MNs leading to increased risk of MN death, I next sought to determine the impact of these astrocytes on morphology of live MNs. Previous studies have shown that co-culturing MNs with ALS astrocytes alters the morphology of MNs, resulting in reduced branching, shorter neurites and smaller soma size139,147. As MNs derived from the HB9::GFP human ESC line have relatively high GFP expression in neurites, this enabled morphologic assessment of these MNs.

Utilising the co-culture experiment setup for the single cell longitudinal survival analysis, the morphology of HB9::GFP positive MNs that had survived to the end of 10 days of live imaging was examined. Of note, only those MNs that consistently expressed the GFP from the beginning of the live imaging (i.e. day 1) were recruited for the morphology analysis. As these MN cultures were not synchronised,
progenitor cells were present in the cultures and could give rise to MNs in different waves, resulting in appearance of HB9::GFP positive MNs at various time points over the 10 days of live imaging. Excluding the late appearing HB9::GFP positive MNs could ensure comparability of MNs cultured in different conditions.

On day 10 of live imaging, an additional imaging at a higher magnification (20x) was performed to ensure visibility of fine neurites, as such structures were difficult to be distinguished at 10x (Figure 6.1A). To assess morphology of survived MNs, the number of neurites projected from each MN soma was quantified, and individual neurites were traced to measure the longest neurite length and total neurite length for each individual MN (Figure 6.4).
Figure 6.4 Morphology analysis of HB9::GFP positive MNs cultured with or without iPSC-derived astrocytes

(A) A representative image of an HB9::GFP positive MN that had survived through the 10 days of live imaging and was subject to morphology analysis. This was the same MN presented in Figure 6.1B as a representative of the single cell longitudinal survival analysis. The image shown here was acquired at 20x to allow detailed morphology assessment, whereas images shown in Figure 6.1B were acquired at 10x. (Scale bar, 50µm)

(B) A screen shot of morphology analysis on this particular MN showed tracing of one neurite (yellow fragmented line) projecting from the cell soma. Number of neurites, longest neurite length and total neurite length of each individual MN were measured for further comparison. (Scale bar, 50µm)
Firstly, the morphology of MNs cultured alone was compared with those co-cultured with iPSC-derived astrocytes (Figure 6.5, data collected from 4 independent experiments). Cumulative frequencies of neurite numbers and neurite length were plotted as a presentation of data distribution and used for comparison. Presence of astrocytes, regardless of their genetic background, clearly enhanced the neurite branching of control MNs and showed a trend in increasing the neurite length of MNs (Figure 6.5A). Pooled data of HB9::GFP positive MNs co-cultured with astrocytes derived from all six iPSC lines showed significant increases in neurite numbers, total neurite length and the longest neurite length in comparison to MNs cultured alone. Together, these data indicate that co-culturing control MNs with iPSC-derived astrocytes provide support to neurite outgrowth, and this positive influence is independent of whether the iPSC-derived astrocytes carry the C9orf72 mutation.
Figure 6.5 Neurite numbers and length of HB9::GFP positive MNs cultured with or without iPSC-derived astrocytes
HB9::GFP positive MNs that survived through 10 days of observation were subject to morphology analysis (n=69, 90, 90, 84, 77, 101, 90 for MNs cultured alone, co-cultured with CTRL1-2, Carrier1-3 and Carrier3-ΔG4C2 astrocytes, respectively; data collected from 4 independent experiments). Cumulative frequencies of their neurite numbers (A) and length (C & E) were plotted for HB9::GFP positive MNs cultured alone and for their counterparts in co-culture with astrocytes derived from all six iPSC lines (insets are corresponding mean values). Co-culturing MNs with astrocytes, regardless of their genotypes, improved the neurite outgrowth of HB9::GFP positive MNs after 10 days of co-culture.

Data of HB9::GFP positive MNs co-cultured with astrocytes derived from all six iPSC lines were pooled together (insets are corresponding mean values). Neurite numbers (B), total neurite length (D) and the longest neurite length (F) all showed significant increase in MNs co-cultured with astrocytes compared to their counterparts cultured alone (Kolmogorov-Smirnov test. ** p<0.01, *** p<0.001).
Next, the morphology of HB9::GFP positive MNs on control astrocytes (pooled data of CTRL1 and 2) was compared with those on C9orf72 mutant astrocytes (pooled data of Carrier1, 2 and 3) after 10 days of co-culture (Figure 6.6B, E & H, data collected from 4 independent experiments). MNs in the latter group displayed significantly shorter neurites, as evidenced by reduction in both total neurite length and longest neurite length (Figure 6.6E & H). Although there was a trend towards a reduction in neurite numbers of HB9::GFP positive MNs on C9orf72 mutant astrocytes, the difference did not reach statistical significance when compared to MNs on control astrocytes. Nevertheless, these data suggest negative impacts of C9orf72 mutant astrocytes on MNs, leading to altered neuronal morphology.

To determine if this negative impact on MN morphology was dependent on the G₄C₂ repeat expansion, the cumulative frequencies of neurite numbers and neurite length of HB9::GFP positive MNs on astrocytes derived from the isogenic pair iPSC lines were directly compared. As shown in Figure 6.6C, F & I, the total neurite length and the longest neurite length of HB9::GFP positive MNs were significantly increased in MNs co-cultured with the Carrier3-ΔG₄C₂ when compared to those co-cultured with the mutant Carrier3 astrocytes (Figure 6.6F & I). Although there was a trend towards an increase in neurite numbers of MNs co-cultured with the gene edited astrocytes, the difference did not reach statistical significance (Figure 6.6C). Together, these data support the hypothesis that mutant astrocytes influence neurite outgrowth of control MNs in vitro.
Figure 6.6 Neurite numbers and length of HB9::GFP positive MNs cultured with iPSC-derived astrocytes of different genotypes

(A, D, G) Cumulative frequencies of neurite number (A) and length (D & G) were plotted for HB9::GFP positive MNs in co-culture with each individual iPSC line-derived astrocytes, showing variance in MN morphology on different lines of astrocytes (insets are corresponding mean values).

(B, E, H) Data of MNs co-cultured with CTRL1-2 astrocytes and data of MNs co-cultured with Carrier1-3 astrocytes were pooled together, respectively. Cumulative frequencies of neurite numbers (B), total neurite length (E) and the longest neurite length (H) were plotted for these two groups (insets are corresponding mean values).

HB9::GFP positive MNs co-cultured with C9orf72 mutant astrocytes showed
significantly reduced neurite length with a trend of reduced neurite numbers. (Kolmogorov-Smirnov test, ** p<0.01, **** p<0.0001).

(C, F, I) Cumulative frequencies were plotted for HB9::GFP positive MNs co-cultured with Carrier3 astrocytes and for those cultured with Carrier3-ΔG4C2 astrocytes (insets are corresponding mean values). MNs co-cultured with the mutant Carrier3 astrocytes showed significantly shorter neurite length (F & I) and a trend of reduced neurite numbers (C) in comparison to MNs co-cultured with the gene edited Carrier3-ΔG4C2 astrocytes. (Kolmogorov-Smirnov test, ** p<0.01, *** p<0.001).
Collectively, findings in this section demonstrate that iPSC-derived astrocytes improve the neurite outgrowth of control MNs, regardless of the presence of the \textit{C9orf72} mutation in astrocytes. More importantly, astrocytes harbouring the \textit{C9orf72} mutation adversely affect morphology of control MNs. Further, this effect can be reversed by exciting the \textit{G}_4\textit{C}_2 repeat expansion in astrocytes, strongly linking the \textit{C9orf72} mutation to the adverse impact of astrocytes on MN morphology. Together with findings in the previous section, the non-cell autonomous role of \textit{C9orf72} mutant astrocytes was extended from negative effects on MN viability to adverse impacts on surviving MNs leading to their altered neuronal morphology.
6.3 Discussion

Results in this chapter demonstrate that astrocytes carrying the C9orf72 mutation manifest adverse impacts on the survival and morphology of control MNs, leading to increased risk of MN death and altered neuronal morphology. Importantly, comparison of paired isogenic lines provides direct evidence that these negative effects are due to the G_4C_2 repeat expansion in astrocytes. Nevertheless, both control and C9orf72 mutant astrocytes provide support to control MNs, as the survival and neurite outgrowth of MNs are improved upon co-culture with iPSC-derived astrocytes when compared to them cultured alone.

These findings support the non-cell autonomous role of astrocytes in C9orf72-mediated ALS. Of note, the C9orf72 mutant astrocytes not only adversely affect MN survival leading to neuronal loss, but also display negative impacts on surviving MNs. In light of these findings, we are collaborating with researchers at St. Andrews University to perform detailed electrophysiological studies of control MNs co-cultured with iPSC-derived astrocytes. This is an important question to address, as the negative impacts of C9orf72 mutant astrocytes on surviving MNs observed here may occur prior to MN loss and reflect early phases of ALS. Further studies are required to define the molecular mechanisms responsible for these phenotypes, which may shed light on potential treatments targeting early phases of ALS to prevent progressive neuronal loss.

Findings of the non-cell autonomous role of C9orf72 mutant astrocytes here are in agreement with a recent study, where reduced survival of control MNs was observed
in co-culture with patient-derived astrocytes carrying the $C9orf72$ mutation$^{201}$. However, several differences are worth noting between these two studies. Firstly, Meyer et al.$^{201}$ generated astrocytes through direct conversion of adult skin fibroblasts into NPCs which were subsequently differentiated into astrocytes in serum-containing media without patterning. In the current study, however, fibroblasts were first reprogrammed into iPSCs before generating spinal astrocytes in chemically defined media. These differences in generation methods can lead to distinct epigenetic and transcriptional patterns in resulting astrocytes, and ultimately alter the behaviour of astrocytes. Hence, astrocytes derived in these two studies are not directly comparable.

Secondly, Meyer et al.$^{201}$ utilised mouse ESC-derived MNs to explore the toxicity of $C9orf72$ mutant astrocytes, whereas a completely humanised co-culture model was established here, which is more pathologically relevant to ALS patients.

Last, but most importantly, an isogenic control iPSC line was generated in the current study, where reversed non-cell autonomous phenotypes were observed in MNs co-cultured with the gene edited astrocytes. These findings provide convincing evidence that the $C9orf72$ mutation is responsible for astrocyte toxicity, while such conclusion cannot be drawn from Meyer et al.’s study$^{201}$ due to a lack of isogenic pairs. Despite all differences listed above, similar toxicity of $C9orf72$ mutant astrocytes is demonstrated in two independent studies, strongly supporting the non-cell autonomous mechanism in $C9orf72$-mediated ALS.
Our group previously have shown that iPSC-derived astrocytes carrying an M337V mutation in *TARDBP* do not affect the survival of MNs *in vitro*\(^1\). Using the same astrocyte generation method and similar experiment settings, here non-cell autonomous toxicity of *C9orf72* mutant astrocytes was observed. This further supports the hypothesis that the *TARDBP*-mediated ALS may have distinct pathogenic pathways from other subgroups of ALS. It is worth noting though, that MNs derived from control human iPSC lines were used in the *TARDBP* study\(^1\), whereas the HB9::GFP human ESC line was applied in the current chapter. The difference in cell lines may be a confounding factor. Additionally, in Serio et al.’s study\(^1\), a rather small number of iPSC lines were recruited, including two independent control lines and two clonal lines derived from one patient, and no isogenic control lines were generated. These limitations may be another reason accounting for the lack of non-cell autonomous toxicity of *TARDBP* mutant astrocytes. However, an independent study also did not observe any toxicity of mouse *TARDBP* mutant astrocytes both *in vitro* and *in vivo*\(^2\), thereby supporting the findings in the human iPSC-based model in Serio et al.’s study\(^1\). Together with the findings in this chapter, these studies strongly indicate distinct mechanisms involved in *TARDBP*- and *C9orf72*-mediated ALS, and it would be interesting to decipher the reasons behind the difference in astrocytes carrying these two ALS-causing mutations in the future.

As previously discussed in Chapter 4 & 5, recruitment of the isogenic control iPSC line not only enables the causality study of the impact of the *C9orf72* mutation on astrocyte, but also facilitates the identification of disease relevant phenotypes *in*
Data in this chapter again show apparent variation across different iPSC lines, which leads to underestimation of the impact of the \(C9orf72\) mutation on astrocytes using pooled data. Specifically, the HR of MNs on mutant astrocytes is 1.36 compared to those on control astrocytes by using pooled data of control and mutant astrocytes respectively, whereas the difference is much greater in direct comparison between the isogenic pair, showing a 1.91-fold difference. In other words, variation across different cell lines leads to underestimation of the toxicity of astrocytes. Instead, direct comparison between isogenic pairs enables uncovering more accurate consequence of harbouring the \(C9orf72\) mutation in astrocytes.

Although negative impacts of \(C9orf72\) mutant astrocytes on survival and morphology of control MNs were observed here, several other interesting questions can be addressed using the current platform in the future. For example, studies in the \(SOD1\)-mediated ALS have suggested that the toxicity of mutant astrocytes is, at least partially, mediated through soluble factors released into extracellular environment\(^{139}\). Whether such mechanism is also applicable to the \(C9orf72\)-mediated ALS can be addressed through culturing MNs in media conditioned by astrocytes with or without the \(C9orf72\) mutation. Moreover, the interaction between astrocytes and MNs is bidirectional, and unknown signals from mutant MNs can exacerbate the toxicity of mutant astrocytes, as previously shown in \(SOD1\)-related ALS\(^ {140}\). Therefore, in addition to human ESC-derived control MNs used in this chapter, it is equally interesting to co-culture \(C9orf72\) mutant MNs with control or mutant astrocytes to evaluate if the non-cell autonomous phenotypes are more sever in these scenarios. Understanding the molecular pathways responsible for the neuronal contribution to
astrocytes’ toxicity would be highly beneficial to develop therapeutic treatments targeting this crosstalk. Finally, although the HB9::GFP line has been widely used in studies to investigate the role of astrocytes in ALS, it is worth noting that HB9 is a transcription factor that is temporarily up-regulated during the early development of MNs, and down-regulated as MNs mature, accompanied by up-regulation of other cell identity markers, such as choline acetyltransferase (ChAT)\textsuperscript{212}. Therefore, in the single cell longitudinal survival analysis of co-culture experiments, it is possible that loss of HB9::GFP positive cells was a result of maturation of MNs rather than cell death. To overcome this problem, characterisation of MNs in co-culture with cell death markers, e.g. the apoptotic marker Caspase-3, or generation of reporter cell lines under mature MN markers, e.g. ChAT, can be conducted in the future.

Overall, findings in this chapter support the non-cell autonomous hypothesis in \textit{C9orf72}-mediated ALS, and an \textit{in vitro} platform has been established to further investigate the molecular mechanisms underlying the astrocytic toxicity as well as testing small molecules to screen for potential therapeutic treatments.
Chapter 7  Discussion
7.1 Summary of findings

In this thesis, an *in vitro* platform was established and subsequently utilised to explore the cell-autonomous and non-cell autonomous effects of harbouring the G₄C₂ repeat expansion in *C9orf72*, causative of ALS, in human iPSC-derived astrocytes.

Results in Chapter 3 demonstrated successful generation of iPSCs from healthy controls and ALS patients, and targeted removal of the G₄C₂ repeat expansion in *C9orf72* through CRISPR/Cas9-mediated gene editing. Highly enriched functional astrocytes were generated from control, *C9orf72* mutant and gene edited iPSCs as evidenced by expression of astrocyte markers, uptake of extracellular glutamate and propagation of calcium waves under stimulation. Notably, harbouring the repeat expansion did not lead to alterations in astrocytes differentiation efficiency or functional capacities.

Following the establishment of pure astrocyte cultures, experiments in Chapter 4 focused on detailed characterisation of pathological features associated with *C9orf72*-mediated ALS in iPSC-derived astrocytes. Harbouring the G₄C₂ repeat expansion in *C9orf72* caused down-regulation of total *C9orf72* transcripts, formation of G₄C₂ repeat RNA foci and RAN translation of the G₄C₂ repeats into poly-GP DPR products, which were reversed by correction of the mutant allele of *C9orf72*. These findings demonstrate that patient iPSC-derived astrocytes are a valid *in vitro* model of *C9orf72*-mediated ALS, and that the G₄C₂ repeat expansion is responsible for development of pathological changes in astrocytes.
In Chapter 5, cell-autonomous effects of the \textit{C9orf72} mutation on astrocyte viability were examined. Under basal culture conditions, although the repeat expansion did not lead to overt change in viability of astrocyte populations, a significant increase of vulnerability was revealed in patient iPSC-derived astrocytes by single cell longitudinal survival analysis. Furthermore, autophagy inhibition triggered increased cytotoxicity that was associated with the \textit{C9orf72} mutation, even though removal of the repeat expansion in one of the patient iPSC line did not rescue such phenotype.

At the end, the non-cell autonomous roles of \textit{C9orf72} mutant astrocytes were explored in Chapter 6. Findings of co-culture experiments illustrated the adverse impacts of \textit{C9orf72} mutant astrocytes on viability and morphology of control human MNs, which could be rescued by correction of the mutation in astrocytes.

Collectively, work in this thesis support the use of patient iPSC-derived cells as a valid \textit{in vitro} model of ALS. In addition, findings here demonstrate that astrocytes, in addition to MNs, are also affected by harbouring the \textit{C9orf72} mutation, leading to development of pathological changes, increased vulnerability and non-cell autonomous toxicity on MNs. Importantly, iPSC-derived astrocytes provide a valuable platform to further investigate the molecular mechanisms responsible for these observed phenotypes and to explore potential therapeutic strategies to modify such impairments both \textit{in vitro} and \textit{in vivo}.
7.2 Discussion and future directions

Work in this thesis not only demonstrate the importance of using human iPSC for modelling neurodegenerative diseases in vitro, but also highlight the crucial roles of astrocytes in C9orf72-mediated ALS. Further studies are required to address several interesting and important questions that have not yet been explored here.

7.2.1 Human iPSC and in vitro disease modelling

Importance of using patient iPSC-derived cells for modelling ALS

Although several animal models have been generated to mimic C9orf72-mediated ALS, conflicting findings or lack of ALS-like phenotypes have been reported. In addition, overexpression of the G_{4}C_{2} repeats raises the question whether these findings are pathophysiologically relevant to ALS patients.

In contrast, patient iPSC-derived cells possess only one mutant allele of C9orf72, and highly pure astrocyte cultures allow the examination of ALS relevant phenotypes with primary focus on one cell type at a time. Using this method, I identified phenotypes in astrocytes that have not yet been reported in animals or ALS patients. For example, the production of DPR proteins has been proposed to be specific to neurons\(^9\), whereas I have found poly-GP translation in patient iPSC-derived astrocytes that are dependent on the presence of the C9orf72 mutation. Moreover, this is the first study reporting an increased vulnerability of astrocytes as a consequence of harbouring the C9orf72 mutation. Together, these findings support the use of human iPSC-derived cells for modelling neurodegenerative disorders.
Use of human genome editing

The biggest challenge and inevitable problem of using patient iPSC-derived cells to model diseases is the variability among individual cell lines. Indeed, inter-cell line variation was observed in nearly all experiments conducted in this thesis, making it sometimes challenging to interpret the findings. To overcome this problem, the CRISPR/Cas9-mediated genome editing was utilised to selectively excise the G₄C₂ repeat expansion in C9orf72, successfully generating an isogenic control for one patient iPSC line. Direct comparison of the isogenic pair allows identification of subtle but ALS relevant phenotypes as a consequence of harbouring the C9orf72 mutation.

It is worth noting, however, that off-target mutagenesis through CRISPR/Cas9-mediated gene editing does occur, although relatively rare, in human cells. The resulting mutations may lead to unknown alterations in gene regulations and cell functions, which is no longer an isogenic control and cannot be used for examination of disease relevant phenotypes. Therefore, it is necessary to perform evaluation of off-target events in the future.

Although patient iPSC-derived astrocytes provide informative evidence of ALS relevant phenotypes, the simplified culture conditions do not fully mimic the complex environment in patients. Hence, it is important to validate in vitro findings in animal models, such as transgenic mice and engraftment of iPSC-derived astrocytes into mice.
**Identification of disease modifiers**

In addition to genetic background variations, the variability in molecular and cellular phenotypes across different iPSC lines may also reflect the significant contributions of disease modifiers. As shown in the result chapters, astrocytes derived from Carrier1 and Carrier2 iPSC lines appeared to be more vulnerable and to manifest more severe pathological phenotypes compared to Carrier3 astrocytes. It is possible that these two lines may carry certain ALS enhancers or be lack of suppressors that may be present in Carrier3. Therefore, it would be interesting to conduct selective genetic and epigenetic characterisations of these lines to determine if such modifiers are responsible for variability, given that a couple of genetic and epigenetic modifying factors have recently been proposed for C9orf72-mediated ALS\textsuperscript{161,162}.

Moreover, targeted editing of potential genetic modifiers and subsequent evaluation of molecular and cellular phenotypes would help to determine whether these modifiers indeed play an enhancing or suppressing roles in C9orf72-mediated ALS. This would be particularly informative, as such questions cannot be addressed by clinical association studies in ALS patients.

**Modelling late onset neurodegenerative disorders**

A long-standing question of using patient iPSC-derived cells to model neurodegenerative disorders is how to mimic the clinical feature of slow development and adult onset, given that the reprogramming process rejuvenate the adult somatic cells and that iPSC-derived cells are more reminiscent of embryonic human cells\textsuperscript{168,213,214}. One argument is that the stringent \textit{in vitro} culture conditions may accelerate the manifestation of disease relevant phenotypes. Evidence to support
such notion is the fact that pathological changes and signs of degeneration do manifest in patient iPSC-derived cells, and stressors can further accelerate the process. More importantly, given that the disease-causing mutations are present throughout embryonic development to adulthood, the young iPSC-derived cells provide an intriguing opportunity to identify early cellular and molecular events that are potentially responsible for pre-clinical stages of neurodegenerative diseases. This may be of help to discover biomarkers and early stage treatments.

Despite all these arguments, it remains interesting whether aged cells manifest more severe phenotypes and what pathways are responsible for these. Increasing evidence starts to show that ageing itself is an important pathogenic contributor to the development of neurodegenerative disorders. For example, primary mouse astrocytes from aged animals are less supportive to neurons than astrocytes prepared from young mice\textsuperscript{215}. In order to model the ageing aspect, molecular and cellular definition of aged cells is required. Indeed, recent studies have started to shed light on such questions, as many transcriptional signatures have been identified as highly associated with ageing\textsuperscript{168,216}. Moreover, manipulation on iPSCs and new derivation methods have shown the possibility to age human cells \textit{in vitro}. Specifically, induced expression of progerin, a protein causing premature ageing, is shown to accelerate and aggravate \textit{in vitro} phenotypes of patient iPSC-derived cells\textsuperscript{217}. In addition, direct conversion of somatic cells of elderly patients into neurons maintains the transcriptional signature of aged cells\textsuperscript{216}, thereby can be used to study the role of ageing in disease development.
Therefore, it would be interesting to age the human iPSC-derived astrocytes through such methods. This will allow the investigation of whether more severe phenotypes would develop, and more importantly what factors are responsible for the severity of phenotypes.

7.2.2 Astrocytes in C9orf72-mediated ALS

The C9orf72 mutation affects non-neuronal cells in the CNS

Recapitulation of pathological features and discovery of the cell-autonomous vulnerability of C9orf72 mutant astrocytes not only provide convincing evidence to support the use of patient iPSC-derived cells as an in vitro model of ALS, but also, more importantly, prove the idea that ALS is a multisystem disease, affecting both neurons and glial cells. It appears that MNs are more vulnerable than astrocytes, as patients manifest clinical symptoms as a consequence of MN death rather than loss of astrocytes. It is particularly interesting to determine what accounts for such selective neuronal vulnerability or astrocytic resistance to ALS. Indeed, astrocytes are distinct from neurons at many aspects, from gene regulations to network functions\textsuperscript{110}. Such diversity may account for differential responses or degeneration threshold under disease conditions. Hence, given that highly pure MNs can be easily derived from iPSCs\textsuperscript{174}, comparisons between MNs and astrocytes carrying the same mutation would enable identification of cell type specific pathogenic pathways.
Astrocytes contribute to the pathogenesis of C9orf72-mediated ALS

In this study, viability and morphology of MNs is shown to be adversely affected by C9orf72 mutant astrocytes, confirming the non-cell autonomous contributions of astrocytes to ALS pathogenesis. This is particularly important, as solely curing MNs in patients may not be effective. Instead, treatments targeting different aspects of ALS are required.

It is also very interesting that such negative impacts are not found in TARDBP-mediated ALS using a similar model system. This suggests that distinct cellular pathways may underlie individual subgroups of ALS caused by different mutations. Hence, investigation of the differences between astrocytes carrying these two mutations would be helpful to acquire a better understanding of astrocyte toxicity and potential neuroprotective pathways.

Astrocytes in FTD and sporadic ALS

It is becoming increasingly clear that astrocytes are a heterogeneous population of cells, and that there are various subtypes of astrocytes with temporal and regional specificity. Therefore, it is likely that different subtypes of astrocytes may contribute to disease development in distinct manners, hence resulting in different disease manifestation. For examples, loss of EAAT2 is proposed underlying the non-cell autonomous contributions from spinal astrocytes, whereas excessive calcium signalling is suggested responsible for their involvement in Alzheimer’s disease. Therefore, it is important to study the non-cell autonomous effects of astrocytes with defined subtypes. Given that cortical astrocytes can be derived from human iPSCs.
and that FTD shares clinical, pathological and genetic features with ALS\textsuperscript{35}, it would be interesting to generate cortical astrocytes carrying the \textit{C9orf72} mutation and determine their roles in FTD. Moreover, it would be important to decipher whether there are differences in the non-cell contributions of astrocytes in these two interlinked diseases even with the same mutation.

Although modelling ALS with a known genetic mutation provides valuable information on pathogenesis in general, the vast majority of ALS patients are sporadic and may have distinct pathogenic pathways. One important application of human iPSC is to model sporadic diseases \textit{in vitro}, which cannot be addressed by animal models due to a lack of genetic abnormalities. Several reports have shown that cells derived from sporadic neurodegenerative disorder patients manifest disease relevant phenotypes similar to cells carrying a disease causing mutation\textsuperscript{219,220}. Establishment of such models for sALS would benefit our understanding of differential and common pathogenic pathways underlying fALS and sALS, and development of subgroup specific therapies.

\textbf{Molecular pathomechanisms and therapeutic targets}

ALS is a multisystem and multifactor disease, with non-neuronal cells and various pathways involved in disease development and progression. Hence, it is likely that the therapeutic treatments targeting different pathways need to be combined. Before achieving this, understanding the pathogenic mechanisms associated with each cell type is important, and iPSC-derived cells provide a powerful tool to achieve this.
Several pathways have been proposed to be involved in astrocyte-mediated toxicity. For examples, focal loss of EAAT2\(^{135}\), possibly caused by mis-splicing\(^{203}\), is proposed to be involved in glutamate-mediated excitotoxicity. Interestingly, overexpression of arginine-containing DPRs in human astrocytes induce the alternative splicing of the EAAT2\(^{102}\). Therefore, it would be interesting to examine whether such aberrant RNA processing occurs in patient iPSC-derived astrocytes carrying the C9orf72 mutation and account for the negative impacts on MNs. Moreover, RNA-sequencing may provide new candidates potentially responsible for cell-autonomy and non-cell autonomy of astrocytes.

The ultimate goal of studying pathomechanisms of ALS is to develop therapeutic medications. Over the past ten years, many pathogenic mechanisms have been proposed and targeted in pre-clinical and clinical trials, but none of them has been successfully translated into licensed drugs due to negative human trials\(^ {37}\). There are several reasons behind the failure. Firstly, most of pathomechanisms are identified in the mutant SOD1 rodent models. Mutations in SOD1 account for merely 10% of fALS and 2% of all ALS cases, whereas the vast majority of patients are sporadic\(^ 8\). Hence, it is not surprising that pre-clinical trials relied on positive findings in the SOD1 models cannot be translated into ALS patients with more diverse genetic backgrounds. Secondly, SOD1-mediated ALS appear to have distinct pathogenic pathways compared to other subgroups of ALS mediated by different mutations. For example, RNA processing is not affected in SOD1-mediated ALS, but is highly relevant to ALS caused by mutations in TARDBP, FUS and C9orf72\(^ {20}\). In addition, TDP-43 proteinopathies are commonly observed in nearly all ALS cases but not in
SOD1-mediated ALS\textsuperscript{26,27}, further suggest distinct pathogenic pathways. Thirdly, most, if not all, ALS rodent models are generated through overexpression of the mutant form of ALS relevant proteins, which does not mimic the pathophysiological conditions in ALS patients\textsuperscript{37}.

Altogether, these highlight the importance of understanding the pathogenesis of individual subgroups of ALS as well as the use of other model systems to validate therapeutic strategies targeting such pathways prior to human clinical trials. Cells derived from iPSCs are an invaluable tool to achieve this, as they are patient specific and a relatively large number of lines can be generated representing the diverse genetic nature of patients. Indeed, patient iPSC-derived neurons have already been used for high throughput screening of small molecules\textsuperscript{175}. Similarly, astrocytes carrying the C9orf72 mutation can be utilised to screen for drugs specifically targeting pathogenic pathways specific to astrocytes.

**Transplantation of astrocyte lineage cells**

Another important potential application of human iPSC-derived cells is cell replacement therapy through transplantation. This is particularly tempting for neurodegenerative diseases, as it offers an option of replacing the lost neurons. However, such therapeutic strategy is highly challenging, as lack of directing signals and cells in adult CNS makes it difficult for the grafted neurons integrating into the existing neuronal networks and establish correct circuits. Although new techniques, such as optogenetics manipulations, offer exciting opportunities to controlled guidance towards peripheral neural circuit developments\textsuperscript{221}, it still requires long time
before application in patients. Furthermore, a toxic glial environment may lead to newly grafted neurons to lose their functions and eventually die.

From a different perspective, transplantation of healthy glial lineage cells can provide support to surviving MNs and prevent further neuronal loss in patients. In light of this idea, several small-scale early-stage clinical trials have shown that focal transplantation of glial restricted progenitor cells into patients’ spinal cords is very well tolerated\textsuperscript{222}. Significant beneficial outcomes are yet to be demonstrated following transplantation. Nevertheless, manipulating human astrocytes to enhance production of neurotrophic factors offers another possibility to lead to positive clinical outcomes\textsuperscript{222}. Therefore, the human iPSC-derived APCs and astrocytes may potentially be used for therapy through transplantation.
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