

Cell targeted phenotyping:
understanding molecular genetic
mechanisms of *C9ORF72* ALS in motor
neurons



Sarah Burley

St-Hugh's College
Department of Physiology, Anatomy and Genetics
University of Oxford
Hilary 2018

A thesis submitted towards the degree of Doctor of Philosophy

Abstract

Motor neuron disease affects 2 in every 100,000 people each year. Amyotrophic lateral sclerosis (ALS), the most prevalent form of motor neuron disease, presents in adulthood with the loss of both upper and lower motor neurons. Approximately 10% of ALS cases are familial with dominant inheritance, while the remaining 90% are sporadic in nature. A large hexanucleotide repeat expansion in the *C9ORF72* gene causes 7% of sporadic and 40% of familial cases, making the *C9ORF72* gene expansion the most prevalent cause of ALS known to date. At present there is no cure for ALS and only treatments such as Riluzole to prolong life for a few months are available. There is a need to better understand disease progression and develop therapeutic approaches.

The differentiation of motor neurons from induced pluripotent stem cells (iPSC), derived from patients with the *C9ORF72* gene expansion, provides a model allowing exploration of the downstream effects of the mutation whilst maintaining the patient's genetic background. However, these neurons are typically immature and may not fully recapitulate disease phenotypes as presented by patients. Therefore, generating mature iPSC-derived motor neurons is critical for the study of adult onset diseases including ALS, as they allow for improved functional assays and form a more physiological model for drug testing. In Chapter 3, two motor neuron differentiation protocols were initially trialled before selection of the final protocol and further optimisation trials to create mature motor neurons.

In Chapter 4, patient and control iPSC-derived motor neurons were analysed for the expression of motor neuron specific markers, excitability and internal calcium phenotypes. Motor neuron marker (SMI-32) analysis detected a significant difference in expression at the early time point, day 40 during MN differentiation, between patient and control cells. A phenotypic difference was also observed at day 40 showing increased excitability and increased internal calcium release in patient versus control cells.

In Chapter 5, iPSC-derived motor neurons were further engineered to express optogenetic tools in a cell specific or sub-compartment specific manner to allow for improved selective functional studies including synaptic vesicle release and intrinsic membrane potential studies. Combining optogenetic and reporter systems particularly for electrophysiological studies as used here, not only allows for selective functional analysis but also tracking and visualisation of live motor neurons in culture. This study generated a dual optogenetic and motor neuron reporter lentiviral vector based on the Cre/Lox system, able to both track and activate live motor neurons in culture.

Analysing the downstream effects of the gene mutation in neurons, as above, is crucial. However, analysing how the gene itself leads to dysfunction is equally important. It is still unknown by which mechanism the *C9ORF72* gene expansion causes disease, three mechanisms including haploinsufficiency, toxic RNA and toxic protein are hypothesised. Fluorescent in situ hybridisation aimed to further explore and add a novel approach to the question of haploinsufficiency. In the final results Chapter 6, the expression of the *C9ORF72* gene itself was probed using fluorescent in situ hybridisation in patient cells to infer information on the expression of the expanded vs non-expanded alleles. One

patient showed a mislocalisation of the expanded allele towards the nuclear periphery compared to the non-expanded allele suggesting reduced *C9ORF72* gene expression. This project overall aims to further elucidate disease pathophysiology to allow future development of therapeutic approaches.

Declaration

I declare the work presented in this thesis was performed by the author at the University of Oxford, except where acknowledgement is made.

This work in this thesis has not been submitted for any other degree in this or any other University.

Acknowledgements

There are so many people I would like to thank for helping me through the DPhil journey!

Firstly, I would like to thank Professor Richard Wade-Martins for giving me the opportunity to join the lab 4 years ago. I have learnt so much in my time in the lab and have enjoyed every minute of being part of the Wade-Martins team! Thank you so much for all your advice and guidance.

Oscar, I cannot thank you enough for being an amazing supervisor! I have really enjoyed working with you and always appreciated all your advice. I honestly could not have asked for a better supervisor!

Dayne, thank you so much for teaching me electrophysiology. I have loved learning everything about Ephys and definitely want to continue learning more!! There was never a dull moment.....I hope you're now a Kygo fan!

Charmaine, your knowledge of flow cytometry was invaluable to me! Thank you so much for persevering and giving me great advice to finally optimise the protocol. It was so much fun working with you! A special thanks to Irene for being a FlowJo expert!

During my DPhil the 2D FISH technique was one of the hardest to optimise, with many failures along the way. Ana, I really appreciated all your advice to get the technique finally working!

My wonderful housemates and lab buddies, Gaby and Becky!! I have really enjoyed sharing this DPhil journey with you both! Becky, I will never forget the first day we met in lab, we looked identical! You're an amazing friend, whether it be sitting at home

watching random TV, going out for drinks or competing in the Lab Bake Off final! It's been wonderful sharing a house and lab with you!

Gaby, thank you so much for always being there for me. I always knew if I needed anything you would be there!

The original tea office crew, Nora and Ali! Sharing an office with you were honestly some of the best months of my DPhil! We had so much fun, laughing all the time!

Ali, thank you for the constant laughs, I'm pretty sure you brought me to tears laughing on more than one occasion! It was just not the same when you left.

Nora, you were always the one I could turn to if I ever needed help or advice or anything, it's so nice to know you would always be there!

Brian, you made me feel so welcome when I joined the lab, it really was not the same when you left! You were my late-night lab buddy, it was always so nice knowing there was someone around to grab a drink with after work! I loved our spontaneous dinner dates, you honestly made the first few years of my DPhil so fun!

Dana, our lab chats were the best, whether it be talking about science or random things, I always loved it.

Brent, thanks for always being a great friend and knowing when it was time to force me out of the lab for food! I'm so glad we became friends, I will really miss sharing a lab with you and all the great/extremely random chats!

I would also just like to thank everyone in the lab past and present for always being so friendly, so helpful and so much fun to hang out with outside of lab!! Siv, Natalie, Tara, Hugo, Fede, Amy, Marta, Joel, Helle, Lisa, Milena, Mel, Kathie, Pip, Deepak, Gabriele, Mindaugas, Peter, Heather, Janine and Uroosa, it has been an amazing 4 years, thank you so much for being part of it!

A massive thank you to my old friends from the University of Bath! I truly love you guys so much! I'm so glad we all manage to meet up so often and I hope we continue. An extra thanks especially to Jaii and Grace for all our girls weekend meet ups, without those I would not have survived!!

A big thank you to my amazing boyfriend Mike for putting up with all my late nights in lab, even the 4am nights and working weekends! I always look forward to our weekends together, your chilled, no worries attitude to everything always makes me feel so much better after a busy week!

Finally, I would like to thank my family for all the love and support they have given me over the last 4 years of my DPhil.

This thesis is dedicated to my wonderful late Auntie Susan, you were always so proud of all that I did and achieved. I always felt your support whether it was when you hand wrote and posted me letters when I was in Minnesota or simply giving me updates over the phone about home with Stewart, Lee and Ozzy. I miss you everyday and you will always be part of my life.

Thanks to my brother Matt, you are the inspiration to why I became a scientist! Also thank you for all the random conversations, they give me endless laughs! A big thank you to my Dad for all your support and really random science quotes to always brighten my day! And a special thank you for making me hundreds of cups of tea a day while writing my thesis. I 100% got my drive to achieve goals from my mum! Your support and encouragement (and sometimes nagging) is always in the back of my mind driving me forward to complete what I set out to do!

Presentations

Burley,S., Cordero Llana,O., Beccano-Kelly,D., Lang,C., Cowley,S. and Wade-Martins,R.

Cell targeted phenotyping: Understanding the molecular genetic mechanisms of C9ORF72 ALS in motor neurons. (Poster presentation) MRC Stem Cell Partnership Workshop – Cardiff, 2018

Burley,S., Cordero Llana,O., Beccano-Kelly,D., Lang,C., Cowley,S. and Wade-Martins,R.

Understanding the molecular genetic mechanisms of C9ORF72 in Motor Neurons: Shining a light on ALS. (Invited Speaker) Max Planck Institute for Metabolism Research – Cologne, Germany, 2018

Burley,S., Cordero Llana,O., Beccano-Kelly,D., Lang,C., Cowley,S. and Wade-Martins,R.

Cell targeted phenotyping: Understanding the molecular genetic mechanisms of C9ORF72 ALS in motor neurons. (Poster presentation) Society for Neuroscience Conference – Washington DC, USA, 2017

Table of Contents

Abstract.....	2
Declaration.....	5
Acknowledgements	6
Presentations	9
Table of Contents.....	10
List of Abbreviations	17
List of Figures	21
List of Tables.....	24
Chapter 1: Introduction.....	25
1.1 Amyotrophic Lateral Sclerosis	26
1.1.1 Introduction	26
1.1.2 Clinical Presentation of ALS.....	27
1.1.3 ALS Genetics.....	28
1.1.4 Pathogenic Mechanisms of ALS Genes	31
1.1.5 Current ALS treatments.....	33
1.2 C9ORF72 Gene	34
1.2.1 C9ORF72 Discovery	34
1.2.2 Hexanucleotide repeat expansion	35
1.2.3 C9ORF72 Function.....	36
1.3 C9ORF72 ALS Disease	37

1.3.1 Disease mechanisms	37
1.3.1.1 Haploinsufficiency	37
1.3.1.2 Toxic RNA Foci	39
1.3.1.3 Toxic Dipeptide Repeat Proteins	40
1.4 C9ORF72 model systems	42
1.4.1 C9ORF72 models	42
1.4.2 iPS cells as a model system	43
1.4.3 Differentiation of iPSCs to motor neurons	44
1.5 C9ORF72 Cellular Dysfunction	47
1.6 Optogenetics Tools	50
1.7 Thesis Aims	51
Chapter 2: Materials and Methods	52
2.1 Cell Culture	53
2.1.1 Induced Pluripotent Stem Cell Culture	53
2.1.2 HEK293T Cell Culture	54
2.1.3 Lymphoblastoid Cell Culture	54
2.2 Motor Neuron Differentiation	55
2.2.1 Qu Differentiation	55
2.2.2 Du Differentiation	56
2.3 Repeat Primed PCR	59
2.3.1 Genomic DNA extraction	59
2.3.2 Repeat primed PCR	60
2.4 Immunostaining Techniques	62
2.4.1 Immunostaining	62
2.4.2 Flow Cytometry	63

2.5 RT-qPCR.....	65
2.5.1 RNA Extraction	65
2.5.2 cDNA	66
2.5.3 qPCR	66
2.6 Whole-Cell Patch Clamp Electrophysiology	68
2.6.1 Internal solution	69
2.6.2 External solution	69
2.6.3 Patch Clamp Protocols	70
2.7 Fura-2 AM.....	71
2.8 Cloning	72
2.8.1 Polymerase Chain Reaction.....	73
2.8.2 Restriction Digestion	74
2.8.3 Gel Electrophoresis and Extraction	74
2.8.4 Gibson Assembly and Hi-Fi builder.....	75
2.8.5 Bacterial Transformation	75
2.8.6 MiniPrep.....	76
2.8.7 Sequencing.....	76
2.8.8 MaxiPrep	78
2.9 Lentiviral packaging	80
2.9.1 Third Generation Lentiviral Packaging	80
2.9.2 Titering	82
2.10 Western Blot.....	83
2.11 2D Fluorescent in situ hybridisation	84
2.11.1 Probe Preparation	84
2.11.2 Slide preparation.....	85
2.11.3 Hybridisation	86

Chapter 3:	87
<i>iPSC-derived Motor Neuron Maturation</i>	87
3.1 Introduction	88
3.1.1 Methods to Determine Motor Neuron Maturation	89
3.1.1.1 Mature Motor Neuron Markers	89
3.1.1.2 Motor Neuron Functional Studies – Electrophysiology	91
3.1.2 Media to Increase iPSC Motor Neuron Maturation	95
3.1.2.1 BrainPhys	95
3.1.2.2 Synptojuice	96
3.1.3 Aims of Chapter	99
3.2 Qu motor neuron differentiation is unsuitable for electrophysiological studies	100
3.3 Du motor neuron differentiation produces motor neurons after 28 days in culture	102
3.4 Adapting Du motor neuron protocol can produce motor neurons with mature phenotypes	108
3.4.1 Flow cytometry to assess SMI-32 Expression	109
3.4.2 Immunocytochemistry and Morphology	111
3.4.3 RT-qPCR for mature motor neuron markers	114
3.4.4 Electrophysiology of motor neurons	116
3.5 Discussion	120
Chapter 4:	125
<i>C9ORF72 iPSC patient derived motor neurons display functional dysregulation</i>	125
4.1 Introduction	126
4.1.1 Expanded <i>C9ORF72</i> functional phenotypes	126
4.1.1.1 Published excitability phenotypes in <i>C9ORF72</i> gene expansion motor neurons.	127
4.1.2 Aims of Chapter	129

4.2 Induced pluripotent stem cell line selection and validation	130
4.3 ALS vs CTRL iPSC-derived MNs show comparable morphology but differing MN marker protein expression.....	134
4.4 Motor Neuron Marker mRNA Expression	140
4.5 iPSC-derived <i>C9ORF72</i> gene expansion Motor Neurons show Hyperexcitability	143
4.5.1 Spontaneous action potential firing shows a trend towards hyperexcitability in <i>C9ORF72</i> gene expansion carriers.	145
4.5.2 Evoked action potential firing hyperexcitability in <i>C9ORF72</i> expansion carriers	148
4.5.3 Sodium and Potassium Channel currents are unaffected by <i>C9ORF72</i> expansion	150
4.6 Calcium Dyshomeostasis is shown in <i>C9ORF72</i> gene expansion iPSC-derived motor neurons	156
4.7 Discussion.....	162
<i>Chapter 5:.....</i>	169
<i>Dual Cre/Lox Optogenetic Systems are Functional in Mature Motor Neurons</i>	169
5.1 Introduction.....	170
5.1.1 Channelrhodopsin for optogenetic experiments in iPSC derived motor neurons	170
5.1.1.1 ChR2 Structure	170
5.1.1.2 ChR2 Iterations.....	171
5.1.2 Motor Neuron Reporter Genes.....	173
5.1.2.1 HB9 Promoter.....	173
5.1.2.2 Cre/Lox systems	175
5.1.3 Gene Delivery.....	177
5.1.3.1 Lentiviral delivery	177
5.1.4 Aims of Chapter.....	179
5.2 F1 Optogenetic Tools	180

5.3 F1 Vector Validation	185
5.4 F1-Lentiviral Packaging Optimisation	187
5.5 F1 transduction of iPSC derived Motor Neurons.....	189
5.6 F2 Optogenetic Tools	194
5.7 Second Generation Lentiviral iPSC derived Motor Neuron Transduction	202
5.8 Discussion.....	206
Chapter 6:.....	211
<i>Differential Allele expression at the C9ORF72 locus in Heterozygous Patients</i>	<i>211</i>
6.1 Introduction.....	212
6.1.1 Lymphoblastoid cell lines	212
6.1.2 Fluorescent in situ hybridisation.....	213
6.1.3 Aims of Chapter.....	215
6.2 LCL Repeat Expansion Verification	216
6.3 Optimisation of Expanded and WT Probes	218
6.4 2D DNA FISH enables detection of both expanded and WT <i>C9ORF72</i> alleles in patient LCLs	223
6.5 Expanded <i>C9ORF72</i> shows a Heterogenous Pattern of Expression in LCLs	227
6.6 Motor Neurons Show Heterogeneous <i>C9ORF72</i> Variant Expression change	231
6.7 Discussion.....	234
Chapter 7:.....	238
<i>General Discussion.....</i>	<i>238</i>
7.1 Overall.....	239

7.2 The reduced PF protocol produced cells suitable for electrophysiology	239
7.3 Patient cells show hyperexcitability and calcium phenotypes	241
7.4 Dual promoter and Cre/Lox lentiviral constructs are successful in motor neuron differentiations	243
7.5 Fluorescent in situ hybridisation highlights the heterogeneous expression levels of <i>C9ORF72</i> in patients	244
7.6 Concluding Remarks	245
<i>References</i>.....	247

List of Abbreviations

Ω	Ohms
2D	2 - Dimensional
A	Amperes
AAV	Adeno-associated viruses
ACSF	Artificial cerebrospinal fluid
ALS	Amyotrophic lateral sclerosis
B2M	Beta-2-microglobulin
BARTs	BamHIA region transcripts
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
bp	Base pairs
C-total	Total C9ORF72
C1	C9ORF72 Variant 1
C2	C9ORF72 Variant 2
C3	C9ORF72 Variant 3
<i>C9ORF72</i>	Chromosome 9 open reading frame 72
Ca^{2+}	Calcium
ChAT	Choline acetyltransferase
ChRs	Channelrhodopsins
Cl^-	Chloride
CNTF	Ciliary neurotrophic factor

CTRL	Control
<i>DCTN1</i>	Dynactin
DENN	Differentially expressed in normal and neoplastic cells
DMH1	dorsomorphin homolog 1
DNA	Deoxyribonucleic acid
DRP	Dipeptide repeat proteins
EBV	Epstein Barr Virus
ES cells	Embryonic stem cells
F1	Fist Generation
F2	Second Generation
FISH	Fluorescent in situ hybridisation
FTD	Frontal temporal dementia
<i>FUS</i>	Fused-in-Sarcoma
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GOI	Gene of interest
GWAS	Genome wide association studies
HB9	MN and Pancreas Homeobox 1
HPRT	Hypoxanthine Phosphoribosyltransferase
HSV	Herpes simplex virus
/	Current
ICC	Immunocytochemistry
IGF	Insulin like growth factor

iPSC	Induced pluripotent stem cell
IRES	internal ribosome entry site
ISH	In situ hybridisation
ISL1	Insulin gene enhancer 1
K ⁺	Potassium
kb	Kilobases
K _v	Voltage-gated Potassium Channel
LCL	Lymphoblastoid cell line
LHX3	LIM homeobox 3
LMN	Lower motor neuron
MN	Motor Neuron
MND	Motor neuron disease
MOI	multiplicity of infection
Na ⁺	Sodium
Na _v	Voltage-gated Sodium Channel
<i>OPTN</i>	Optineurin
PAX	Paired box gene
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pF	PicoFarad
Pur	Puromorphamine
PURO	Puromycin
PYFU	Person-years of follow-up

qPCR	Quantitative polymerase chain reaction
<i>R</i>	Resistance
RA	Retinoic acid
RAN	Repeat-associated non-ATG
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription PCR
SNPs	Single nucleotide polymorphism
SOCE	Store operated Ca^{2+} entry
<i>SOD1</i>	Superoxide dismutase
SOX	SRY-related HMG-box
<i>SQSTM1</i>	Sequestosome-1
<i>TARDBP</i>	TAR DNA Binding Protein
TUJ1	Beta III tubulin
<i>UBQLN2</i>	Ubiquilin-2
UMN	Upper motor neuron
V	Voltage
V	Volts
VACht	Vesicular acetylcholine transporter
WT	Wild type

List of Figures

Figure 1.1: C9ORF72 transcript variants	35
Figure 1.2: Stages of motor neuron development compared to stages of differentiation.	45
Figure 3.1: Whole cell patch clamp configuration.....	92
Figure 3.2: Diagram of an action potential	94
Figure 3.3: Optimised Qu iPSC to motor neuron differentiation protocol	101
Figure 3.4: Preliminary characterisation of control iPSC line CTRL 2 differentiated using the Du protocol	103
Figure 3.5: Preliminary flow cytometry of control iPSCs CTRL 1 using the Du protocol	105
Figure 3.6: RT-qPCR of motor neurons marker expression throughout Du differentiation	107
Figure 3.7: Flow cytometry analysis of Adaptations to the Du MN differentiation protocol.....	110
Figure 3.8: Immunostaining and morphology of Control iPSC lines with the adapted Du MN differentiation protocol.	113
Figure 3.9: RT-qPCR of motor neurons marker expression throughout Du differentiation	115
Figure 3.10: Preliminary electrophysiological study of maturation conditions.....	117
Figure 3.11: Electrophysiological Action Potential data of maturation conditions	119
Figure 4.1: iPSC lines selection	131

Figure 4.2: Presence/absence <i>C9ORF72</i> gene repeat expansion	133
Figure 4.3: Optimised 'Reduced PF' Du motor neuron protocol.....	135
Figure 4.4: Flow cytometry analysis of patient vs control optimised Du differentiated MNs.	136
Figure 4.5: Immunocytochemistry at day 40 on iPSC-derived motor neurons.....	138
Figure 4.6: Immunocytochemistry at day 47 on iPSC-derived motor neurons.....	139
Figure 4.7: RT-qPCR of motor neurons marker expression throughout Du differentiation	141
Figure 4.8: Intrinsic properties of motor neurons at day 40 and 47.	144
Figure 4.9: Spontaneous AP activity in MNs at resting membrane potential	147
Figure 4.10: Evoked Action Potential Firing at day 40 to day 47.	149
Figure 4.11: Voltage-gated channel current at day 40 and 47.	151
Figure 4.12: Voltage-gated sodium channel current at day 40 and 47.....	152
Figure 4.13: Voltage-gated A-type and delayed rectifier Potassium current at day 40 to day 47.	155
Figure 4.14: Fura-2 AM assay at day 40 and day 47 with ionomycin injection.....	159
Figure 4.15: Fura-2 AM assay at day 40 and day 47 with FCCP injection	161
Figure 5.1: Channelrhodopsin opening in response to blue light.	171
Figure 5.2: HB9 promoter optimisations/truncations	174
Figure 5.3: Recombination of CRE at lox sites.....	176
Figure 5.4: Generation of first generation optogenetic tools	182
Figure 5.5: Generation of first generation optogenetic tools	184
Figure 5.6: Initial test of optogenetic/reporter in HEK 293T cells.....	186

Figure 5.7: Lentiviral transduction of iPSC derived motor neurons using the Qu protocol	190
Figure 5.8: Lentiviral transduction of iPSC derived motor neurons using the optimised Du protocol	193
Figure 5.9: CRE/LOX second generation optogenetic tool/reporter	195
Figure 5.10: Generation of second generation optogenetic tools – insertion of HB9 CRE.	198
Figure 5.11: Generation of second generation optogenetic tools – insertion of EF1 cassette.	200
Figure 5.12: Generation of second generation optogenetic tools – insertion of Hb9 CRE-M.	201
Figure 5.13: Second generation optogenetic tool/reporter lentiviral transduction into iPSCs.....	204
Figure 5.14: Validation of Second generation optogenetic tool/reporter expressing GFP and mCherry.....	205
Figure 6.1: Repeat expansion present in LCL samples.....	217
Figure 6.2 Labelling C9ORF72 fosmid for 2D FISH	220
Figure 6.3: Optimisation of FISH oligos and probes in LCLs	222
Figure 6.4: <i>C9ORF72</i> expression in LCLs using FISH and qPCR	226
Figure 6.5: <i>C9ORF72</i> expression in LCLs using 2D FISH and qPCR	228
Figure 6.6: <i>C9ORF72</i> expression in LCLs using 2D FISH and qPCR	230
Figure 6.7: <i>C9ORF72</i> expression in iPSC derived motor neurons using qPCR.....	233

List of Tables

Table 1: Genes associated with ALS	30
Table 2.1: Primers used for Repeat Primed PCR	61
Table 2.2: PCR set up for Repeat Primed PCR	61
Table 2.3: Primary Antibodies used for Immunostaining	62
Table 2.4: Primary Antibodies and IgG controls used for Flow Cytometry	64
Table 2.5: RT-qPCR optimised primers	67
Table 2.6: Internal Solution components for electrophysiology.....	69
Table 2.7: External Solution components for electrophysiology	69
Table 2.8: PCR primers used to clone the successful second generation optogenetic/report construct pHb9-CREM-EF1-floxGFP-floxChR2mCherry.....	73
Table 2.9: Primers used for Big dye Sequencing of CREM-EF1-floxGFP-floxChR2mCherry	77
Table 2.10: BigDye Terminator v3.1 PCR set up.....	78
Table 2.11: Mix 1 Lipofectamine transfection	81
Table 2.12: Mix 2 Lipofectamine transfection	81
Table 2.13: Ca ₃ (PO ₄) ₂ transfection reagents.....	81
Table 3.1: Components of Synaptojuice	98
Table 4.1: Two- way RM ANOVA posthoc Sidak's multiple comparisons results	142

Chapter 1: Introduction

1.1 Amyotrophic Lateral Sclerosis

1.1.1 Introduction

Amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease, is the most common form of adult-onset motor neuron disease (MND) with a crude global incidence of approximately 1.75/100,000 person-years of follow up (PYFU) (Marin *et al.*, 2017).

Globally, incidence is consistent with the exception of geographical foci on the Pacific island of Guam (Bradley *et al.*, 2009) and Kii peninsula in Japan (Ishiura *et al.*, 2012).

ALS affects both the upper and lower motor neurons as well as extramotor systems including temporal, behavioural and executive frontal circuits in certain patients (Barson *et al.*, 2000; Lomen-Hoerth *et al.*, 2003). The age of symptom onset ranges from 50 to 65 years old, over 75 years old there is a decline in disease incidence (Barson *et al.*, 2000; Lomen-Hoerth *et al.*, 2003). At present there is no cure for ALS, and disease progression leads to increasing paralysis and often death from respiratory failure within 2-3 years of symptom appearance/diagnosis. Only 10-20% of patients survive 5 years and 5-10% of patients 10 years following diagnosis due to the rapid progression of the disease (Chiò *et al.*, 2009).

1.1.2 Clinical Presentation of ALS

ALS characteristically impacts the upper motor neurons (UMN) and lower motor neurons (LMN) including brainstem and spinal cord regions, however, presentation is varied among patients and characteristic UMN and LMN loss may not be present in early stages of disease leading to difficulty in diagnosis (Davenport *et al.*, 1996).

Disease phenotype is important for predicating prognosis and survival, the site of symptom onset is used to define the disease phenotype. Initial presentation of limb symptoms is the most common onset shown by 70% of patients. 25% of patients present with bulbar onset with either dysarthria or dysphagia and 5% of patients present with respiratory onset (Testa *et al.*, 2004; Kiernan, 2007; Knibb *et al.*, 2016). Patients with limb-onset ALS have a better prognosis than bulbar onset, while respiratory onset suggests the worst prognosis for patients (de Carvalho *et al.*, 1996; Louwerse *et al.*, 1997; Del Aguila *et al.*, 2003; Testa *et al.*, 2004). Improved survival is associated with younger age of diagnosis, patients may survive as long as 10 years if diagnosed before 40 years old (Preux *et al.*, 1996; Louwerse *et al.*, 1997; Del Aguila *et al.*, 2003; Testa *et al.*, 2004).

Motor symptoms dominate disease progression; however, non-motor symptoms are present in some patients including: sensory, autonomic and extrapyramidal symptoms.

Several small and large scale clinical studies have confirmed that cognitive function is impaired in a proportion of ALS patients (Gallassi *et al.*, 1989; Kew *et al.*, 1993; Massman *et al.*, 1996; Abe *et al.*, 1997; Abrahams *et al.*, 1997, 2000, 2005; Ringholz *et al.*, 2005; Witgert *et al.*, 2010). 50% of patients show signs of cognitive impairment and 10-15%

have diagnosed frontal temporal dementia (FTD) (Ringholz *et al.*, 2005; Phukan *et al.*, 2012).

1.1.3 ALS Genetics

Genetically inheritable ALS known as Familial ALS accounts for 5-10% of all ALS cases, the remaining cases are known as sporadic ALS. The basis of sporadic ALS is unclear, however, twin studies have shown as high as 60% heritability suggesting a large proportion of cases involve a genetic component (Al-Chalabi *et al.*, 2010; McLaughlin, Vajda and Hardiman, 2015). These statistics suggest genetics to be a main contributor and important to study for ALS.

Many early ALS genes were discovered using familial linkage analysis which is a form of genetic mapping. Linkage analysis was first described in 1913, parental crosses are observed which vary at marker sites (polymorphic variants) and a Mendelian trait. Markers and traits are thought to lie close in the genome if (due to meiotic recombination) they display correlated segregation (Sturtevant, 1913; Altshuler, Daly and Lander, 2008).

As technology advanced, whole genome and exome sequencing was possible which in combination with linkage analysis, are valuable tools for discovering ALS associated genes. Genome wide association studies (GWAS) are now commonly used to study sporadic ALS as it is thought to result from multiple genetics variants. GWAS usually focus on single-nucleotide polymorphisms (SNPs) and are based on associations between (disease) traits and genetic loci. GWAS do not typically identify causal variants but

identify regions of linkage disequilibrium in which causal variants are located (Bush and Moore, 2012).

Since the discovery in 1993 of the first ALS causative mutations in Superoxide Dismutase (SOD1) using linkage analysis, more than 50 other potential genes have been discovered (Rosen *et al.*, 1993). Table 1 includes a list of genes with mutations known to be causative factors for ALS (Taylor, Brown and Cleveland, 2016; Chia, Chiò and Traynor, 2018).

Locus	Year identified	Gene	Protein function	Mode of Inheritance	Contribution to familial or Sporadic ALS (%)		References
					familial	sporadic	
21q22.1	1993	<i>SOD1</i>	Superoxide dismutase	Dominant, recessive, de novo	12	1-2	(Rosen <i>et al.</i> , 1993)
2p13	2003	<i>DCTN1</i>	Component of dynein motor complex	Dominant, risk factor	1	1	(Puls <i>et al.</i> , 2003)
20q13.3	2004	<i>VAPB</i>	Involvement in proteostasis	Dominant	-	-	(Nishimura <i>et al.</i> , 2004)
14q11	2006	<i>ANG</i>	Ribonuclease	Risk factor	<1	<1	(Greenway <i>et al.</i> , 2006)
Q36	2008	<i>TARDBP</i>	RNA-binding protein	Dominant, recessive, de novo	5	<1	(Kabashi <i>et al.</i> , 2008; Sreedharan <i>et al.</i> , 2008)
16p11.2	2009	<i>FUS</i>	RNA-binding protein	Dominant, recessive, de novo	5	<1	(Kwiatkowski <i>et al.</i> , 2009; Vance <i>et al.</i> , 2009)
9p13.3	2010	<i>VCP</i>	Ubiquitin segregase	Dominant, de novo	1-2	<1	(Johnson <i>et al.</i> , 2010)
10p15-p14	2010	<i>OPTN</i>	Autophagy adaptor	Dominant, recessive	4	1	(Maruyama <i>et al.</i> , 2010)
9p21-22	2011	<i>C9ORF72</i>	Suggested guanine nucleotide exchange factor	Dominant	40	7	(DeJesus-Hernandez <i>et al.</i> , 2011; Renton <i>et al.</i> , 2011)
Xp11.23-Xp13.1	2011	<i>UBQLN2</i>	Autophagy adaptor	X-linked dominant	<1	<1	(Deng <i>et al.</i> , 2011)
5q35	2011	<i>SQSTM1</i>	Autophagy adaptor	Dominant	<1	-	(Fecto <i>et al.</i> , 2011; Teyssou <i>et al.</i> , 2013)
17p13.2	2012	<i>PFN1</i>	Actin-binding protein	Dominant	<1	<1	(Wu <i>et al.</i> , 2012)
12q13.1	2013	<i>HNRNPA1</i>	RNA-binding protein	Dominant, de novo, risk factor	<1	<1	(Kim <i>et al.</i> , 2013; Q. Liu <i>et al.</i> , 2016)
5q31.2	2014	<i>MATR3</i>	RNA-binding protein	Dominant	<1	<1	(Johnson <i>et al.</i> , 2014)
2q36.1	2014	<i>TUBA4A</i>	Microtubule subunit	Dominant	<1	<1	(Smith <i>et al.</i> , 2014)
22q11.23	2014	<i>CHCHD 10</i>	Mitochondrial protein	Dominant	<1	<1	(Bannwarth <i>et al.</i> , 2014)
12q14.1	2015	Serine/threonine-protein kinase TBK1	Regulates autophagy and inflammation	Dominant, de novo	-	-	(Freischmidt <i>et al.</i> , 2015)

Table 1: Genes associated with ALS

Adapted from:(Taylor, Brown and Cleveland, 2016; Chia, Chiò and Traynor, 2018)

1.1.4 Pathogenic Mechanisms of ALS Genes

Many ALS pathogenic mechanisms have been hypothesised, a combination of which are the likely cause of ALS neurodegeneration. Mechanisms hypothesised to be involved (some of which will be discussed below) include: mitochondrial dysfunction, oxidative stress, excitotoxicity, dysfunctional RNA processing, altered axonal transport as well as other mechanisms such as dysfunctional protein degradation and autoimmunity. Often the known functions of ALS genes are used to infer potential motor neuron vulnerability. Morphologically disrupted mitochondria, observed as vacuolated and dilated with disorganised cristae, have been reported in both sporadic and familial ALS (Higgins, Jung and Xu, 2003; Sasaki and Iwata, 2007). Increased levels of mutated mitochondrial DNA but lower overall mitochondrial DNA levels were reported in spinal cords of ALS patients as well as decreased activity of citrate synthase as well as other respiratory chain complexes (Wiedemann *et al.*, 2002). Damaged mitochondria can lead to many downstream detrimental effects in cells as seen in SOD1 mice and patients including ionic imbalance due to deficiency of ATP needed to drive ATP-dependent ion pumps, abnormal ROS (reactive oxygen species) production, perturbed calcium buffering and apoptosis triggering (Pasinelli *et al.*, 1998; Mattiazzi *et al.*, 2002; Damiano *et al.*, 2006). ALS studies using patient motor cortex and spinal cord samples have also linked glutamate excitotoxicity to reduced glutamate clearance from the synaptic cleft by astrocytes (Lin *et al.*, 1998). Increased glutamate in the synaptic cleft leads to overstimulation of neurotransmitter receptors and increased firing of motor neurons as well as an influx of calcium (Vucic, Rothstein and Kiernan, 2014).

Thirdly, RNA processing dysfunction is reported in ALS; processing can be affected at many levels including: transcription, splicing, mRNA transport, RNA stability, translation to protein and RNA degradation. Many forms of ALS and FTD show ubiquitin-positive, tau- negative, TAR DNA-binding protein 43 (TDP-43) positive inclusions throughout the central nervous system neurons and glia. TDP-43 is a nuclear RNA binding protein known to regulate alternative splicing, which becomes mis-localised from the nucleus in most forms of ALS (excluding SOD1 and FUS associated ALS) to form these hallmark cytoplasmic inclusions (Buratti and Baralle, 2001; Arai *et al.*, 2006; Neumann *et al.*, 2006; Mackenzie *et al.*, 2007; Vance *et al.*, 2009). Several studies have reported splicing dysfunction and aberrant RNA including processing of the astrocyte *EAAT2* transporter resulting in aberrant *EAAT2* mRNA, which is suggested to be the cause of perturbed glutamate clearance from the synaptic cleft (Lin *et al.*, 1998; Rabin *et al.*, 2010).

Motor neurons rely heavily on effective axonal transport to transport organelles, RNA, proteins, lipids and other cellular components from cell bodies to synapses which can be as far as 1 metre in length from the cell body in humans. Dysfunctional axonal transport is reported in ALS patients, slowing of retrograde transport is seen in *SOD1* ALS and dynactin (*DCTN1*) ALS as well as slowing of anterograde transport in *SOD1* ALS (Williamson and Cleveland, 1999; Puls *et al.*, 2003; Perlson *et al.*, 2009). Altered axonal transport can lead to an accumulation of three main cargos including mitochondria, autophagosomes and neurofilaments which in turn leads to motor neuron death (reviewed (Ikenaka *et al.*, 2012)).

Overall It is clear there are multiple pathways of disease converging in ALS creating a complex disease phenotype.

1.1.5 Current ALS treatments

Although ALS was first described in 1869, the treatment options are still very limited (Goetz, 2000) likely due to the highly complex nature of ALS. Riluzole is currently the only approved drug in Europe and the USA for ALS treatment. In human trials it demonstrates modest improvement in slowing disease progression by approximately 3-6 months with highest efficiency seen in later stages of disease (Bensimon, Lacomblez and Meininger, 1994; Lacomblez *et al.*, 1996; Miller, Mitchell and Moore, 2012; Fang *et al.*, 2018).

Riluzole's mechanism of action is however still uncertain, many pathways are suggested including (but not limited to): altered fat metabolism, mitochondrial function, effects on axonal sodium channels and modulation of excitotoxicity by anti-glutamatergic affects (Fang *et al.*, 2018). Non-invasive ventilation to support weak respiratory muscles and gastrostomy feeding to circumvent dysphagia are surgical options that are used/offered to prolong life in patients (Bourke *et al.*, 2006).

Currently treatment options only moderately extend the life of patients, no cure or long-term treatments are available to allow patients a normal life span emphasising the need for continued research into disease mechanisms and drug trials.

1.2 *C9ORF72* Gene

1.2.1 *C9ORF72* Discovery

Linkage analysis combined with GWAS and next generation sequencing lead to the discovery of a hexanucleotide mutation in chromosome 9 open reading frame 72 (*C9ORF72*) (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011).

Strong links between ALS and FTD highlighted a 3.7 Mb region on the short arm of chromosome 9 (9p21) as an area of disease interest using linkage analysis of ALS, FTD and ALS-FTD cases (Morita *et al.*, 2006; Vance *et al.*, 2006; Valdmanis *et al.*, 2007; Boxer *et al.*, 2011; Pearson *et al.*, 2011). Several large ALS and FTD genome wide-association studies confirmed the importance of region 9p21 (van Es *et al.*, 2009; Laaksovirta *et al.*, 2010; Shatunov *et al.*, 2010; Van Deerlin *et al.*, 2010). A study of the Finnish population further refined the region of interest to a 232 kb block of linkage disequilibrium, with the identification of a founder haplotype also present in European-ancestry populations (Mok *et al.*, 2012).

Next generation, deep resequencing lead to the discovery of the GGGGCC hexanucleotide repeat expansion within this region, which was confirmed using repeat primed PCR (polymerase chain reaction) and fluorescent in situ hybridisation (FISH) (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011).

Since its discovery in 2011, the *C9ORF72* expansion is still the most common form of both sporadic ALS and FTD, accounting for 5-7%. *C9ORF72* ALS also accounts for 40% of

familial ALS and 25% familial FTD (Laaksovirta *et al.*, 2010; Byrne *et al.*, 2012; Mahoney *et al.*, 2012; Majounie *et al.*, 2012).

Studies have shown that patients with the *C9ORF72* gene expansion tend to have increased bulbar-onset ALS incidence, accelerated disease progression, increased risk of cognitive impairment and earlier disease onset (Byrne *et al.*, 2012; Chio *et al.*, 2012; Stewart *et al.*, 2012; Irwin *et al.*, 2013).

1.2.2 Hexanucleotide repeat expansion

The *C9ORF72* gene yields three predicted mRNA transcript variants that code for a short and long form of the protein (Figure 1.1). Variant 1 and 3 both encode the long 481 amino acid form of the protein encoded by exons 2-11. Variant 2 encoded by exons 2-5, is the shorter form, only 222 amino acids in length. In variant 2 the repeat expansion is located in the promoter whereas in variants 1 and 3 the repeat expansion is located intronically between two alternatively spliced exons (DeJesus-Hernandez *et al.*, 2011).

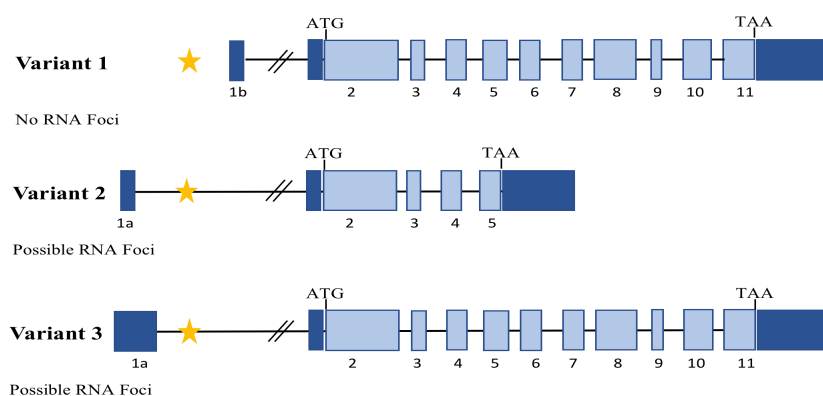


Figure 1.1: C9ORF72 transcript variants

Light blue boxes denote coding regions and dark blue non-coding. The hexanucleotide expansion is shown as a yellow star. Adapted from DeJesus-Hernandez *et al.*, 2011.

Healthy individuals generally have *C9ORF72* gene expansion repeat lengths of no longer than 2-23 repeats, whereas patients can show repeat lengths up to 1000s of repeats (DeJesus-Hernandez *et al.*, 2011).

1.2.3 C9ORF72 Function

Understanding the normal function of a disease-causing gene can have great advantages to drug discovery and understanding disease mechanisms. Unfortunately, the function of *C9ORF72* protein is poorly understood. *C9ORF72* contains a DENN (DENN – differentially expressed in normal and neoplastic cells) domain, which is known to interact with RABs typically as guanine-nucleotide exchange factors (GEF) in the activation/exchange of GDP for GTP (Levivier *et al.*, 2001; Yoshimura *et al.*, 2010). Therefore the *C9ORF72* protein is thought to also act as a guanine-nucleotide exchange factor for RABs which has been confirmed using RAB8a and RAB39b (Sellier *et al.*, 2016). RABs are the largest group of small GTPases and are involved in the process of membrane trafficking by recruiting effectors e.g. tethering factors and motors (Stenmark, 2009). Studies have also shown *C9ORF72* vesicles colocalising with early endosome proteins RAB5 and EEA1 (Shi *et al.*, 2018). Research needs to continue to fully understand the relationship between RABs and *C9ORF72* and its role in membrane trafficking.

1.3 C9ORF72 ALS Disease

1.3.1 Disease mechanisms

The mechanism by which expanded *C9ORF72* ultimately causes ALS remains unclear, there are three non-mutually exclusive mechanisms of pathophysiology hypothesised: haploinsufficiency, toxic RNA and toxic dipeptide repeat proteins. The contribution of each to disease causation is unknown.

1.3.1.1 Haploinsufficiency

Haploinsufficiency refers to the mechanism of disease presented if one copy of a gene does not produce enough functional protein to maintain normal processes in an organism if the second copy is non-functional (Deutschbauer *et al.*, 2005).

Although the function of C9ORF72 protein is unclear some studies have suggested that the loss of C9ORF72 protein by haploinsufficiency could lead to the disease phenotypes observed.

Post-mortem tissues and blood samples from patients with expanded *C9ORF72* have shown reduced levels of *C9ORF72* mRNA (DeJesus-Hernandez *et al.*, 2011; Gijssels *et al.*, 2012; Belzil *et al.*, 2013; Ciura *et al.*, 2013; Donnelly *et al.*, 2013). Reduced mRNA levels are also observed in vitro in lymphoblastoid cells and iPSC derived neurons (DeJesus-Hernandez *et al.*, 2011; Ciura *et al.*, 2013; Donnelly *et al.*, 2013; Shi *et al.*, 2018). Additionally reduced protein levels are found in the prefrontal cortex of patients

(Waite *et al.*, 2014). The reduced mRNA expression is thought to be due to abortive transcription resulting from the formation of G-quadruplex and R-loop structures in the DNA repeats (Fratta *et al.*, 2012; Reddy *et al.*, 2013; Haeusler *et al.*, 2014).

Hypermethylation as seen in Friedreich's ataxia and Fragile-X syndrome could also cause the silencing of the expanded allele by hypermethylation of CpG islands (Al-Mahdawi *et al.*, 2007; Xi *et al.*, 2013; Mor-Shaked and Eiges, 2018). The reduced levels of mRNA and protein could lead to haploinsufficiency however, this phenotype is not consistently reported, some studies have shown downregulation of some variants but upregulation of others (Almeida *et al.*, 2013; Cohen-Hadad *et al.*, 2016).

Functionally, loss of zC9ORF72 in Zebra Fish embryonic development leads to motor phenotypes and behavioural deficits which are recovered with *hC9ORF72* mRNA overexpression, supporting the C9ORF72 loss of function hypothesis (Ciura *et al.*, 2013). However, haploinsufficiency has been refuted by several studies in mice where antisense oligos targeting endogenous wild type (WT) *C9ORF72* mRNA do not produce motor or behavioural phenotypes (Lagier-Tourenne *et al.*, 2013). Conditional knockout of C9ORF72 in neurons and glia in mice also does not show hallmarks of ALS including motor neuron death, TDP-43 or gliosis (Koppers *et al.*, 2015).

Finally, complete C9ORF72 knockout (KO) mice develop abnormal macrophages, microglia, age-related neuroinflammation, autoimmunity, splenomegaly and lymphadenopathy (Atanasio *et al.*, 2016; Burberry *et al.*, 2016; Jiang *et al.*, 2016; ORourke *et al.*, 2016) and yet do not show any motor deficits or ALS symptoms. This suggests *C9ORF72* ALS could have a distinct inflammatory component.

These results suggest that loss of C9ORF72 alone does not cause disease but do not exclude that C9ORF72 loss of function could cause other phenotypes within the organism such as immune dysfunction or modulate disease onset, duration and severity.

1.3.1.2 Toxic RNA Foci

RNA foci can be seen in the brain and spinal cord of *C9ORF72* patients (DeJesus-Hernandez *et al.*, 2011, 2017). These foci can be composed of sense or anti-sense RNA as bidirectional transcription is evident (Gendron *et al.*, 2013; Zu *et al.*, 2013; DeJesus-Hernandez *et al.*, 2017). RNA foci sequester RNA binding proteins and splicing factors which recognise the repeat sequences in a similar disease mechanism to myotonic dystrophy type 1 (Taneja *et al.*, 1995; Davis *et al.*, 1997; Mankodi *et al.*, 2001; Jiang *et al.*, 2004).

In *C9ORF72* ALS several RNA-binding proteins have been suggested as sequestered by the RNA foci including several heterogeneous nuclear ribonucleoproteins (hnRNPs) (hnRNP H1/F, hnRNPA3, hnRNPA1 and hnRNP-H) (Kohji Mori, Lammich, *et al.*, 2013; Lee *et al.*, 2013; Cooper-Knock *et al.*, 2014) as well as several other proteins including ADARB2 and ASF/SF2 (Donnelly *et al.*, 2013; Reddy *et al.*, 2013).

Again, it appears that RNA foci alone cannot cause *C9ORF72* ALS. A *Drosophila* repeat expansion model containing the hexanucleotide repeat expansion with stop codons to prevent repeat-associated non-ATG (RAN) translation to allow study of purely RNA foci, exhibited no phenotypes (Mizielinska *et al.*, 2014).

Additionally if RNA foci burden was a determining disease factor it would be anticipated that FTD patients would have a higher foci load in the frontal cortex compared to MND

patients, however this is not observed (DeJesus-Hernandez *et al.*, 2017). Finally as discussed below mouse models displaying RNA foci do not always show neurodegeneration or motor neuron loss (O'Rourke *et al.*, 2015; Peters *et al.*, 2015).

1.3.1.3 Toxic Dipeptide Repeat Proteins

Toxic proteins are the final suggested mechanism of disease. Toxic protein is translated from the bidirectionally transcribed repeats by repeat-associated non-ATG (RAN) translation (Ash *et al.*, 2013; Zu *et al.*, 2013). RAN translation causes the production of 6 dipeptide repeat proteins (DRPs) including: glycine-alanine (GA), glycine-arginine (GR) from the sense strand, proline-arginine (PR), proline-alanine (PA) from the antisense strand and glycine-proline produced from both. DRPs accumulate and form aggregates throughout the central nervous system (Ash *et al.*, 2013; K. Mori *et al.*, 2013; Kohji Mori, Arzberger, *et al.*, 2013). However DRPs are usually not present in cells with RNA foci (Gendron *et al.*, 2013).

In vitro studies have shown that GA DPRs cause neurotoxicity evidenced by active caspase-3, ER stress and reduced proteasome activity within cells without the presence of RNA foci (Zhang *et al.*, 2014). In vivo studies in mice looking at GA in the central nervous system showed that the inclusions sequester ubiquitin-proteasome related proteins such as HR23 and other nucleocytoplasmic proteins such as RanGAP1 and Pom121 (Y.-J. Zhang *et al.*, 2016). Recently GR and PR DPRs are increasingly studied as these are thought to be the most toxic repeats. When applied exogenously in vitro to cells they cause global deficits in mRNA splicing and biogenesis of ribosomal RNA leading to cell death (Kwon *et al.*, 2014).

In addition to neuronal toxicity DPRs also cause mis-splicing of glutamate transporter 'excitatory amino acid transporter 2' (EAAT2) in astrocytes which could contribute to excitotoxicity phenotypes seen in neurons (Kwon *et al.*, 2014).

Although seemingly toxic in model systems there is a lack of evidence to support this toxicity in humans. It has been suggested, as seen for RNA inclusions, that it would be expected that these inclusions accumulate in the most affected areas of disease or level of accumulation correlate with disease severity however, post mortem tissue does not show this (Davidson *et al.*, 2014; Mackenzie *et al.*, 2015; Schludi *et al.*, 2015).

1.4 *C9ORF72* model systems

1.4.1 *C9ORF72* models

As human motor neurons cannot easily be studied in vivo and cannot be used as primary cultures there is a clear need for model systems to study the effects of the expansion, disease mechanisms and allow for drug testing.

The first repeat expansion mouse model was created in 2015 carrying 66 G4C2 repeats and partial surrounding regions of the human *C9ORF72* gene transduced into the CNS of C57BL/6J mice. RNA foci were seen in these mice as well as RAN translation, TDP-43 inclusions and motor deficits, however no motor neuron loss in the spinal cord was described (Chew et al., 2015).

Other models using bacterial artificial chromosome (BAC) technology to clone a large portion (exons1-6) of the human *C9ORF72* gene with 500 repeats in SJL/B6 mice (Peters et al., 2015) or full length *C9ORF72* with 100-1000 repeats into C57BL/6J mice (O'Rourke et al., 2015), again showed RNA foci as well as RAN translation. However, no other ALS associated phenotypes were present including TDP-43 inclusions, neuroinflammation or neuronal cell death. The full-length BAC did however show evidence of nucleolar stress. Since these first models other mouse models were created again with limited success in recapitulating human ALS phenotypes, the most successful again using a full *C9ORF72* gene BAC in FVB/NJ mice displayed hallmark pathology including RNA foci, TDP- 43 inclusions and motor neuron loss (Y. Liu et al., 2016).

1.4.2 iPS cells as a model system

As well as mouse models, induced pluripotent stem cell (iPSC) models as discussed below are becoming increasingly utilised to study ALS.

Differentiating iPS cells to the cell of interest allows not only the study of the cell type of interest but also allows for study of the mutation of interest while maintaining the genetic background of the patient. Maintaining the genetic background of patients and controls can be important to study disease and gene interplay.

iPS cells were created to resemble the properties of embryonic stem cells. Embryonic stem cells are able to differentiate into all three germ layers and grow indefinitely in culture while maintaining pluripotency (Evans and Kaufman, 1981; Martin, 1981). Studies of embryonic stem cells showed their benefit for disease modelling and potential for treatments however there were several issues to their usage. Embryonic stem (ES) cells are part of the inner cell mass of blastocysts (Evans and Kaufman, 1981; Martin, 1981), which creates ethical issues in terms of using human embryos, additionally transplantation of ES cell derivatives could also cause tissues rejection issues. The use of iPS cells circumvents both of these issues as these cells can be generated from the adult cells of the patients themselves.

Several areas of research lead to the creation of iPS cells. Firstly, nuclear transfer reprogramming showed that differentiated cells do not lose the genetic information needed for the development of complete organisms but also that oocytes have the ability to reprogram cells. Nuclear transfer reprogramming was first achieved by

Gurdon's group in 1962 in tadpoles (Gurdon, 1962) then shown in mammals by the birth of Dolly the sheep (Wilmut *et al.*, 1997).

Following these studies iPS cells were created in 2006 from mouse fibroblasts by retroviral expression of 4 transcription factors, Klf4, Sox2, c-Myc and Oct-4. These 4 transcription factors were found from screening 24 candidate pluripotency factors using an elegant drug resistance screening assay. A β geo cassette was inserted in the Fbx15 locus (a gene expressed in ES cells and embryos) therefore cells were only drug resistant if presenting an ES-like state (Takahashi and Yamanaka, 2006).

In 2007 the creation of human iPSCs was achieved by two groups using slightly different combinations of factors: KLF4, SOX2, c-MYC and OCT-4 (Takahashi *et al.*, 2007) or OCT4, SOX2, NANOG, and LIN28 (Yu *et al.*, 2007).

1.4.3 Differentiation of iPSCs to motor neurons

Since the first generation of human ES cells they have been differentiated into many cell types and used extensively as disease models. The first ES-derived neurons with motor neuron-like characteristics were generated in 1998 using mouse ES cells (Renoncourt *et al.*, 1998). Following the 1998 study, more defined mouse ES differentiation protocols were developed based on developmental signalling factors (Wichterle *et al.*, 2002) shortly followed by the development of human ES cell MN differentiation protocols (Miles *et al.*, 2004; Li *et al.*, 2005; Shin, Dalton and Stice, 2005; Di Giorgio *et al.*, 2008; Karumbayaram *et al.*, 2009).

However, due to ethical issues as discussed Chapter 1.4.2, the use of iPSCs is now favoured in place of ES cells. Since the generation of iPSCs in 2007 many iPSC motor neuron differentiation protocols have emerged with varying lengths of differentiation and final percentages of motor neurons produced (Lee *et al.*, 2007; Dimos *et al.*, 2008; Hu and Zhang, 2009; Patani *et al.*, 2011; Amoroso *et al.*, 2013; Sareen *et al.*, 2013; Qu *et al.*, 2014; Devlin *et al.*, 2015; Du *et al.*, 2015; Maury *et al.*, 2015). As with ES cell differentiation protocols, iPSC protocols share a common basis of recapitulation of the developmental pathway of motor neurons Figure 1.2.

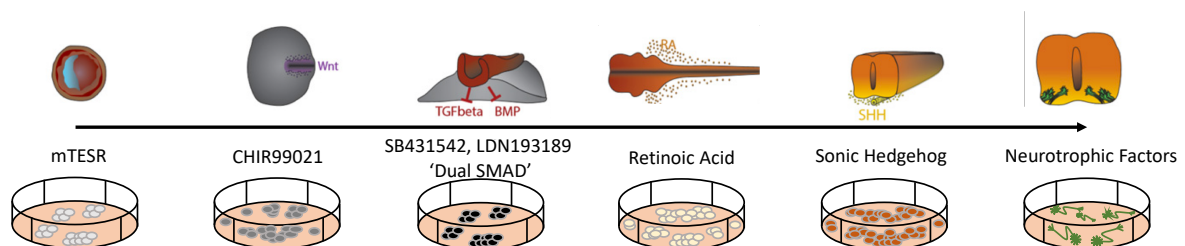


Figure 1.2: Stages of motor neuron development compared to stages of differentiation.

Adapted from Sances *et al.*, 2016.

Development and specification of lower motor neurons begins during gastrulation when ectodermal cells are initially anterior-posterior patterned by fibroblast growth factors (FGFs) and Wingless-type MMTV integration site group members (Wnts), followed by neuroectoderm specification through mesoderm and endoderm factor inhibition (Waddington, 1936; Hamburger, 1989; Hemmati-Brivanlou and Melton, 1997). In differentiation protocols the initial Wnt/FGF patterning is recapitulated using GSK3 inhibitor CHIR99021 (Wnt activator) followed by the neuroectoderm phase of inhibition of mesoderm/endoderm factors, bone morphogenic proteins (BMPs) and transforming

growth factor beta (TGFbeta) in a common stage called dual-SMAD inhibition. Chambers in 2009 were the first group to apply the dual SMAD inhibition method using small molecules SB431542 (TGF beta inhibitor) and LDN193189 (BMP inhibitor) to produce an enriched neural ectoderm population evidenced by high percentages of cells expressing paired box protein 6 (PAX 6) and sex determining region Y-box 2 (SOX2) (Chambers *et al.*, 2009).

Following dual SMAD inhibition neuroectoderm is specified rostro-caudal and dorso-ventral by signalling gradients. Retinoic acid (RA) is produced by the enzyme Aldehyde Dehydrogenase 1 Family member A2 (Aldh1a2) which is expressed by presomitic mesoderm. RA with Wnt signalling, induces caudal hindbrain subtypes and rostral spinal cord neuronal subtypes. RA decreases down the spinal cord as inhibitory FGFs increase and Aldh1a2 decrease (Liu, Laufer and Jessell, 2001; Nordström, Jessell and Edlund, 2002). Sonic Hedgehog (SHH) is a key signalling molecule involved in dorsoventral patterning and is critical for motor neuron development (Roelink *et al.*, 1995; Ericson *et al.*, 1996). It follows that most differentiation protocols after dual SMAD inhibition follow with a caudalization phase using retinoic acid and a ventralisation phase with SHH.

Newer protocols have tried to make the process more efficient by changing systematically the timing of addition of compounds, the use of synthetic compounds in place of protein and the concentrations and combinations used (Du *et al.*, 2015; Maury *et al.*, 2015). The protocols can achieve yields of 95% neurons and 60% motor neurons (Sances *et al.*, 2016; Selvaraj *et al.*, 2018)

1.5 C9ORF72 Cellular Dysfunction

Although as discussed above it is not clear how mutant C9ORF72 protein causes disease directly it is clear it that causes changes to normal cellular function, particularly neuronal excitability, neurotransmitter receptor accumulation and calcium dyshomeostasis.

Given C9ORF72's suggested normal function as a guanine-nucleotide exchange factor for RABs it is unsurprising that studies have suggested cells with mutant the *C9ORF72* gene expansion exhibit abhorrent vesicle trafficking. A recent study by Shi *et al* showed C9ORF72 protein colocalization in vesicles with early endosome makers RAB5 and EEA1. The group also reported dysfunctional mannose-6-phosphate receptor vesicles in iPSC-derived motor neurons harbouring the *C9ORF72* gene expansion which usually traffic lysosomal proteins from the *trans*-golgi to endosomes for lysosome formation (Kornfeld and Mellman, 1989). Subsequently iPSC-derived motor neurons contained fewer lysosomes which is consistent with *C9ORF72* conditional knockout models (Koppers *et al.*, 2015; Shi *et al.*, 2018).

In connection with abhorrent lysosomal degradation, this study as well as others, have shown increased glutamate receptors in *C9ORF72* gene expansion neurons. In cortical neurons the lysosomal pathway is crucial for synaptic plasticity and leads to the degradation of glutamate receptors from chronic signalling (Wang, Gilbert and Man, 2012). Therefore, dysregulation of this pathway could lead to glutamate receptor accumulation. Increased glutamate receptors NR1 and AMPA GluR1 are described in iPSC derived motor neuron studies, in *C9ORF72* gene null mice and in patient spinal cord and motor cortices (Selvaraj *et al.*, 2018; Shi *et al.*, 2018). In association with increased

glutamate receptors, studies have shown excitotoxicity of neurons in response to glutamate (Donnelly *et al.*, 2013; Selvaraj *et al.*, 2018; Shi *et al.*, 2018).

As well as increased glutamate receptors, dysfunctional clearance of excess glutamate from the synaptic cleft by astrocytes (via the excitatory amino acid transporter 2 (EAAT2)) has been suggested as contributing to excitotoxicity, as seen in SOD1^{G93A} mutant rodent models and patient post-mortem motor cortex samples (Rothstein *et al.*, 1995; Howland *et al.*, 2002).

Although excitotoxicity causes excessive firing of motor neurons, there remains disagreement in the field in relation to the excitability of *C9ORF72* gene expansion iPSC-derived motor neurons. Many factors contribute to the excitability of neurons including inhibitory inputs from interneurons, sodium and potassium channel function, as well as extracellular potassium homeostasis (Do-Ha, Buskila and Ooi, 2018). Both hyper- (Wainger *et al.*, 2014) and hypo- (Sareen *et al.*, 2013) excitability has been described in iPSC patient derived *C9ORF72* gene expansion motor neurons, as well as models showing a shift from a hyper- to hypo- excitable state (Devlin *et al.*, 2015).

Calcium also plays a huge role in synaptic function including regulating neurotransmitter release (Harvey and MacIntosh, 1940). Overload of calcium in the cytoplasm by excitotoxicity mechanisms has been suggested to cause endoplasmic reticulum (ER) stress as shown by a recent study by Dafinca *et al* reporting ER calcium dysfunction leading to activation of cell death pathways in *C9ORF72* gene expansion patient derived iPSC motor neurons (Dafinca *et al.*, 2016).

As described above, there is clear cellular dysfunction in *C9ORF72* gene expansion ALS however the type and severity of dysfunction is still in debate.

1.6 Optogenetics Tools

Tools to control and manipulate model systems are important for both in vivo and in vitro work. In neuroscience optogenetics has emerged as a useful and widely-used tool for studying neurons. Optogenetics uses light of different wavelengths to cause either loss or gain of function in effectors within cells i.e. activation of ion channels (Deisseroth, 2010). Previous tools such as ion channel blockers do not allow for the same degree of temporal and cellular precision in a fast timescale within intact neurons. Optogenetics addresses the need to manipulate events in selected cell types at certain time points in intact cells. The idea of using light to control neurons is however not a new concept, Francis Crick was the first to suggest the idea in 1979 (Crick, 1999). The first single-component microbial light activated ion pump called bacteriorhodopsin was discovered in 1971 (Oesterhelt and Stoeckenius, 1971), followed by halorhodopsin in 1977 (Matsuno-Yagi and Mukohata, 1977) and channelrhodopsin (ChR2) in 2002 (Nagel *et al.*, 2002). In 2005 ChR2 was used in neurons successfully, followed by bacteriorhodopsin and halorhodopsin (Boyden *et al.*, 2005).

1.7 Thesis Aims

The overall theme of this project is to explore the impact of the hexanucleotide *C9ORF72* gene expansion, both at the gene level and downstream functional impact in patient vs control cells. The project is divided into four non-mutually exclusive aims to explore different aspects of *C9ORF72* disease pathophysiology:

1. To generate and characterise mature iPSC derived motor neurons to model ALS in *C9ORF72* expansion carriers and control cells. Motor neuron maturity will be confirmed by gene expression and immunocytochemical profiling as well as electrophysiological characterisation. (Chapter 3)
2. To investigate electrophysiological excitability phenotypes and calcium dyshomeostasis in ALS and control iPSC-derived motor neurons. (Chapter 4)
3. To generate a motor neuron-specific bifunctional vector enabling live-detection and optogenetic activation of HB9 positive motor neurons. The reporter function will be used to enable electrophysiological characterisation of labelled cells, while the optogenetic tool is intended for downstream applications beyond the scope of this thesis. (Chapter 5)
4. To characterise the nuclear localisation of both expanded and WT *C9ORF72* alleles as well as the expression of *C9ORF72* transcript variants in human lymphoblastoid cell lines. (Chapter 6)

Chapter 2: Materials and Methods

2.1 Cell Culture

2.1.1 Induced Pluripotent Stem Cell Culture

All iPSC lines were generated and bulked for cryopreservation in the Cowley Laboratory at Oxford University. Vials were requested and brought to the Wade-Martins Laboratory for thawing and further bulking.

Two/three weeks prior to motor neuron differentiation, iPSCs were rapidly thawed in a 37°C water bath, before dilution in PBS (Life Technologies: 10010-015) and centrifugation at 400g to remove freezing media. iPSCs were subsequently cultured at 37°C with 5% CO₂ in mTsr1 medium (StemCell Technologies: 85870, 85851, 85852) with 1% Pen/Strep (Life Technologies: 15070-063) on hESC-qualified Matrigel (Corning: 354277) diluted in KnockOut DMEM (Life Technologies: 10829-018). iPSCs were routinely colony passaged at a typical passage ratio of 1:4 using 0.5 mM EDTA (Life Technologies 15575-038, 0.5 M pH 8 diluted 1000x) in PBS without calcium and magnesium (PBS-/-) (Life Technologies: 10010-015). PBS-/ was used to lift cells, as the addition of calcium and magnesium promotes cell adhesion. Colonies were passaged when they reached ~80% coverage of the well and formed dense colony centres. Single cell passaging of iPSCs using TrypLE (Life Technologies:12604-013) was used only for viral transduction. ROCKi (Bio-technique:1254, Y27632 dihydrochloride) was added to the media at 10 µM after single cell passaging or colony passing with clumps of less than 4 cells. ROCKi is added to reduce dissociation-trigger apoptosis (Watanabe *et al.*, 2007).

2.1.2 HEK293T Cell Culture

Human embryonic kidney 293T (HEK293T) cells were cultured in T-175 flasks (Corning: 431080) in 25ml media composed of DMEM-high glucose (Sigma D6546-500ML) with 1% L-glutamine (Life Technologies: 25030-024), 1% Pen/Strep (Life Technologies: 15070-063) and 10% Fetal Bovine Serum (FBS) (Life Technologies: 26140-079) at 37°C with 5% CO₂. Once confluent, HEK293Ts were passaged using Trypsin-EDTA (Sigma: T3924-100ML) for 5 mins at 37°C at a typical ratio of 1:10 1-2 times per week. For cryopreservation 5 X 10⁶ cells per ml were frozen per vial (1ml) to thaw into a T-75 flask (Corning: 430641). Freezing media included the above complete growth media supplemented with 5% Dimethyl Sulfoxide (DMSO) (Sigma).

2.1.3 Lymphoblastoid Cell Culture

Four Lymphoblastoid cell (LCL) lines (ND06769, ND06751, ND07612 and ND07332) were obtained from Coriell Cell Repositories. Cells were cultured in RPMI-1640 (Life Technologies: 31870-025) supplemented with 2mM Glutamax (Life Technologies: 35050-038), 15% Fetal bovine serum (FBS) (Life Technologies: 26140-079) and 1% Pen/Strep (Life Technologies: 15070-063), upright in T-25 flasks (Corning: 4306039) in a humidified 37°C incubator with 5% CO₂. LCLs were cultured in 20 ml of medium, at a starting density of 2 x 10⁵ cells/ml. Every 3-4 days depending on cell growth, assessed by media colour and LCL clumping, cells were either re-fed or passaged. Flasks were passaged before reaching 10⁶ cells/ml. To passage, LCL clumps were broken apart by gentle pipetting and

either passaged at 1:3 or counted bi-weekly using the automated NucleoCounter NC-250 to seed at 2×10^5 cells per ml. To cryopreserve LCLs, 5 million cells per ml were frozen per vial (1ml) in RPMI-1640 with 30% FBS (Life Technologies: 26140-079) and 5% DMSO (Sigma).

2.2 Motor Neuron Differentiation

2.2.1 Qu Differentiation

The first protocol used was adapted from (Qu *et al.*, 2014) with modifications optimised by Dr Ruxandra Dafinca. To begin differentiation, iPSCs were plated on Matrigel (Corning: 354277) diluted in KnockOut DMEM (Life Technologies: 10829-018) and left to become confluent. Once confluent, cells were washed with PBS with calcium and magnesium (Life Technologies: 14040) and media changed to basal media containing: DMEM/F12 with HEPES (Life Technologies), 1 x Anti Anti (Life Technologies: 15240-062), 1 x N2 (Life Technologies: 17502048), 1 mM L-Glutamine (Life Technologies: 25030-024), 1 x Non-essential amino acids (Life Technologies: 11140-050) and 0.2% heparin (Sigma: H3149). Media was changed daily and freshly supplemented with 1 μ M Compound C (EMD Biosciences: 171261-1MG) for 3 days (Day 0-3). On day 3 media was changed to basal media supplemented with 1 μ M Compound C and 100 nM Retinoic acid (Sigma: R2626) and changed daily thereafter. Day 6 media was changed to basal media including 1 x B27 (Life Technologies: 12587010), supplemented with 100 nM Retinoic acid, 1 μ M SAG (Enzo Life Sciences: ALX-270-426-M001) and 1 μ M Puromorphamine (MERK: 540220)

and changed daily. On day 9 cells were dissociated using Accutase (Life Technologies: A11105-01) and passaged 1:6 on to Geltrex (Life Technologies: A1413302) coated plates in basal media supplemented with 100 nM Retinoic acid, 1 μ M SAG, 1 μ M Puromorphamine, 10 ng/ml BDNF (Peprotech:450-02), 10 ng/ml GDNF (Peprotech: 450-10), 10 ng/ml IGF-1 (Life Technologies: PHG0078) and 0.1 μ M cAMP (Sigma: D0627). 10 μ M ROCKi (Bio-techne:1254, Y27632 dihydrochloride) was supplemented to the media for plating but removed thereafter. Day 14 cells were dissociated again using Accutase and plated on nitric acid etched coverslips spotted with 40 μ l Geltrex at 25,000 cells per coverslip in basal media supplemented with 100 nM Retinoic acid, 1 μ M SAG, 1 μ M Puromorphamine, 10 ng/ml BDNF, 10 ng/ml GDNF, 10 ng/ml IGF-1 and 0.1 μ M cAMP. 10 μ M ROCKi was added on the day of plating but removed thereafter. Media was changed alternate days until the end of the protocol at day 27. For virus application to coverslips, virus was diluted in 300 μ l media per coverslip, then topped up to 500 μ l after 3-4 hours or after overnight incubation.

2.2.2 Du Differentiation

Protocol published by (Du *et al.*, 2015) with modifications I optimised. iPSCs, when confluent were colony passaged to give a plating of 30% (typically 1:6 passage ratio) coverage using 0.5 mM EDTA (Life Technologies 15575-038, 0.5 M pH 8 diluted 1000x) in PBS-/- (Life Technologies: 10010-015). Colonies were plated onto Matrigel (Corning: 354277) coated plates (diluted in KnockOut DMEM (Life Technologies: 10829-018)) in mTesr1 media (StemCell Technologies: 85870, 85851, 85852). The following day, cells

were washed with PBS with calcium and magnesium (Life Technologies: 14040) and media changed to basal media containing: 1:1 DMEM/F12 (Life Technologies: 21331020) and Neurobasal (Life Technologies: 21103049), 0.5 x N2 (Life Technologies: 17502048), 0.5 x B27 (Life technologies: 12587010), 2 mM Glutamax (Life technologies: 35050-038) and 1 x Anti-Anti (Life technologies: 15240-062) and supplemented with 3 μ M CHIR99021 (Bio-technique: 4423), 2 μ M DMH1 (Tocris: 4126), 2 μ M SB431542 (Tocris: 1614) and 0.1 mM ascorbic acid (Sigma) for 6 days with media changes on alternate days. After 6 days, cells were colony passaged using Dispase (Life Technologies: 17105-041 1.79 units/mg) dissolved in PBS/- (Life Technologies: 10010-015) to give a 30% density after plating. For dispase passaging, a 10 mg/ml stock concentrate is added directly to fresh media to give a working concentration of 1 mg/ml, before incubation at 37°C for 5 minutes. Colonies are plated onto Matrigel coated plates in basal media supplemented with 1 μ M CHIR99021, 2 μ M DMH1, 2 μ M SB431542, 0.1 μ M retinoic acid (Sigma-R2625), 0.5 μ M Puromorphamine (MERK: 540220) and 0.1 mM ascorbic acid. On day 12 cells were lifted, again using dispase dissolved in PBS/- and plated on ultra-low attachment plates (Corning: 3471) for 6 days in basal media with 0.5 μ M Retinoic acid, 0.1 μ M Puromorphamine and 0.1 mM ascorbic acid. After 6 days in suspension on ultra-low attachment plates, neurospheres were collected in 37 μ M reversible cell strainers (StemCell Technologies: 27250) to remove dead/single cells, then dissociated with Accumax (Millipore: SCR006) and plated on nitric acid etched coverslips, first flooded with Matrigel then re-spotted with 40 μ l Matrigel. Cells are plated in 40 μ l spots at 10^5 cells per spot. Cells plated for western or RNA were plated in 12 well plates at 1.25×10^5 cells/well, or in full-size 96 well plates (Greiner: 655090) at 10^5 cells for immunostaining or Calcium assays. Cells were matured for 10 days in basal media with 0.5 μ M Retinoic

acid, 0.1 μ M puromorphamine, 0.1 μ M Compound E (Merk 565790), 10 ng/ml IGF (Life Technologies: PHG0071), 10 ng/ml CNTF (Peprotech: 450-13), 10 ng/ml BDNF (Peprotech: 450-02) and 0.1 mM ascorbic acid.

For the maturation study the following conditions were added to the above Du protocol.

- Standard: At day 28 the cells were maintained in the same media and fed alternate days.
- Reduced PF: At day 28, the media was changed to basal media with 10 ng/ml IGF, 10 ng/ml CNTF, 10 ng/ml BDNF and 0.1 mM ascorbic acid. All other patterning factors were removed. Media was changed three times per week.
- DAPT: At day 18 after re-plating, the standard media was applied with 10 μ M DAPT (Abcam: ab120633-50mg) in place of Compound E. The reduced PF method was then followed from day 28 onwards.
- Synaptojuice: After plating on day 18 cells were plated in Synaptojuice A (gift from Nick Allen's Laboratory at Cardiff University) supplemented with 0.5 μ M Retinoic acid and 0.1 μ M Puromorphamine. Media was half-changed every three days until day 26 when the media was full-changed to Synaptojuice B (gift from Nick Allen's Laboratory at Cardiff University). The media was then half-changed three times a week until day 40 when Synaptojuice was removed and replaced with BrainPhys (StemCell Technologies: 05790) supplemented with 1x N2, 1x B27 and 1x Anti-Anti.

For viral transductions, vectors were added to the full media volume for the first generation viruses and to half media volume for second generation viruses, media was changed after 24 hours. Cells used for second generation lentiviral transduction were treated with Mitomycin C (Sigma: M4287) for 1 hour at 37°C to kill proliferating cells.

2.3 Repeat Primed PCR

Repeat Primed PCR is used in this thesis to assess the presence or absence of the hexanucleotide repeat expansion in the *C9ORF72* gene. Two wells of a 6 well plate of colony passaged iPSCs; one well of a 12 well plate of neurons seeded at 1.25×10^6 cells/well or 5×10^6 LCLs were used for genomic DNA extraction.

2.3.1 Genomic DNA extraction

DNA was extracted using either the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma: G1N70) following the kit guidelines and eluting in 200 μ l or using Phenol: Chloroform extraction.

To Phenol: Chloroform extract DNA, cells were lysed in lysis buffer containing: 0.6% SDS (Sigma), 100 mM NaCl (Sigma), 50 mM Tris.Cl (pH 8) (Sigma) and 20 mM EDTA (diluted from Life Technologies: 15575-038) with 50 μ g/ml RNase A (Qiagen: 19101) for 20 minutes at room temperature. Cell solution was left overnight at 37°C, then Proteinase K (Sigma) at 100ug/ml was added before another overnight incubation at 37°C. Lysate was then extracted twice with Phenol: Chloroform: Isoamyl Alcohol (Sigma: P2069) and twice with Chloroform (Sigma) using Light Phase Lock Gel tubes (Eppendorf: 0032007961).

DNA was then precipitated with 5 M NaCl (Sigma) and absolute Ethanol (Sigma) and centrifuged at 17949g at 4°C for 30 minutes. The pellet was then washed with 70% ethanol and spun for another 30 minutes at 4°C. After removing the 70% ethanol and drying the pellet the DNA was resuspended in 75 μ l TE.

2.3.2 Repeat primed PCR

The repeat primed PCR method was based on (DeJesus-Hernandez *et al.*, 2011) and optimised for use here, for both motor neuron samples and LCL samples. The repeat primed PCR reaction was set up using the DeJesus published primers Table 2.1 with the Taq DNA polymerase set up using Table 2.2.

The PCR was performed in a thermocycler using the published cycling conditions including: Denature for 10 minutes at 98°C, followed by 10 cycles of denaturing at 97°C for 35 seconds, annealing at 64°C for 2 minutes and extension for 8 minutes at 68°C. This was followed by 25 cycles of denaturing at 97°C for 35 seconds, annealing at 64°C for 2 minutes and extension at 68°C for 8 minutes with 20 seconds added to each cycle. The ramp rate was set at 64% (0.5 °C/second) for denaturing and extension steps and 25% for annealing (0.5 °C/ second).

1 µl of the reaction was then transferred to a clean 96 well PCR plate (AB-1400) for fragment analysis using the Liz-500 ladder. This analysis was completed at the sequencing facility at the Weatherall Institute of Molecular Medicine (WIMM), University of Oxford. Results of the fragment analysis were analysed using Geneious.

Primer Name	Sequence
DejesusForward	FAM-TGTAAAACGACGGCCAGTCAAGGAGGGAAACAACCGCAGCC
DejesusReverse	CAGGAAACAGCTATGACCGGGCCCGCCCGACCACGCCCGGCCCGGCCCGG
DejesusAnchor	CAGGAAACAGCTATGACC

Table 2.1: Primers used for Repeat Primed PCR

PCR Mix (20 μ l)	Volume (μ l)
10x Qiagen buffer	2
DNTP Mix (5 mM of each dCTP, dATP, dTTP)	1
7-deaza-2-deoxy GTP 5 mM (NEB N0445L)	1
3 Primer Mix 10 μ M each	2
DMSO (Sigma)	1
Betaine (Sigma: B0300-1VL)	4
Taq DNA Polymerase (Qiagen 201205)	0.5
Water	7.5
DNA (kit- 100-200 ng/ μ l, Phenol: Chloroform- 200 ng/ μ l)	1

Table 2.2: PCR set up for Repeat Primed PCR

2.4 Immunostaining Techniques

2.4.1 Immunostaining

Cells were fixed for 20-30 minutes at room temperature in 4% PFA and washed 3x in PBS with calcium and magnesium (Life Technologies: 14040). Cells were permeabilised for 10 minutes with 0.1% triton (PBS-T) (Sigma: T8787), blocked for 1 hour in PBS-T with 5 % serum (serum from the animal the secondary is raised in) and 2% bovine serum albumin (BSA) (Sigma: A9085). Primary antibodies (Table 2.3) were applied overnight rocking at 4°C in block before washed 3x with PBS-T. Samples were then blocked for another 30 minutes at room temperature. Alexa secondary antibodies (all Life Technologies) diluted 1:1000 were incubated for 1 hour at room temperature. Cells were finally washed with PBS with calcium and magnesium and counterstained with 1 µg/ml DAPI for 10-20 minutes then mounted in Vectashield (Vector Laboratories: H-1000) for coverslips or washed and stored in PBS with calcium and magnesium for 96 well plates (Sodium Azide at 0.01% may be added for long term storage to prevent bacterial growth).

Primary Antibody	Dilution	Supplier
ChAT	1:100	Millipore: Ab144p
ISL1	1:500	DSHB: 40.2D6-c (stock diluted 1:1 with glycerol)
TUJ1	1:1000	Abcam: ab52623 rb clone EP1569Y
SMI-32	1:1000	Biolegend: 801701
GFAP	1:1000	Abcam: ab4674

Table 2.3: Primary Antibodies used for Immunostaining

2.4.2 Flow Cytometry

Two wells of a 12 well plate seeded with 1.25×10^6 iPSC-derived MNs at day 18 were used for flow cytometry analysis. Primary antibodies and IgG controls used for flow cytometry listed table 2.4.

Cells were washed with PBS-/- (Life Technologies: 10010-015) then dissociated using Trypsin-EDTA (Sigma: T3924-100ML) for 5 minutes at 37°C then inactivated with Defined Trypsin Inhibitor (DTI) (ThermoFisher: R007100) supplemented with 3U/ml DNase I (NEB: M0303L). Dissociated neurons were then passed through a pre-wetted (pre-wet with PBS+ 3U/ml DNase I) 40µm cell strainer (Corning), then washed with cold PBS+ 3U/ml DNase I.

After centrifuging at 300g for 5 minutes at 4°C, cells are transferred in PBS-/- to (Protein or DNA) Lo-Bind tubes (Sigma: Z666505, Z666548) with 10% BSA. Cells were spun at 300g for 3 minutes at 4°C, before fixing cells in 4% PFA on ice for 10 minutes. A solution of 1 M Glycine (Sigma) is added after fixation with a solution of PB (0.1% Saponin (Sigma), 2% BSA, PBS, Normal Donkey IgG 1:500 2µg/ml (ProSci: 5087). Cells are spun again at 300 g for 3 minutes at 4°C to remove PFA before blocking for 10 minutes on ice in glycine and PB. After, blocked samples are split into two or three tubes each. One sample used for the IgG control and one for primary antibodies. The third tube, if selected, is the unstained control. After centrifuging at 300g for 3 minutes at 4°C cells are resuspended in PB with either primary antibodies or IgG controls (concentration matched to primary antibody) for 1 hour spinning at 4°C. After centrifuging at 300 g for 3 minutes cells are resuspended in Alexa secondary antibodies 1:2000 for 15 minutes in ice. Cells were

washed in PBS then kept as a pellet on ice until flow cytometry when the cells were diluted in PBS.

The unstained control sample was used first to gate the population of interest using FSC and SSC, then single cells gated using SSC-H and SSC-A. IgG controls for each cell line were analysed and gated for negative cells. Primary stained samples were then compared against their IgG control for positive cells. After flow cytometry, samples were analysed using FlowJo.

Antibody	Dilution	Supplier
Normal Donkey IgG (1 mg/ml)	1:500	ProSci: 5087
Normal Mouse IgG (1 mg/ml)	1:1000	ThermoFisher: 02-6100
Normal Rabbit IgG (1.646 mg/ml)	For TUJ1: 1:76923 For mCherry: 1:1639	Abcam: ab172730
TUJ1	1:2000	Abcam: ab52623 rb clone EP1569Y
SMI-32	1:1000	Biolegend: 801701
mCherry	1:1000	Abcam: ab167453

Table 2.4: Primary Antibodies and IgG controls used for Flow Cytometry

2.5 RT-qPCR

2.5.1 RNA Extraction

For iPSC-derived MN RNA extraction, one well of a 12-well plate seeded with 1.25×10^6 neurons was used per sample.

Wells were washed with PBS-/- (Life Technologies: 10010-015) and Trizol reagent (Ambion Life technologies: 15596018) added straight to the well and incubated while swirling for 10-15 minutes before collection and storage at -80°C until processing.

To process samples, Trizol pellets were thawed on ice before addition of Chloroform (200 μl : 1ml Trizol). After vigorous shaking and incubation for 2 minutes at room temperature, samples were centrifuged at 10,621g for 10 minutes at 4°C . The aqueous phase was then transferred to new tubes and an equal volume of 70% ethanol added. The solution was then added to RNase Easy Mini Columns (Qiagen: 74104) and spun for 1 minute at 10,621g at room temperature. The RNase Easy kit RW1 buffer was applied to the column and centrifuged at 17949g for 1 minute. DNase I treatment (Qiagen: 79254) was then incubated on the columns for 15 minutes at room temperature, followed by RW1 buffer and centrifugation at 10,621g for 1 minute. Finally, two washes of RPE are centrifuged through the columns before elution in 30 μl RNase free water.

2.5.2 cDNA

RNA was quantified using a Nanodrop 1000 (ThermoScientific), then cDNA was produced using SuperScript VILO MasterMix (Invitrogen: 11755-050) as per instructions using 1 µg of RNA.

2.5.3 qPCR

Fast SYBR green (Applied biosystems: 43856120) was used for the qPCR reaction using the StepOnePlus Real-Time PCR System (Applied biosystems). cDNA was diluted 1:4 and efficiency of qPCR primers checked using serial dilution of cDNA. Optimised primers were then used for qPCR reactions (Table 2.5), with each sample run in triplicate on each plate. Gene samples to be compared were run on the same plate or a calibrator sample run on both plates if run separately. This calibration was done by correcting CT values according to the following calculation:

Corrected $C_a^{\text{Sample}} = (C_a^{\text{Sample}} - C_a^{\text{calibrator}}) + \text{average } C_a^{\text{calibrator}}$ of all plates.

Where C_a is the average of triplicate CT values for each sample.

Gene	Sequence
C9ORF72 Total	F: CCGGAAAGGAAGAATATGGATGC R: TTCCATTCTCTCTGTGCCTTCT
C9ORF72 Variant 1	F: CCACGTAAAAGATGACGCTTGATA R: AAAGCAATCTCTGTCTTGGCA
C9ORF72 Variant 2	F: CAGGCGGTGGCGAGT R: AATCTCTGTCTTGGCAACAGC
C9ORF72 Variant 3	F: GTTGCTTCTCTTTGGGGGCG R: ATGGCGGTGGGCAAAGAGT
SMI-32	F: ACCGTCATCAGGCCGACATT R: ATCTCCCACTTGGTGTTCTC
ISL1	F: TCAGTATTTGGACGAGAGCTGTA R: CGTTCTTGCTGAAGCCGATG
ChAT	F: CTGAGCACGTCATCGTAGCC R: TGAGTGAACAGATCCCCCTCA
HB9	F: GTCATGCTCACCGAGACCC R: TCTTTGGCCTTTTGTGCTGCGT
HPRT	F: GCTATAAATTCTTTGCTGACCTGCTG R: AATTACTTTTATGTCCCCTGT TGA CTGG
B2M	F: AGATGAGTATGCCTGCCGTG R: TCAAACCTCCATGATGCTGCT

Table 2.5: RT-qPCR optimised primers

2.6 Whole-Cell Patch Clamp Electrophysiology

Electrophysiology was completed on iPSC-derived motor neurons plated on 11 mm nitric acid etched coverslips at a density of 10^5 cells/coverslip. Cells were selected for patching based in neuronal morphology.

The rig consisted of an Axon Digidata 1550A (Molecular Devices), MultiClamp 700B Amplifier (Molecular Devices), temperature controller with peltier heater (Scientifica: SM-4600) and PatchStar micromanipulator (Scientifica) on an air table (TMC) to reduce vibrations with a Faraday cage to reduce external electromagnetic interference. For optogenetic stimulation a CoolLED pE-300 was fitted and controlled via multiclamp. An inverted microscope (Scientifica) with 10x and 60x objectives was used to visualise the neurons to be patched.

Glass micropipettes for whole cell patch clamp were pulled using a vertical puller (Narishighe: PP-83) from Borosilicate glass with filament for backfilling (outer diameter: 1.2mm, inner diameter 0.69mm, length 10cm Warner instruments: G120F-4). Tips had a final resistance of 5-8 M Ω .

2.6.1 Internal solution

The internal solution used to fill the micropipettes included the components in Table 2.6 and adjusted to a final pH of 7.3 using KOH and osmolarity of 290 mOsm.

Components	Concentration (mM)	Supplier
Potassium Gluconate	140	Sigma: D4500
NaCl	6	Honeywell: S9625
EGTA	1	Sigma: E3889
Hepes	10	Sigma: H3375
MgATP	4	Sigma: A9187
Na ₃ GTP	0.4	Sigma: G8877

Table 2.6: Internal Solution components for electrophysiology

2.6.2 External solution

The external solution used to run through the rig and record cells included the components in table 2.7 and adjusted to a final pH of 7.4 and osmolarity of 300 mOsm. Temperature was maintained at 25°C with a flow rate of ~1.5 ml/minute for recordings.

Components	Concentration (mM)	Supplier
NaCl	167	Honeywell: S9625
KCl	2.4	Sigma: P9541
MgCl ₂	1	Fluka BioChemika: 68475
Glucose	10	Sigma: G7528
Hepes	10	Sigma: H3375
CaCl ₂	2	Fluka: 21114

Table 2.7: External Solution components for electrophysiology

2.6.3 Patch Clamp Protocols

The whole cell patch clamp method was used to record cells. As discussed in Sub-chapter 3.2.1.2 a $G\Omega$ seal was obtained and then ruptured to allow equilibrium and ion exchange between the pipette internal solution and the cell intracellular fluid. Cells were held at -70mV in voltage clamp for 2 minutes to allow solutions to dialyse before recordings using Clampex 10. Cells with an access resistance greater than 40 $M\Omega$ were rejected and recordings with a change in access resistance or cell capacitance greater than 10% over the duration of the recording were also omitted.

To record voltage-gated sodium and potassium channels, voltage clamp was used at gain 1, with a sampling rate of 10,000 Hz. The protocol used gave a 2 second sweep duration for 15 sweeps. Cells were held at -70mV between sweeps and stepped from -70 mV by 10 mV each sweep for 100 ms each step. Leak subtraction was applied before recording for 4 sub-sweeps with a settling time of 250 ms.

Induced action potential firing was recorded in current clamp at gain 1 with a sampling rate of 10,000 Hz and total sweep duration of 1 second. A biased current was applied throughout the recording to manually lower the resting membrane potential of each cell to -70 mV between sweeps. A square current step was applied for 500 ms with the first step at -10 pA followed by a 5 pA increase each step. All traces were analysed offline using Clampfit. Any cell lines post stained with less than 30% motor neurons were excluded from analysis as the chance of patching a non-motor neuron is increased.

2.7 Fura-2 AM

Fura-2 AM is a plate reader assay using the Fura-2 QBT Calcium Kit: Component A (Molecular devices: R7540). Neurons were plated at a density of 10^5 cells per well on flat bottom black walled 96-well plates (Greiner: 655090) for analysis.

To one vial of Fura-2 QBT Calcium Kit: Component A, 10 ml of room temperature HBSS (Life technologies: 14175-053) with 10 mM Hepes (Sigma: H3375-500G) were added.

This was then diluted 1:1 with cell culture media and supplemented with F127 (Life Technologies: P6866) at 1:5000. Media was removed from cells and replaced with 100 μ l of 50:50 media: Fura-2 AM mix and incubated at room temperature for 1 hour in the dark. Ionomycin at 25 μ M (Sigma: I0634) and Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma - C2920) at 250 μ M were diluted in HBSS plus Hepes and supplemented with F127 (1:5000).

After 1-hour, plates were read using the FlexStation system with the following set up: Fluorescence was read at 340 nm excitation, 510 nm emission and 380 nm excitation, 510 nm emission with a sensitivity setting of 4. Recording was set to 120 seconds at 5 second intervals, 50 μ l of Ionomycin or FCCP were injected after 30 seconds with a pipette height of 230 μ l at a rate of 3 (78 μ l/second). Readings were exported using SoftMax Pro and analysed on excel and GraphPad Prism.

2.8 Cloning

Vectors were constructed using Gibson assembly/NEBuilder, with the exception of the IRES and Puromycin construct which was constructed using the traditional restriction/ligation method. The generation of constructs is discussed Chapter 5 but the methods of each technique used will be described here.

In general, to generate the constructs via Gibson/NEBuilder assembly a similar work flow was followed including:

- PCR amplification of fragments to be inserted
- Restriction digestion of the parent plasmid
- Gel purification of the fragments and digested backbone
- Gibson assembly of the fragments and backbone
- Transformation into chemically competent *Escherichia coli* (*E.coli*)
- Pick colonies, grow overnight and miniprep
- Screening digestion of minipreps to check for correct colonies
- Sequencing of correct colonies
- Re-transform in chemically competent Stbl3 *E.coli* suitable for lentiviral maxiprep
- Pick colonies, grow overnight and miniprep
- If colonies are correct use overnight culture to grow maxiprep
- Generate DMSO stock from maxiprep culture

2.8.1 Polymerase Chain Reaction

Vectors were constructed by PCR amplification of fragments required from parent plasmids (20 ng) using either the Kappa HiFi Hotstart PCR kit (Kapa Biosystems: KK2501) or Roche Long template PCR system (Roche: 11681834001). PCR reactions were set up following manufacturer's instructions and cycling conditions. Initial PCR amplification of fragments used a gradient PCR until the correct annealing temperature was determined. PCR primers used for the successful second generation optogenetic tool/reporter are reported Table 2.8

Target	Primer Name	Sequence
Cppt	CPPPTF CPPPTR	F: attagtgaacggatctcgac R: TCGATAAAATTTGAATTTTG
EF1	NENV1EF1F NENV1EF1R	F: acaaattacaaaaattcaaaattttatcgagtttaaacgtgaggctccggtgccgctc R: gtcgactcacgacacctgaaatggaag
GFP	NENV1GFPF NENV1GFPR	F: ttcttcatttcaggtgtcgtgagtcgacATAACTTCGTATAAaGTATcCTATACGAAGTTATcgccaccatggtgagcaagg R: ttactgtacagctcgtccatg
ChR2-mCherry	NENV1MCHCASF NV1MCHCASR	F: cggcatggacgagctgtacaagtaaATAACTTCGTATAGCATACATTATACGAAGTTATactagtTCACACTTCGTTCTCGTAGCAGAAG R: gccaccATGGACTATGGCGGCGCTTTG
Lox sites	LOXF LOXR	F: CCGCCATAGTCCATggtggc cccgggATAACTTCGTATAGGATACTTTATACGAAG TTATATAACTTCGTATAATGTATGCTATACGAAGTTATatgcatcaatcaacctctggattaca R: tgtaatccagaggttgattgatgcataATAACTTCGTATAGCATACATTATACGAAGTTATATAACTTCGTATAAAG TATCCTATACGAAGTTATccggggccaccATGGACTATGGCGG
HB9 + beta globin	NV1BHB9F NV1BHB9R	F: ctataggcggaattgggtaccgctCGCCGGCGGATCCTGTCTCCCTGGCCAG R: CACCTTCCTCTCTCTCTGG
Cre-M	NV1BCREMF NV1BCREMR	F: GGCACCCAAGAAGAAGAGGAAGGTGGCGAATTTACTGACGGTACC R: ttcacttctcaattcgccctcctttaattaaCTAATCGCCATCTTCAGCAG

Table 2.8: PCR primers used to clone the successful second generation optogenetic/report construct pHb9-CREM-EF1-floxGFP-floxChR2mCherry

2.8.2 Restriction Digestion

Restriction digestion was used to linearize the parent backbone in order to insert the PCR amplified fragments; to digest PCR amplified fragments to prepare for ligation; or to screen colonies after fragment insertion. NEB restriction enzymes were used for the majority of digestions in a total volume of 50 μ l with 0.5 μ l enzyme (with the exception of backbone digestion using 1 μ l). Where possible High-Fidelity versions of the enzymes were used to reduce star activity. For backbone digestion in general 5 μ g of DNA was digested per reaction. For miniprep screening, as the DNA was not quantified, due to large numbers (<30) of colonies being screened, 4 μ l of miniprep was used from the 50 μ L elution.

Again, for fragment digestion for ligation the DNA was not quantified, once fragments were gel purified and eluted in 30 μ l, 15 μ l was then used for the digestion.

In addition to restriction digestion mung bean nuclease (NEB: M0250S) was used to digest the end of the parent backbone for the second generation optogenetic tool (discussed Chapter 5) following manufacturer guidelines.

2.8.3 Gel Electrophoresis and Extraction

PCR products were run on 1% agarose gels (Sigma: A9539) in 1x Tris-Borate-EDTA (Sigma: T4415), the correct size band cut out and purified using the QiaQuick gel extraction kit (Qiagen: 28704, 28706) following the manufacturer's instructions. DNA was eluted in 20 μ l.

2.8.4 Gibson Assembly and Hi-Fi builder

Gibson assembly (NEB: E2611S) or NEBuilder HiFi DNA assembly mix (NEB: E2621S) was used to ligate fragments into the backbone.

The Gibson assembly mix is optimised to join fragments with ~ 20 bp overlaps and NEBuilder HiFi DNA assembly mix fragments with ~25 bp overlaps. The reactions were set up at 50°C for 15 minutes for 2-3 fragment assemblies or for 60 minutes for more fragments. The ratio of insert to fragments was optimised for each assembly but ranged from 1:2 to 1:8.

2.8.5 Bacterial Transformation

Following Gibson or NEBuilder assembly, the mix was diluted 4 fold with distilled water (Life Technologies: 10977-035) and 2 µl transformed into One Shot TOP10 chemically competent *E.coli* (Life Technologies: C4040-10).

For sequenced minipreps 1 µl of DNA was transformed into One Shot Stbl3 chemically competent *E.coli* (Life Technologies: C7373-03). Stbl3 are recommended for cloning of unstable vectors such as lentiviral backbones that contain long terminal repeat sequences. Heat shock to induce uptake of DNA was followed using manufacturer's instructions. After heat shock and recovery *E.coli* were spread on LB Agar (Millipore: 1.10283.0500) plates with the appropriate antibiotic selection and grown overnight inverted at 37 °C.

2.8.6 MiniPrep

Following transformation into *E.coli* colonies were picked, grown overnight in 2 ml LB broth (Thermo Fisher: 1280-1660) and then harvested for miniprep. If TOP10 *E.coli* were used miniprep was completed without a kit using an optimised protocol from Richard Wade-Martins.

The overnight culture was pelleted and resuspended in STET (8% Sucrose, 5% Triton, 50 mM EDTA and 50 mM Tris.HCL (pH 8)) before lysis in a 1% SDS, 0.2 M NaOH solution. Lysis was neutralised using 7.5 M Ammonium acetate before centrifugation at 17,900 g for 10 minutes at 4°C. The supernatant was transferred into a fresh tube with isopropanol and spun again at room temperature for 10 minutes at ~10,500 g. The pellet was washed with 70% ethanol before resuspension in 50 µl TE (10 mM Tris and 1 mM EDTA) with 5 µg/ml RNase A.

Plasmid DNA transformed into Stbl3 *E.coli* was miniprep using the Qiagen Spin Miniprep kit (Qiagen: 27104, 27106) as per manufacturer's instructions. The kit was used for Stbl3 bacteria as they are Endonuclease I positive (*endA1+*) which the 'in house' method does not inactivate.

2.8.7 Sequencing

Following check digestions, correct minipreps were sequenced to ensure there were no point mutations within the vector inserts. Sequencing primers used for successful pHB9-CREM-EF1-floxGFP-floxChR2mCherry construct are reported Table 2.9.

Primer	Sequence
HB9F1	AACAGACACTGCAGGTCC
HB9F2	TAGAGGTCCCTGGACTCAG
HB9F3	GAGGATGATTGGTCCAAC
HB9F4	CGCCGATTGCACCGAAATTG
HB9F5	TGGTCTGAAGCCCAATGCCG
HB9F6	AATTTCCAGATGGGCCAAG
HB9F7	TTGGTCTGTCCGTCCATCTG
HB9F8	TTAGTACGCCCTGATTCACC
HB9F9	CACCTTCAAAGCAGACCC
NV1LENT BF1	AAC CCT CTA TTG TGT GCA TC
HB9R9	CACCGGGAAACAATGCAATTC
HB9R8	GACGACAGGTGAGAGTTGGG
HB9R7	CATCTTTGCCTTAGTGTTCC
HB9R6	GGAAGTGTTGCGAGAAAGGC
HB9R5	AGACATCGTAGATTGTTGAGG
HB9R4	CAAGTCACAGATCAGCACC
HB9R3	TCACTTCTACTCTCAGCCAAG
HB9R2	GA CTCTGCCTGCACCAATCAC
HB9R1	AGGGAGACAGGATCTCTAG
NV1HBBR1	CTC TCT GCA CCA CTC TTC TC
lentibF1	tatcgtttcagaccacctc
EF1F1	gtttgccgccagaacacagg
EF1F2	agaatcggacggggtagtc
EF1R2B	aagcgtcgcagcaggtcatc
EF1F3B	ttgagtttgatcttggttc
EF1R1	ctctaggcaccggttcaattg
GFPR2	cttggtccccaggatgttg
EF1R3	gggaagcggccatcttc
GFPF1	accatcttctcaaggacg
GFPF2	tgctgccgcacaaccactac
GFPR1B	tttacgtcgccgtccagctc
MCHER2C	TCACCTGTAGATGAACTCG
CHR2R2	CGTGAATCAATATGTGCTCG
CHR2R1	AGGCGGATAAGGATGACAGG
MCHEF1	CCATCATCAAGGAGTTCATG
MCHEF2	CCTGAAGGGCGAGATCAAG
CHR2F1	GACCGCGTCAAATGTCCTGC
CHR2F2	TGTGCTATGGCGCGAACAC
chr2CASF1B	CAGTAACATTGATCCTCAGG
LentiR3	agaatccaggtggcaacacag
lentiR1	agcaatagcatgatacaaggc
CREMF2	ATGCTGTTTCACTGGTTATG
CREMF3	TCAACTCGCGCCCTGGAAGG
CREMR	ACCGGCAAACGGACAGAAGC
CREMR2	CCGTCAGTACGTGAGATATC
CREMR3	CCATCTTCCAGCAGGCGCAC

Table 2.9: Primers used for Big dye Sequencing of CREM-EF1-floxGFP-floxChr2mCherry

Sequencing was completed using the BigDye Terminator v3.1 (Applied Biosystems) system using the PCR reaction set up Table 2.10 and the following cycling conditions: denature for 1 minute at 96°C followed by 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

The DNA was then purified and precipitated by addition of 125 mM EDTA, 3M CH₃CO₂Na and 100% Ethanol and centrifugation for 30 minutes at 3000 g at 4°C. The DNA was then washed with 70% Ethanol and spun for a further 15 minutes at 4°C at 1650 g.

Precipitated DNA was then taken to the zoology sequencing facility for sequencing.

Component	Volume (μl)
Big Dye	0.5
Buffer	1.75
Primer (10 μM)	1
DNA (Miniprep)	1
Water	6.25

Table 2.10: BigDye Terminator v3.1 PCR set up

2.8.8 MaxiPrep

Finally, correct minipreps are maxipreped to generate enough DNA for lentiviral production. Maxiprep's to be used for viral production were DNA extracted using the NucleoBond Xtra Maxi endotoxin free (Macherey-Nagel: 740420.10) kit following the manufacturer's instructions with the 'Finalizer' method of DNA concentration. Other maxipreps were carried out using the Qiagen Maxiprep kit with slight adjustments to the buffer volumes. 1.5 ml DMSO stocks were made from all maxipreps by combining

1350 µl of overnight bacterial culture with 150 µl of DMSO then snap-freezing on dry ice before storage at -80°C.

2.9 Lentiviral packaging

2.9.1 Third Generation Lentiviral Packaging

The Third-Generation Lentiviral packaging protocol used here is a week-long protocol aimed to create a large volume of high titer virus.

Day 1: HEK293T cells are seeded at 8×10^6 cells per 15cm dish in standard HEK media as listed Chapter 2.1.2 and allowed to adhere overnight. 12 dishes are used per virus packaged.

Day 2: The HEK293T cells are transfected using either Lipofectamine LTX (Life Technologies: 15338100) or 2 M CaCl_2 (Sigma) with pH 7.05 HBS (50mM Hepes, 280 mM NaCl and 1.5 mM Na_2HPO_4). After optimisation it was found Lipofectamine was the most effective for large vectors (>10 kb). For lipofectamine LTX transfection two mixes are made, Mix 1 and 2 are reported tables 2.11 and 2.12. These mixes are incubated at room temperature for 5 minutes, then combined and incubated for a further 20 minutes at room temperature before overnight incubation with cells.

Mix 1	Per plate
Gag/Pol	10 µg
Genome	10 µg
Env	3.4 µg
Rev	2 µg
Plus Reagent	25.4 µl
Optimem	1.25 ml

Table 2.11: Mix 1 Lipofectamine transfection

Mix 2	Per plate
Lipofectamine LTX	38.1 µl
optimem	1.25 ml

Table 2.12: Mix 2 Lipofectamine transfection

Alternately $\text{Ca}_3(\text{PO}_4)_2$ transfection used components in table 2.13. The packaging plasmids, CaCl_2 and water were mixed, then HBS (50 mM HEPES, 280 mM NaCl and 1.5 mM Na_2HPO_4) bubbled in, before incubating for 20 minutes. The mix was then applied to cells overnight.

Mix	Per Plate
Gag/Pol	10 µg
Genome	10 µg
Env	3.4 µg
Rev	2 µg
H ₂ O	Up to 1.1 ml
CaCl_2	140 µl
HBS	1.1 ml

Table 2.13: $\text{Ca}_3(\text{PO}_4)_2$ transfection reagents

Day 3: Transfection media was changed to media containing 10 mM sodium butyrate (Sigma), after 6 hours media was harvested in 500ml Conical flasks (Corning: 431123) and replaced with fresh medium.

Day 4: The second media harvest was collected and combined with first before spinning at 1000 g for 5 minutes, then filtered through a 0.45 µm filter. Supernatant was spun overnight at 6000 g at 4 °C, then resuspended in PBS pH 7.4 without calcium and

magnesium (Life Technologies: 10010-015) and ultracentrifuged for 90 minutes at 20,000 rpm (SW 32.1 Ti rotor). The subsequent pellet was incubated with 100 µl TSSM (20 mM Tromethamine, 100 mM NaCl, 10 g/l Sucrose and 10 g/l D-Mannitol) on ice for 2 hours, then the final volume adjusted to 192 µl before spinning at 1000 g for 5 minutes at 4°C then aliquoting.

2.9.2 Titering

Titer was established using flow cytometry on transduced HEK293T cells then calculated using the equation (Where i = dilution factor and 0.5 ml is the transduction volume):

$$\frac{\text{TU}}{\text{ml}} = \frac{1}{N} \sum_{i=3}^{i=6} \frac{(\% \text{GFP}^+ \text{ cells})_i \times (\text{total number of cells}) \times 100}{0.5 \text{ ml} \times 10^{-i}}$$

2.10 Western Blot

Cells were lysed in RIPA buffer and quantified against Bovine Serum Albumin Standards using the Bicinchoninic acid (BCA) assay. Samples were prepared by addition of 5x Laemmli Buffer and boiling at 95°C for 10 minutes. 25 µg of protein was loaded into a 10% polyacrylamide gel and run at 90 V. Proteins were manually transferred onto a PVDF membrane which was activated in methanol, then stacked on top of the gel and run at 220 mA for 90 minutes on ice. The membrane was blocked in TBS with 0.1% triton and 5% milk for 1 hour before application of primary antibody mCherry 1:2000 (Abcam, ab167453) overnight at 4°C. Membrane was washed 3 x in TBS with 0.1% Triton before incubation with secondary antibody goat anti-mouse HRP conjugate 1:2000 (Biorad) in TBS with 0.1% triton and 5% milk for 90 minutes. The membrane was then washed 3x in TBS with 0.1%Triton before developed with Immobilon Chemiluminescent HRP substrate (Millipore) and imaged on a Gel Doc XR+ System (Bio-Rad).

2.11 2D Fluorescent in situ hybridisation

2.11.1 Probe Preparation

Two probes were used for the 2D FISH analysis:

- Oligonucleotide (oligo) probe to recognise the expanded *C9ORF72* gene expansion. The successful probe was 21 nucleotides long and was 5' Alexa 555 labelled, sequence: GGCCCCGGCCCCGGCCCCGGC. The probe was HPLC purified.
- Fosmid to label both the expanded and WT *C9ORF72* gene alleles. Fosmid clone (WI2-2071M15 (G248P86104G8)) was ordered from BACPAC Resources.

The fosmid probe was checked to be correct by restriction digestion using AfeI (NEB: R0652S) and a second digest using XhoI (NEB: R0146S) and PmeI (NEB: R0560S).

500 ng of fosmid was digested for 2 hours at 37 °C using 0.5 µl of restriction enzyme, 1.5 µl digestion buffer and topped up to 15 µl total reaction volume with distilled water (Life Technologies: 10977-035). The digestion was loaded onto a 1% Agarose (Sigma: A9539) gel made with 0.5x Tris Borate EDTA (TBE) (Sigma: T4415) and run on the Pulsed Field gel CHEF-DR II system (Bio-Rad) in 0.5x TBE running buffer for 16 hours at 6 V using the 14°C cooling module. The gel was post-stained in Ethidium Bromide before UV visualisation.

The fosmid probe was quantified using the Qubit (ThermoFisher), then digoxigenin (DIG) labelled using a DIG-Nick Translation Mix (Roche:11745816910) supplemented with 80 U DNase I (NEB: M0303L) and incubated for 90 minutes at 15°C, before stopping the reaction with 1 µl 0.5M EDTA (Life Technologies 15575-038, 0.5 M pH 8). The probe was

then cleaned using the illustra ProbeQuant G-50 Micro Columns kit (GE Healthcare: 28-9034-08) following the manufacturer's instructions and eluting in 50 µl.

For probe precipitation, labelled fosmids were mixed with 3 µg of human Cot-1 DNA (per slide) (ThermoFisher: 15279-011), approximately 20µg salmon sperm DNA (ThermoFisher: 15632-011), 0.1 volumes of 3 M Sodium Acetate pH 5.3 (Sigma: S7899) and 2.25 volumes of ice cold 100% Ethanol (AnalaR NORMAPUR VWR) for 1 hour at -80 °C. Fosmids were then spun at 20,817 g for 30 minutes at 4°C before washing the pellet in 70% ethanol and centrifuging again for 10 minutes. The fosmid was then denatured in FISH hybridisation buffer (Leica Biosystems: KBI-FHB) for 8 minutes at 90 °C before preannealing at 37 °C for 8 minutes. Biotin labelled oligos for optimisation rounds (200 ng, 100 ng, 50 ng or 10 ng) or Alexa 555 GGCCCCGGCCCCGGCCCCGGC oligo were denatured in FISH hybridisation buffer for 8 minutes at 90 °C before cooling on ice.

2.11.2 Slide preparation

2D FISH was completed on expanded LCL patient lines ND06769 and ND06751 from Coriell. Cells were treated with hypotonic solution containing 0.56% Potassium Chloride (Sigma: P9541) for 20 minutes at 37°C before fixing in ice cold 3:1 Methanol: Acetic acid for 30 minutes at -20 °C before placing on methanol cleaned slides. Slides can be stored at 4°C for 1-2 weeks with Silica desiccant beads (Millipore: 1.07735.1000) to remove moisture.

2.11.3 Hybridisation

Slides were incubated with 100 µg/ml RNase for 1 hour at 37 °C, then washed in 2x SSC (diluted from 20x SSC Sigma: S6639) with agitation followed by dehydration by ethanol series. Chromosomes were then denatured in 70 % formamide (Ambion: AM9342, AM9344)/2x SSC at 72 °C for 2 minutes followed by dehydration by ethanol series.

Once the slides were pre-treated as above and probe prepared, these were hybridised with the oligo and fosmid overnight at 37 °C. Slides were then washed with 2 x SSC + 0.1% Igepal (Sigma: I3021) followed by 0.4 x SSC + 0.3 % Igepal for 2 minutes, then for 1 minute.

Slides were blocked for 30 minutes in 3 % BSA fraction V (Sigma: 05482) in 4 x SSC before detection. Optimisation biotin labelled oligo probes were detected using streptavidin Cy3 1:200 (1 mg/ml Jackson Labs: 016-160-084) and DIG was initially double detected using sheep anti-dig FITC 1:50 (200 µg/ml Roche: 11207741910) and rabbit anti-sheep FITC 1:100 (Vector laboratories: F1-6000). Slides were then mounted in Vectashield (Vector Laboratories: H-1000) with 1 µg/ml DAPI added.

Cells were imaged either using a standard wide-field fluorescent microscope with 100 x objective for the optimisation rounds or using spinning disk confocal at 60 x. Images were then analysed using a CellProfiler pipeline that I designed to detect and measure cells positive for two fosmids with one co-localised oligo spot.

Chapter 3:

iPSC-derived Motor Neuron

Maturation

3.1 Introduction

iPSC-derived motor neuron (MN) models provide great benefits to the study of ALS in vitro (See Chapter 1.4.2). These cells allow studies to be conducted on the most disease relevant in vitro model without the need for additional genetic manipulation. iPSCs and their derived neurons not only contain the genetic mutation of interest, but also the entire genetic background of the patient which may contain other unidentified SNPs which could be important for disease progression. Although this study focuses on a known genetic cause of ALS - *C9ORF72* gene hexanucleotide repeat expansion - the use of iPSCs also allows for the study of sporadic diseases, as cells can be taken directly from patients, reprogrammed into iPSCs (see Chapter 1.4.2) then differentiated into the cell type of interest.

All iPSCs used in this Chapter and the patient vs control study in Chapter 4 were generated by Sally Cowley's Laboratory at the University of Oxford. As described in the Introduction Chapter 1.4.3. there are many published protocols available for generating iPSC-derived motor neurons. These protocols follow a general pattern of small molecule or growth factor addition, but the concentration, timing and duration of the protocols vary considerably. This chapter assesses the suitability of two differentiation protocols based on final cell maturation status and potential for electrophysiological studies. The protocols used are based on Qu *et al.*, 2014 and Du *et al.*, 2015.

3.1.1 Methods to Determine Motor Neuron Maturation

For adult onset diseases such as ALS, Parkinson's and Alzheimer's the maturation of iPSC-derived neurons is important when asking questions regarding disease phenotypes.

Patients with these diseases harbour the mutation for many years without any symptoms. Therefore, studying immature neurons may not recapitulate the functional phenotypes seen in patients.

3.1.1.1 Mature Motor Neuron Markers

The first stage of validating iPSC-derived cell types of interest is to evaluate what maturation markers are expressed, these include both protein and RNA. Maturation markers can be detected using several technical methods including:

- RT-qPCR to check the overall RNA expression of a selection of markers.
- RNA-Seq to look at the transcriptome of a bulk sample of cells or at the single cell level.
- Immunocytochemistry to visually assess the presence or absence of protein markers and in some cases their location within cells.
- Flow cytometry to assess protein markers rapidly in a disassociated culture, as used in this thesis to assess the motor neuron marker SMI-32 in high density neuronal cultures. Close cell to cell contacts and overlapping projections make counting individual cells using immunocytochemistry challenging, whereas flow

cytometry eliminates this issue by dissociating the clumps and analysing cells individually as they run through the cytometer.

- Western blot to determine bulk protein levels of selected markers.

The best panel of markers to identify MNs is an ongoing debate in the ALS field as no single marker is specific to solely MNs, as reviewed in Sances et al 2016. Briefly, some potential markers are discussed below.

A group of transiently expressed transcription factors including: insulin gene enhancer 1 (ISL1) (Thor *et al.*, 1991; Ericson *et al.*, 1992), MN and Pancreas Homeobox 1 (HB9) (Arber *et al.*, 1999) as well as LIM homeobox 3 (LHX3) (Sharma *et al.*, 1998) are expressed in MNs and are often used in combination to identify MNs. Alone, none of these markers are truly specific as they are often expressed in different types of interneurons (LHX3 in V2, ISL1 in D2 and HB9 in ventromedial interneurons (Ericson *et al.*, 1997; Liem, Tremml and Jessell, 1997; Hinckley *et al.*, 2005)) as well as other cell types. Using each marker alone could lead to an over- or underestimation of motor neurons as these genes are only transiently expressed.

To circumvent the issue of down regulation, multiple mature MN markers can be used. Choline acetyltransferase (ChAT), the enzyme needed for acetylcholine biosynthesis, is expressed in all mature cholinergic neurons together with the mature marker, vesicular acetylcholine transporter (VACHT). In fact, *VACHT* gene is encoded within the first intron of the *ChAT* locus (Erickson *et al.*, 1994). However, again both ChAT and VACHT are present in interneurons (Krosnowski *et al.*, 2012). SMI-32 is often used in many studies as a marker for motor neurons and colocalises with acetylcholine in large neurons (Carriedo, Yin and Weiss, 1996). SMI-32 stains non-phosphorylated neurofilament heavy

chain found to be enriched but not exclusive to lower motor neurons (Carriedo *et al.*, 1995).

Overall, there are no truly specific motor neuron markers. Therefore, a combination of several markers is the most effective method to identify motor neurons in mixed cultures. Some studies suggest micro-RNAs, such as miR-218, may form more specific motor neuron markers for future studies (Amin *et al.*, 2015). All markers discussed above broadly label motor neurons but do not determine which subtype of motor neuron is present, this requires further transcriptional profiling, an area of study beyond the scope of this thesis but reviewed in Stifani, 2014.

3.1.1.2 Motor Neuron Functional Studies – Electrophysiology

Interrogating the functionality of neurons is an area of interest, with many studies using either calcium imaging or electrophysiology as an indicator of functional cellular machinery and neuronal maturation (Qu *et al.*, 2014; Du *et al.*, 2015; Maury *et al.*, 2015; Dafinca *et al.*, 2016).

Electrophysiology is a branch of functional neuroscience concerned with the measurement of ion flow across cell membranes and the techniques used to study this. The first electrophysiological studies looking at sodium and potassium ion channel properties were conducted in giant squid axons in 1952, for which Hodgkin and Huxley were merited the Nobel prize in Physiology or Medicine (Hodgkin and Huxley, 1990). Techniques were further developed using micropipettes to form high resistance seals on cell membranes to allow study of single ion channels in 1976 by Neher and Sakmann, also recipients of the Nobel prize in Physiology or Medicine (Neher and Sakmann, 1976).

The technique now generally referred to as 'Patch Clamp' is reviewed in a number of publications (Hamill *et al.*, 1981; Sachs and Auerbach, 1983; Kornreich, 2007) but a brief description is included here for reference.

There are several configurations to the patch clamp technique including: whole cell patch clamp, inside-out, outside-out and perforated. For all studies in this thesis I used whole cell patch clamp (Figure 3.1A-C). A whole cell patch clamp is generated by initially achieving a cell attached configuration (Figure 3.1B) with a high resistance gigaohm seal, followed by negative pressure or a high voltage pulse to rupture the cell membrane (Figure 3.1C). Rupture of the membrane inside of the micropipette allows for ion and electron exchange between the pipette internal solution and cell contents.

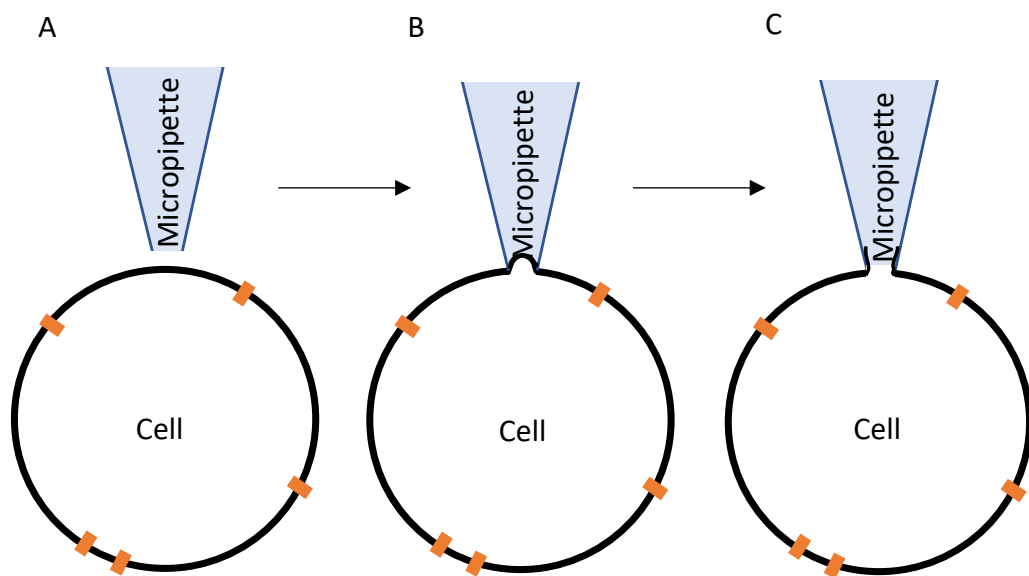


Figure 3.1: Whole cell patch clamp configuration

A) Approach to cell of interest applying positive pressure to ensure tip does not block. B) Cell attached configuration is generated when a gigaohm seal is formed between the glass electrode and the cell membrane. C) Whole cell configuration where the cell membrane is ruptured in the micropipette. Orange squares represent ion channels.

Once patched the electrical activity in cells can be assessed. Electrical activity in neurons is a result of the movement of sodium (Na^+), potassium (K^+), chloride (Cl^-) and calcium (Ca^{2+}) across cell membranes typically through ion channels. The movement of the charged ions is known as the current (I) measured in Amperes (A), and the work needed to move ions/charge is the voltage (V) measured in volts (V) and the ease at which the ions move through paths for current flow (which in neuronal systems are often ion channels) is the resistance (R) measured in ohms (Ω). The relationship between the terms V , I and R form Ohm's Law in which $V = IR$. Whole patch clamp as described above allows for measurement of multiple ion channels simultaneously within the patched cells through voltage clamp mode and membrane potential variation by current clamp. Ohm's Law forms the basis of the voltage clamp configuration of whole cell patch clamping. When voltage is held constant, any fluctuations in current across the cell membrane will indicate changes in resistance across the cell membrane (including opening/closing of ion channels).

During motor neuron maturation the properties measured using electrophysiology change. Studies assessing electrophysiology, as a test for maturation, usually present the resting membrane potential, evoked action potentials and the presence of Na^+ currents, some studies also show calcium transients. Often only sample traces are displayed and no formal quantification of these is presented. However, several studies have assessed the functional maturation of mouse and human ES-derived and iPSC-derived motor neurons (Miles *et al.*, 2004; Takazawa *et al.*, 2012; Lam *et al.*, 2017). During maturation the cell capacitance should increase (inferring an increase in cell size), the resting membrane potential of MNs should hyperpolarize to approximately -70 mV and neurons

should be able to repetitively fire action potentials in response to depolarization (Martin-Caraballo and Greer, 1999).

Action potentials are typically observed as a rapid change in voltage across the cell membrane initiated when a stimulus causes enough depolarization to reach threshold and trigger the opening of voltage-gated sodium channels (Hodgkin and Huxley, 1990) causing the upstroke of the action potential (Figure 3.2). The inactivation of the voltage-gated sodium channels (Na_v) and activation of voltage-gated potassium channels (K_v), including A-type and delayed rectifier, cause the downstroke of repolarisation/hyperpolarisation of the neuron before resting membrane potential is re-established (McLarnon, 1995)(Figure 3.2).

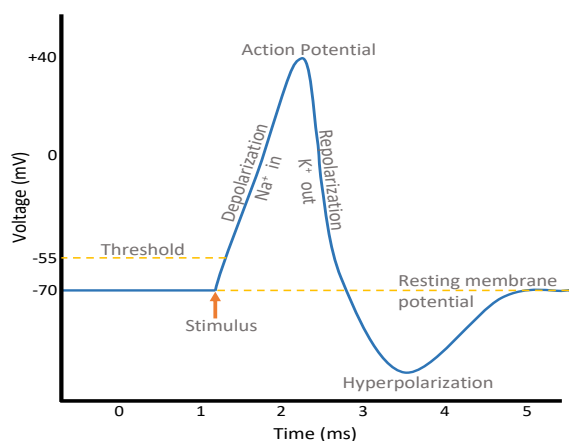


Figure 3.2: Diagram of an action potential
Adapted from (Molecular Devices, 2018)

The ability to repetitively fire action potentials relies on the presence of these voltage gated channels to depolarise and repolarise the neuron, as the cell matures these currents increase and the duration (half-width) of the action potential decreases (Takazawa *et al.*, 2012; Lam *et al.*, 2017). Additionally, as MNs mature they should become responsive to excitatory glutamate through NMDA and non-NMDA receptors and inhibitory GABA and glycine neurotransmitter through functional receptors (Westbrook and Mayer, 1984; Wu, Ziskind-Conhaim and Sweet, 1992; Gao and Ziskind-Conhaim, 1995).

3.1.2 Media to Increase iPSC Motor Neuron Maturation

Most differentiation protocols use common basal medias which include standard KO DMEM, DMEM-F12, Neurobasal or a combination of these which are then supplemented with various factors including serums, proteins or small molecules to aid patterning or maturation (Hu and Zhang, 2009; Qu *et al.*, 2014; Du *et al.*, 2015; Maury *et al.*, 2015). While standard protocols may produce cells with the markers for maturity, this does not necessarily indicate functional maturity of cells. Several groups have attempted to optimise this basal media to increase the functional activity seen by electrophysiology assessment of the cells in vitro.

3.1.2.1 BrainPhys

BrainPhys is a commercially available media from StemCell Technologies based on the paper by Fred Gage's group in 2015 (Bardy *et al.*, 2015). BrainPhys is aimed at increasing the maturation and activity of neurons in culture, particularly cells in the brain.

The BrainPhys study focused on the functionality of neurons by assessing their electrophysiological properties, as well as calcium imaging. Assessment of this media was based on comparison with 'Artificial cerebrospinal fluid' (ACSF), the optimal solution used in electrophysiology to record neuronal activity. ACSF alone cannot sustain cultures, therefore, BrainPhys was designed to combine the activity benefits of ACSF with the ability to sustain cells in culture of DMEM/F12 and Neurobasal media. It was shown that there was reduced calcium activity and reduced spontaneous action potential firing as

well as increased initial depolarisation of cells patched in the DMEM/F12 culture media compared to ACSF. Neurobasal A did not cause a depolarisation of the resting membrane potential, but did show reduced spontaneous and evoked action potential firing and reduced sodium/potassium channel currents.

The creation of BrainPhys focused primarily on optimising the concentration of inorganic salts Na^+ , K^+ and Cl^- as these are essential for action potential firing. BrainPhys includes 120 mM NaCl which is close to physiological levels, as well as Ca^{2+} at 1.1 mM again close to physiological levels. Amino acids such as glutamate, glycine, alanine and serine were removed/reduced as these influence both glutamatergic and GABAergic synaptic activity. The glucose levels were also reduced to more physiological levels of 2.5 mM, while the osmolarity was also increased to 300 mOsmol/L again to mimic cerebrospinal fluid. Overall with these changes BrainPhys (with supplements) can maintain neurons in culture and enhanced neuronal activity. However, this media was not designed to accelerate maturation but rather to increase neuronal activity in standard culture conditions (Bardy *et al.*, 2015).

3.1.2.2 Synaptojuice

Synaptojuice is another medium formulated with a similar aim to the development of BrainPhys, but with more emphasis on accelerating maturation of cells. Although not commercially available this media is based on a paper published in 2016 by Paul Kemp's and Nicholas Allen's groups (Telezhkin *et al.*, 2016). These groups aimed to create a defined media which when applied to neural progenitor cells would produce mature neurons in 21 days without the use of astrocyte co-culture. The media produced is based

on enhancing neurogenesis and synaptogenesis by manipulation of Notch inhibition, cell cycle exit and activation of GABA_A, CREB and WNT. The media is a two-phase system with media A (Table 3.1) applied for 7 days to encourage early neurogenesis and media B applied from day 8 until day 21 to support synaptogenesis (Table 3.1). BDNF and LM22A4 were included in both A and B to increase neuronal outgrowth and promote survival and DAPT and PD0332991 were included to promote cell cycle exit as mature neurons are post-mitotic. The combined media showed increased neurite extension, increased cell capacitance, hyperpolarized resting membrane potential, increased sodium currents and large GABA and glutamate postsynaptic currents when compared to standard differentiation methods.

The above two media described, BrainPhys and Synptojuice, are just two ways in which groups have tried to optimise the maturation of neurons in culture, other groups have explored areas such as differentiating cells on hydrogels (Z.-N. Zhang *et al.*, 2016), overexpression of the pre-mature aging gene Progerin (mutated Lamin A) (Miller *et al.*, 2013) or directly reprogramming somatic cells to neurons therefore, maintaining age-related epigenetic marks (Mertens *et al.*, 2015).

Synaptojuice A	Synaptojuice B
Advanced DMEM:F-12	1:1 Advanced DMEM/F-12:Neurobasal A
Glutamax	Glutamax
1% penicillin/streptomycin	1% penicillin/streptomycin
2% NeuroBrew21	2% NeuroBrew21 with RA
2 μ M PD0332991	2 μ M PD0332991
10 μ M DAPT	3 μ M CHIR 99021
0.6 mM CaCl ₂ (to give 1.8 mM total CaCl ₂ in final media)	0.3 mM CaCl ₂ (to give 1.8 mM total CaCl ₂ in final media)
200 μ M ascorbic acid	200 μ M ascorbic acid
10 ng/ml BDNF	10 ng/ml BDNF
1 μ M LM22A4	1 μ M LM22A4
10 μ M forskolin	
3 μ M CHIR 99021	
300 μ M GABA	

Table 3.1: Components of Synaptojuice

Adapted from Telezhkin *et al.*, 2016 to shows the components of synaptojuice A and B.

3.1.3 Aims of Chapter

The maturation chapter of this thesis aims:

- To establish a motor neuron differentiation protocol based on iPSCs as a starting population.
- To optimise the selected differentiation protocol to form cultures with high percentages of motor neurons
- To generate electrically active motor neuron cultures for subsequent patient vs control studies.

3.2 Qu motor neuron differentiation is unsuitable for electrophysiological studies

An optimised version of the Qu motor neuron differentiation published in 2014 was initially assessed to establish its suitability for patient vs control electrophysiology studies (Qu *et al.*, 2014). The published protocol follows a slightly different starting phase to most protocols reviewed in the Introduction Chapter 1.4.3, with the use of Compound C alone to inhibit Activin and BMP for the dual SMAD inhibition phase (Zhou *et al.*, 2010). The Qu protocol aimed to replace undefined media supplements with defined alternatives, such as replacing KO serum replacement with N2 media. Through these changes, the protocol reports ~87.5% PAX6⁺ neural progenitors 6 days after induction, and a final HB9 percentage of ~69.5%. Co-culture with mice myotubes gave evidence of neuromuscular junction formation and whole-patch clamp electrophysiology reported 69% of cells were able to fire evoked action potentials.

A control iPSC line, referred to as NHDF1, was initially differentiated here using a modified Qu protocol for 27 days. The modified Qu protocol was previously optimised by Dr R Dafinca in the Wade-Martins Laboratory and estimated to yield 90% neurons; 30% of which were MNs (Figure 3.3A). I captured phase contrast images throughout the differentiation and showed the cells growing as a dense monolayer (Figure 3.3B). Day 27 neurons were immunostained for MN marker ChAT and general neuronal marker beta III tubulin (TUJ1) (Figure 3.3C). TUJ1 positive neurons were present, however, it was not possible to distinguish ChAT positive neurons from background staining.

Initial phase contrast images and immunocytochemistry suggest that the Qu differentiation protocol is unlikely to be suitable for electrophysiological studies due to the dense packing of the cells, even after a low initial plating density of 25,000 cells per 11 mm coverslip. Although the yield of motor neurons was not analysed for this thesis, a predicted yield of only 30% motor neurons without a reporter system would cause difficulty when patching cells as it is most likely non-motor neurons would be patched. Overall cell density and motor neuron percentage gave non-optimal conditions for this protocol to be used for patient vs control iPSC derived motor neuron studies.

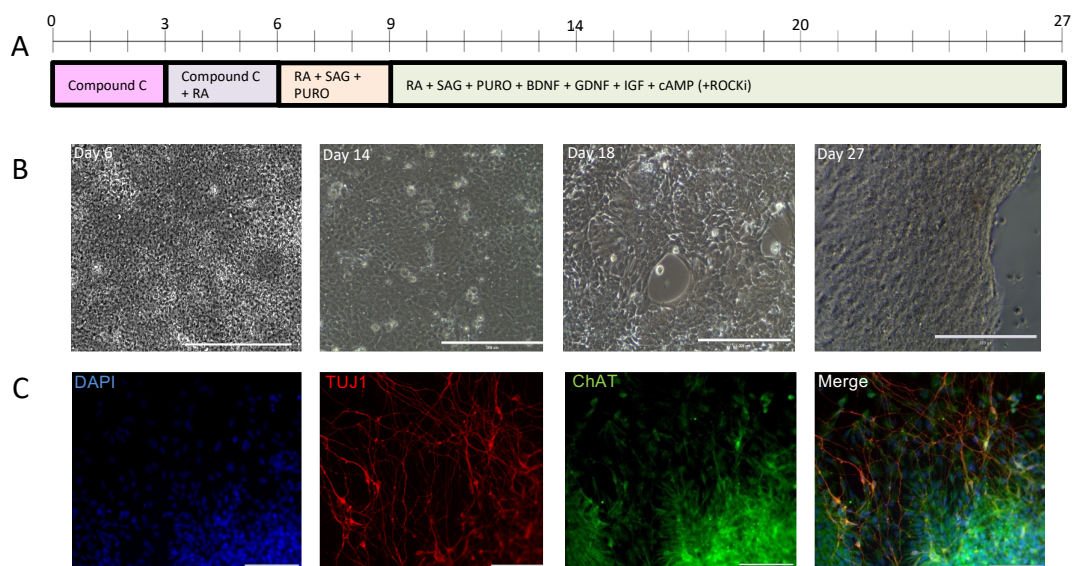


Figure 3.3: Optimised Qu iPSC to motor neuron differentiation protocol

A) Timeline of 27-day protocol published by Qu et al. 2014 and optimised in the Wade-Martins lab. Cells were re-plated on day 9 and 14. B) Phase contrast images capture the differentiation progression at day 6, 14, 18 and 27. Scale bars = 200 μ m C) Representative day-27 ChAT and TUJ1 staining on control iPSC line NHDF1. Scale bars = 100 μ m

3.3 Du motor neuron differentiation produces motor neurons after 28 days in culture

Subsequent to trialling the Qu protocol, two further protocols were published (Du *et al.*, 2015 and Maury *et al.*, 2015) in quick succession. The Du protocol was selected for this study as it provided a defined protocol whereas the Maury paper formed an analysis of supplementation application to promote differentiation.

The Du protocol follows the same general method of differentiation as outlined in the Introduction Chapter 1.4.3. However, it uses small molecule patterning factors in a defined media (Figure 3.4A). SB431542 (an inhibitor of Activin-Nodal signalling (Chambers *et al.*, 2009)) and DMH1 (BMP inhibitor (Neely *et al.*, 2012)) are initially used in the dual 'SMAD' inhibition phase along with CHIR99021 (Wnt activator) to promote neural induction (Li *et al.*, 2011). CHIR99021 was combined with SB431542 and DMH1 to allow for a single step induction and caudalization of neural progenitors, resulting in a reported 98% SOX1 positive cells (Figure 3.4A). This initial phase was followed by 6 days patterning using CHIR99021, SB431542, DMH1, Retinoic acid (RA) and Puromorphamine (Pur, sonic hedgehog signalling agonist) to give 95% OLIG2 positive cells, then 6 days in RA and Pur alone to give 90% HB9 positive cells. Finally, the cells were matured using RA, Pur, Compound E, IGF, BDNF and CNTF. Compound E, similarly to DAPT, is a gamma secretase inhibitor (NOTCH inhibitor), included to halt proliferation of cells resulting in 91% of neurons expressing the mature MN marker ChAT. Throughout the protocol cells are colony passaged every 6 days until day 12 when they are cultured as neurospheres

before dissociation and plating as single cells. To assess functionality, Du *et al.* cultured the neurons with C2C12 mouse myotubes and reported the formation of neuromuscular junctions. Neurons were also reported to be electrophysiologically active shown by the ability to elicit evoked action potentials.

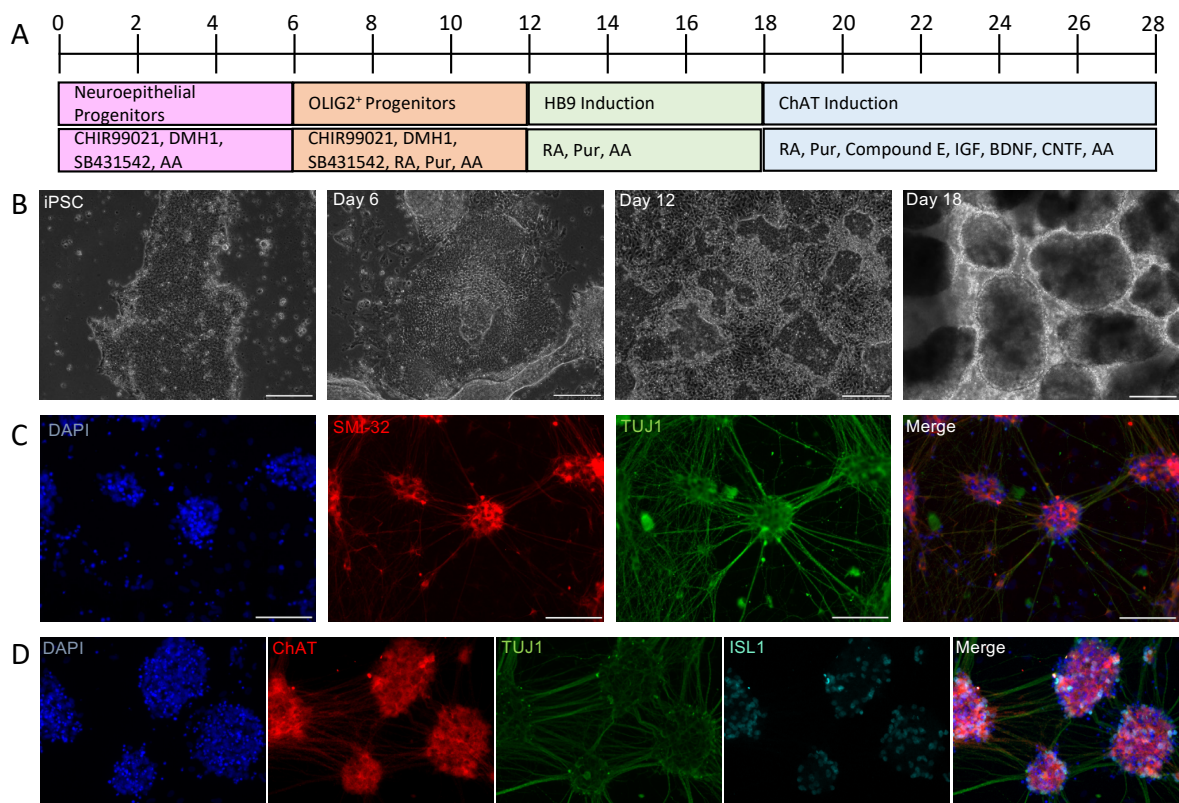


Figure 3.4: Preliminary characterisation of control iPSC line CTRL 2 differentiated using the Du protocol

A) 28-day timeline of Du *et al.* 2015 published protocol used here to differentiate CTRL 2 iPSCs to spinal motor neurons. Cells were re-plated at iPSC stage, day 6, day 12 and day 18. B) Phase contrast images of CTRL 2 were captured during the differentiation progression. Scale bars = 200 μ m. C) Co-localisation of SMI-32 and TUJ1 immunostaining in day 28 cells. Scale bars= 100 μ m. D) Co-localisation of ChAT, TUJ1 and ISL1 in day 28 cells. Scale bars =100 μ m

The preliminary study reported here aimed to establish the suitability of the Du protocol using two control lines, 856-03-04 referred to as CTRL 1, and AH017-3 referred to as CTRL 2. The protocol was tested for its suitability before use on three patient lines and three control lines to study differences in cellular dysfunction.

Several parameters were optimised in the initial trial rounds of differentiation including cell density after colony passaging at day 0, day 6 and day 12; the surface used to prevent cell attachment during neurosphere formation day 12-18; the enzymatic solution to dissociate neurospheres to single cells at day 18, and the single cell plating density. Passaging at a density of 20-30% gave good cell coverage and space for expansion between passaging as shown by phase contrast images after 6 days of growth (Figure 3.4B). Ultra-low attachment plates also led to neurosphere formation without attachment (Figure 3.4B day 18). Dissociation using Accumax and a plating density of 1.25×10^6 per well of a 12 well plate or 10^5 cells per 11 mm coverslip gave optimal survival and density for electrophysiological studies when observing DAPI immunostaining (Figure 3.4C-D). In addition to DAPI, further immunostaining at day 28 also indicated the presence of MN markers ChAT, ISL1, SMI-32 and general neuronal marker TUJ1.

Neuronal cultures generally form clumps of cells (Kriks *et al.*, 2011; Du *et al.*, 2015) which can cause inaccurate MN marker quantification by immunostaining counting. To overcome this issue I optimised and adapted a dopaminergic flow cytometry staining protocol previously established in the Wade-Martins laboratory (Sandor *et al.*, 2017) for use with MN cultures. MN flow cytometry was optimised and tested here on day 39 using control iPSC line CTRL 1 with TUJ1 to generally label neurons in the culture and SMI-32 to label motor neurons. Neurons at day 39 were gated using an unstained sample

to establish forward and side scatter parameters, this population was further gated using cells incubated with concentration matched IgG control antibodies (Figure 3.5A). After gating, cells showing fluorescence over threshold are counted as positive. This initial successful trial (Figure 3.5) revealed that approximately 80% of cells at day 39 expressed neuronal marker TUJ1 with ~67% of neurons also positive for SMI-32. This trial further confirmed the protein expression of TUJ1 and SMI-32 in iPSC derived MNs differentiated using the Du protocol and the suitability of the flow cytometry technique for quantifying MNs in clumpy cultures.

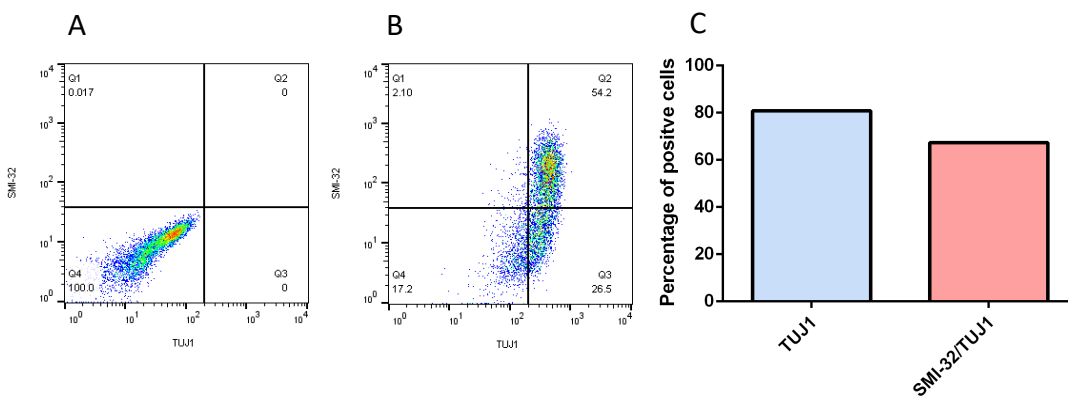


Figure 3.5: Preliminary flow cytometry of control iPSCs CTRL 1 using the Du protocol

A) Flow cytometry completed using cells stained with IgG control antibodies. B) Flow cytometry completed using cells stained with the neuronal marker TUJ1 (x axis) and the motor neuron marker SMI-32 (y axis). C) Graph showing the % of neurons and motor neurons from flow cytometry analysis expressing TUJ1 and SMI-32/TUJ1.

In addition to assessing protein marker expression, mRNA levels were evaluated by RT-qPCR at day 0 (iPSC stage), day 6, day 12, day 18 and day 28 for both CTRL 1 and CTRL 2. mRNA expression of MN markers *HB9*, *ISL1*, *ChAT* and *SMI-32* were analysed using the Livak method (Livak and Schmittgen, 2001) to calculate fold change over time compared to day 0 iPSCs. At the iPSC stage it would not be expected for *HB9*, *ISL1*, *ChAT* or *SMI-32* to be expressed. Both CTRL 1 and CTRL 2 gave differing patterns of expression for all four MN markers (Figure 3.6).

HB9 expression is expected to increase and then decrease over time, as this marker is transiently expressed in vivo (Arber *et al.*, 1999), this was seen for CTRL 1 but not CTRL 2 which gave an overall increase over time (Figure 3.6A). *ISL1* expression should show the same transient pattern as *HB9* (Thor *et al.*, 1991; Ericson *et al.*, 1992), both CTRL 1 and CTRL 2 showed the same patterns of expression as seen for *HB9* respectively (Figure 3.6B). *SMI-32* should increase across the time course and eventually plateau. An increase without plateau was observed for CTRL 2 but not for CTRL 1 which did not show much increase from iPSC levels. Finally *ChAT* expression should follow the same pattern of expression as *SMI-32*, however, both CTRL 1 and CTRL 2 showed an increase in *ChAT* expression until day 18, then a decrease at day 28.

Overall the general pattern of mRNA expression is similar to that expected but should be noted that these results are from two iPSC control lines with only one round of differentiation each. Increasing the number of control lines and the number of differentiations would increase the robustness of the data.

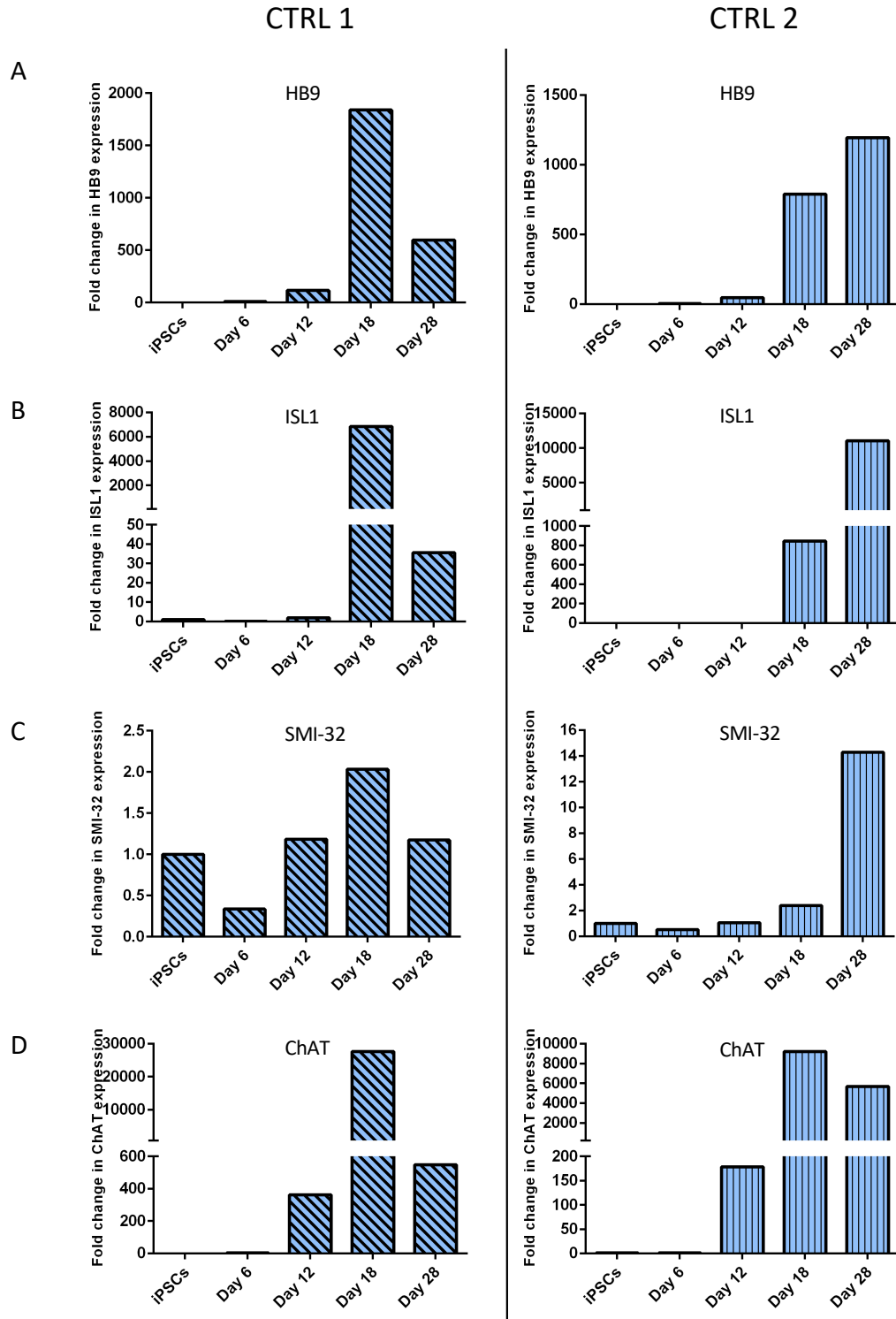


Figure 3.6: RT-qPCR of motor neurons marker expression throughout Du differentiation

A) SYBR green reagent was used to run RT-qPCRS for *HB9* expression in CTRL 1 and CTRL 2 and analysed using the Livak method of analysis. N = 1 differentiation (3 technical repeats). B) RT-qPCRS for *ISL1* expression in CTRL 1 and CTRL 2 and analysed using the Livak method of analysis N = 1 differentiation (3 technical repeats). C) RT-qPCRS for *SMI-32* expression in CTRL 1 and CTRL 2 and analysed using the Livak method of analysis N = 1 differentiation (3 technical repeats). D) RT-qPCRS for *ChAT* expression in CTRL 1 and CTRL 2 and analysed using the Livak method of analysis N = 1 differentiation (3 technical repeats). Fold change compared to iPSC expression level in all samples.

3.4 Adapting Du motor neuron protocol can produce motor neurons with mature phenotypes

Following the confirmation that the Du differentiation protocol produced MNs with mature MN markers, accessible for electrophysiological studies, the next stage was to assess whether the maturity of the cells could be pushed by adapting the maturation phase of the protocol.

Four adaptations to the original Du protocol were tested after day 18 re-plating including (Figure 3.7A):

- Extending the standard Du protocol past day 28 to timepoint 1 referred to as day 35 and timepoint 2 referred to as day 42. Experiments for each time point were completed in a window of 1-2 days either side of the timepoint. All supplements added to media in the day 18-28 phase are included (Figure 3.7A1). Condition referred to as 'Standard'.
- Removal of patterning factors after day 28 and again extending the time in culture as above. Patterning factors are removed, particularly Compound E as gamma secretase inhibitors have been shown to have detrimental effects to cellular function if maintained in media for an extended time (Shen, 2014; Kemp *et al.*, 2016) (Figure 3.7A2). Neurotrophic factors IGF, BDNF and CNTF are maintained in the media. Condition referred to as 'Reduced PF'.

- The same conditions as above but Compound E exchanged for the more commonly used DAPT, to assess whether this has stronger anti-mitotic effects (Figure 3.7A3). Condition referred to as 'DAPT'.
- Synaptojuice (applied from day 18 until day 40) with subsequent BrainPhys culture was tested as the final condition to assess whether this increased motor neuron maturation (Figure 3.7A4). Condition referred to as 'Synaptojuice'.

3.4.1 Flow cytometry to assess SMI-32 Expression

To establish the effects of these four conditions in altering the maturation phase of the protocol, the percentage of mature motor neurons – given by the number of SMI-32 positive cells - was determined at day 42 (Figure 3.7B-D). After the successful flow cytometry trial (Figure 3.5), this technique was used here to detect the percentage of TUJ1 positive cells and SMI-32 positive cells in all 4 conditions for both CTRL 1 and CTRL 2. The same gating procedure was applied as for the trial round. Representative flow cytometry plots (Figure 3.7B) show very few cells were found SMI-32 positive and TUJ1 negative as expected for this staining.

Based on TUJ1 percentage, all conditions produced high percentage neuronal cultures >70% and above. Synaptojuice yielded the highest percentage of TUJ1 positive cells, significantly increased compared to all other conditions (All statistics: Standard One-Way ANOVA with Post-hoc Tukey multiple comparisons, Standard 79.97% $\pm$ 1.73 vs Synaptojuice 93.67 $\pm$ 1.176, $p=0.0162$ N=2 lines, DAPT 71.56% $\pm$ 2.56 vs Synaptojuice 93.67 $\pm$ 1.176, $p=0.0027$ N=2 lines and Reduced PF 71.77% $\pm$ 0.87 vs Synaptojuice 93.67 $\pm$ 1.176, $p=0.0028$ N=2 lines). However, Synaptojuice resulted in a low SMI-32/TUJ1

percentage, significantly lower than the DAPT and reduced PF conditions (All statistics: Standard One-Way ANOVA with Post-hoc Tukey multiple comparisons, DAPT $44.32\% \pm 7.91$ vs Synaptojuice $3.64\% \pm 0.57$, $p= 0.0214$ N=2 lines and Reduced PF $44.65\% \pm 7.14$ vs Synaptojuice $3.64\% \pm 0.57$, $p= 0.0208$ N=2 lines). Both DAPT and reduced PF showed similar percentages of SMI-32 positive neurons ($p>0.05$) which were also increased by 2-fold compared to the standard condition, although this increase failed to reach statistical significance.

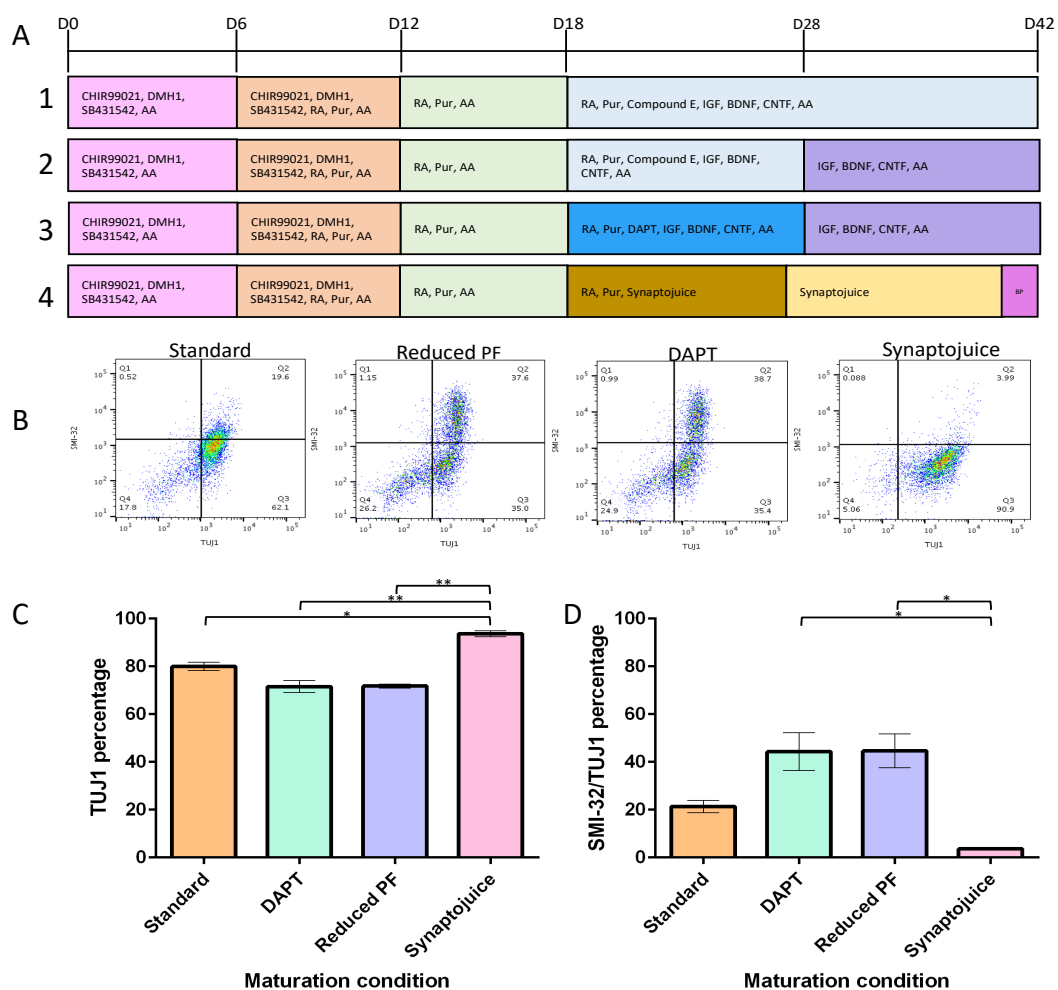


Figure 3.7: Flow cytometry analysis of Adaptations to the Du MN differentiation protocol

A) Multiple adaptations to the maturation phase of the Du MN protocol including; an extension of maturation time, removal of patterning factors, replacement of compound E for DAPT and Synaptojuice/BrainPhys supplementation. B) Flow cytometry completed at day 40 using the neuronal marker TUJ1 (x axis) and the motor neuron marker SMI-32 (y axis). C/D) Summaries of TUJ1 and SMI-32/TUJ1 flow cytometry percentages (N=2 control lines, 1 differentiation) (Mean $\pm$ SEM, Statistics: one-way ANOVA with Tukey multiple comparisons).

3.4.2 Immunocytochemistry and Morphology

To validate the presence of MN markers in the standard, DAPT and reduced PF conditions, as well as lack of markers in the Synaptojuice condition, immunocytochemistry using ISL1 and SMI-32 as well as the pan-neuronal marker TUJ1 was performed for day 42 cells. ChAT immunostaining could no longer be used, as several batches of the commonly used Millipore ChAT antibody failed to produce specific staining. All 4 conditions showed the presence of SMI-32 (Figure 3.8) and ISL1 (Figure 3.8). The Synaptojuice condition showed apparent high percentage positive ISL1 and SMI-32 cells, which was unexpected considering that flow cytometry showed only 3% positive SMI-32 cells. Therefore, immunostaining suggests that Synaptojuice does produce MNs. It should be noted that the morphology of Synaptojuice cultures is very different to the, standard, DAPT and reduced PF conditions (Figure 3.8). Synaptojuice cultures contain many more processes, which as explained in the discussion, could have affected the results obtained by the flow cytometry which was optimised for the cell morphology seen in the other conditions. Furthermore, Synaptojuice cultures also appear far less clumpy than the other conditions, forming instead a dense monolayer across the culture surface. This monolayer would be particularly useful for electrophysiological studies and imaging, although the plating density would need to be optimised as the cells are currently very dense.

Overall there appears to be little difference between DAPT and reduced PF conditions in terms of mature MN markers (SMI-32 and ISL1) and both conditions give a trend towards higher levels of mature markers than the standard protocol. Given the disparity between

ICC and flow cytometry, further investigation is required to establish whether Synaptojuice aids motor neuron maturation.

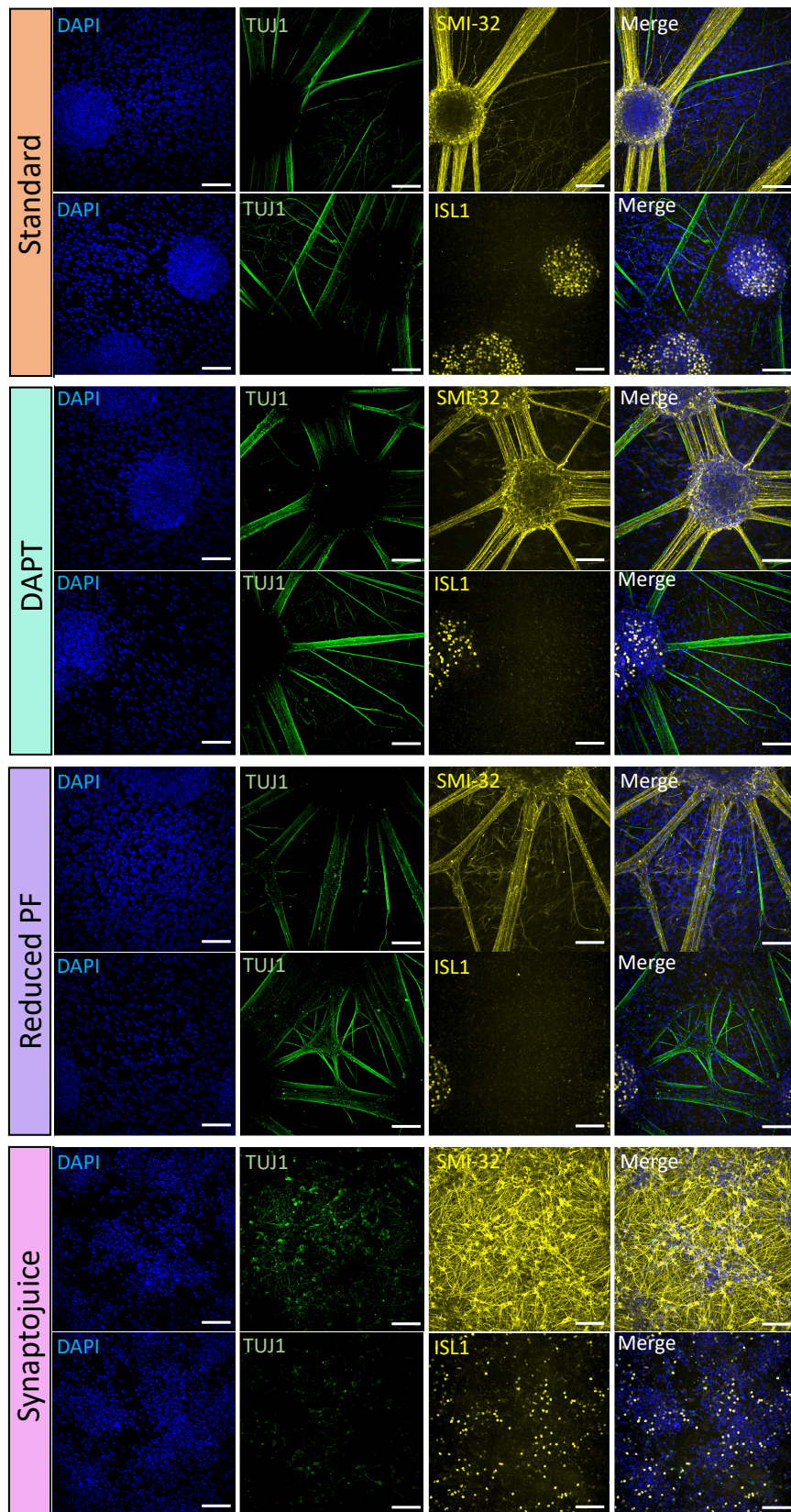


Figure 3.8: Immunostaining and morphology of Control iPSC lines with the adapted Du MN differentiation protocol.

Co-localisation of SMI-32 and TUJ1 immunostaining in day 42 cells for each condition. Scale bars= 100 μ m and co-localisation of TUJ1 and ISL1 in day 42 cells for each condition. Scale bars =100 μ m

3.4.3 RT-qPCR for mature motor neuron markers

In addition to flow cytometry and immunostaining, mRNA expression was analysed using RT-qPCR using the Livak method of analysis with iPSC cells day 0 as the internal control to compare fold change (Figure 3.9, iPSC control bar set to 1 not shown). Synaptojuice treated cultures expressed all four MN markers, *HB9* (Figure 3.9A), *ISL1* (Figure 3.9B), *ChAT* (Figure 3.9C) and *SMI-32* (Figure 3.9D) at both day 35 and 42. Under these conditions, expression of all 4 markers increased from day 35 to 42 in CTRL 1 cells. However, this was not observed for CTRL 2 which displayed a slight decrease over time for *HB9* and *ChAT* expression but a slight increase for *ISL1* and *SMI-32* expression. DAPT and reduced PF conditions behaved very similarly in CTRL 1 for all four MN markers. A decrease in *HB9* and *ISL1* expression was detected from day 35 to 42 for these conditions and a moderate decrease was also observed for *ChAT* from day 35 to 42. *SMI-32* expression remained unchanged from day 35 to 42. As before, CTRL 2 gave a more varied pattern of expression for the DAPT and reduced PF conditions. The standard condition for both control iPSC lines showed a decrease in *HB9* expression from day 35 to 42 but increase in expression over time for all other markers. Overall, it is clear that the RT-qPCR results give a varied pattern of expression for MN markers over time for each condition and control iPSC line. No clear pattern of expression is observed with no clear maturation condition expressing higher MN markers than the other conditions. It should be considered that this analysis was completed on two control iPSC lines differentiated once therefore, with more controls and additional differentiations a clearer pattern may have emerged. However, this data does support the ability of the Synaptojuice protocol to form neurons expressing mature MN markers.

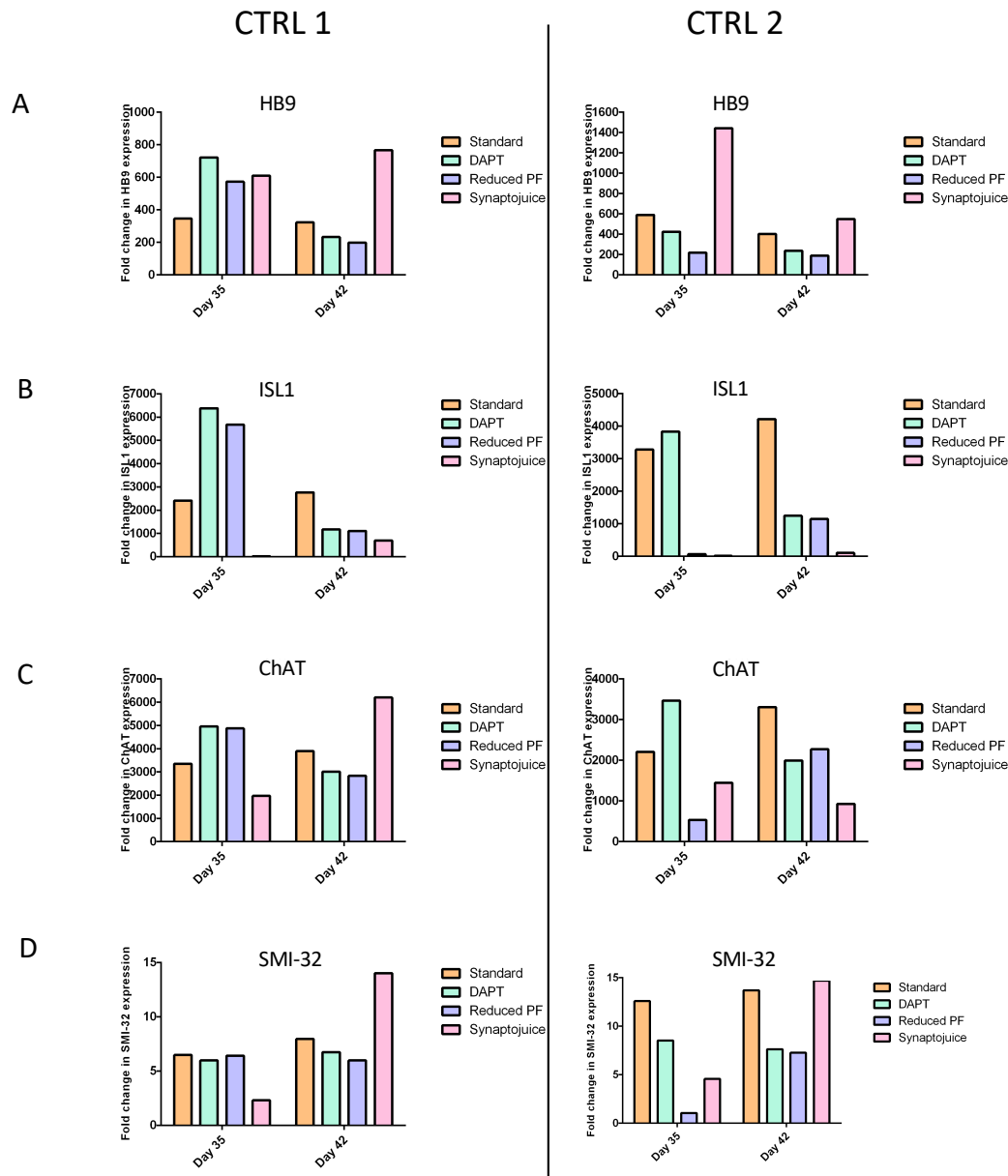


Figure 3.9: RT-qPCR of motor neurons marker expression throughout Du differentiation

A) SYBR green reagent was used to run RT-qPCRS for *HB9* expression in CTRL 1 and CTRL 2 N = 1 differentiation (3 technical repeats). B) RT-qPCRS for *ISL1* expression in CTRL 1 and CTRL 2 N = 1 differentiation (3 technical repeats). C) RT-qPCRS for *ChAT* expression in CTRL 1 and CTRL 2 N = 1 differentiation (3 technical repeats). D) RT-qPCRS for *SMI-32* expression in CTRL 1 and CTRL 2 N = 1 differentiation (3 technical repeats). All analysed using the Livak method of analysis with fold change compared to iPSC expression level in all samples (iPSC bar not shown but set to 1).

3.4.4 Electrophysiology of motor neurons

Finally, the ability of the different maturation conditions to produce active neurons suitable for future electrophysiological analysis was studied. Electrophysiological characterisation was completed on three of the conditions including: standard, DAPT and reduced PF. Patching of Synaptojuice cultured cells was not possible as the cultures were fragile and prone to peeling if disturbed as discussed further Chapter 3.5.

Neuronal capacitance and membrane resistance were recorded at day 35 and day 42 for both iPSC control lines CTRL 1 (Figure 3.10A) and CTRL 2 (Figure 3.10B). The membrane of a cell behaves like a capacitor therefore, cell capacitance (measured in picofarads pF) is proportional to cell surface area. Cells in the standard condition appeared to have a smaller surface area than the DAPT and reduced PF conditions (Figure 3.10A-B). The membrane resistance (R_m , measured in gigaohms, $G\Omega$) gives an indication of membrane permeability to current. A low resistance may indicate the presence of more open ion channels, although this would require further validation by ICC or immunopharmacological blockade. R_m seems to decrease over time in both control iPSC lines for both the standard and DAPT conditions, which could indicate greater functional insertion of ion channels into the neuronal membrane. Although, the reduced PF condition appears to show a slight increase in R_m compared to the other conditions, the number of cells patched for these assessments is too low to draw a firm conclusion. No significant difference was observed in resting membrane potential (Figure 3.10C) between the conditions over time (Two-way R_m ANOVA), although the standard condition showed the most depolarized resting membrane potential at both time points.

The most hyperpolarized resting membrane potential was observed at ≈ -40 mV for DAPT day 35 and reduced PF day 42.

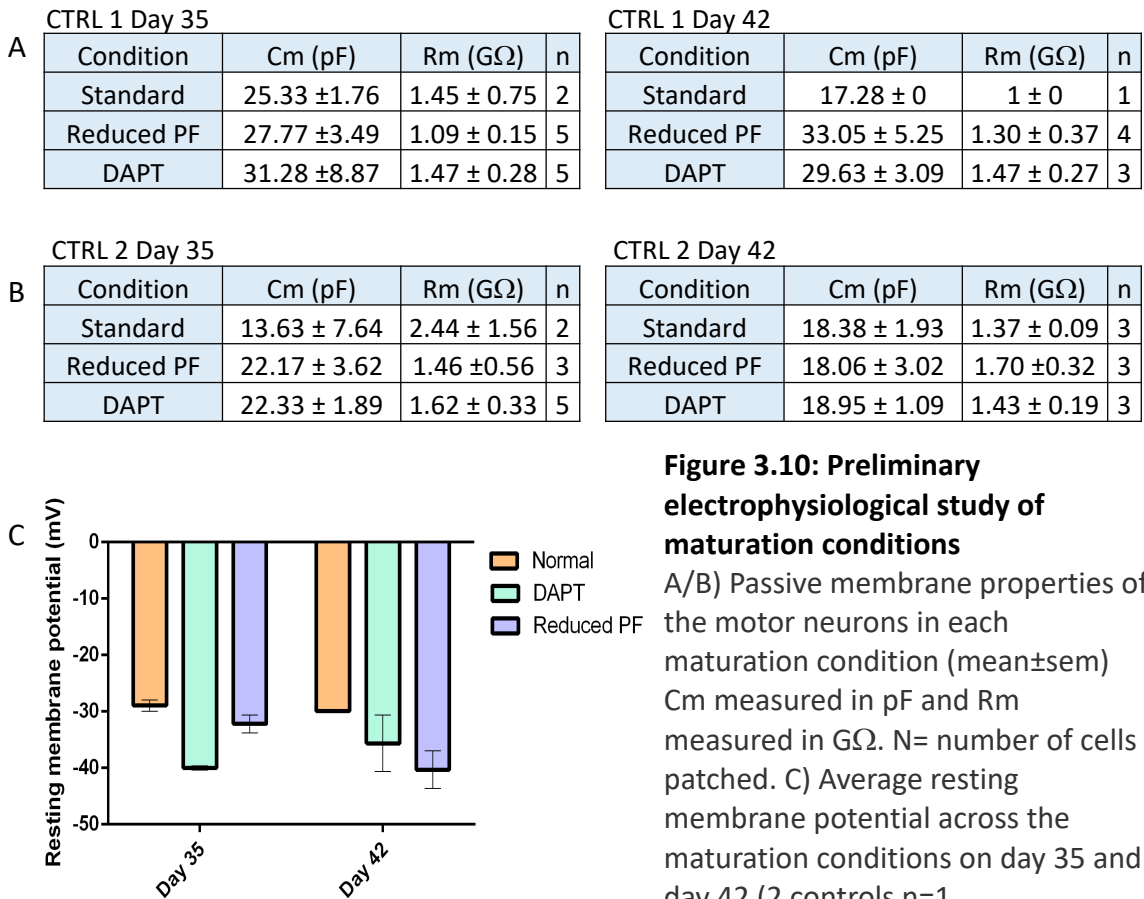


Figure 3.10: Preliminary electrophysiological study of maturation conditions

A/B) Passive membrane properties of the motor neurons in each maturation condition (mean $\pm$ sem) Cm measured in pF and Rm measured in G Ω . N= number of cells patched. C) Average resting membrane potential across the maturation conditions on day 35 and day 42 (2 controls n=1 differentiation) (mean $\pm$ sem).

Considering previous observations, it was decided either the reduced PF or DAPT conditions would be used in future patient vs control studies. Therefore, action potential firing was quantified for these conditions only (Figure 3.11). Additionally, the standard protocol produced less than 30% motor neurons, meaning the majority of cells patched would not be motor neurons, therefore, giving unrepresentative data for the motor neuron population. The standard condition was not used for further comparisons.

Assessing spontaneous motor neuron action potential activity at day 35 showed clearly that cells were not as active at this time point compared to day 42. Preliminary data from 16 total cells patched across the two control iPSC lines and both conditions (8 DAPT and 8 reduced PF) showed only 2 cells with spontaneous action potential firing over 40 mV in amplitude (this is 12.5% active cells in both conditions). However, at day 42 both DAPT and Reduced PF showed increased spontaneous action potential firing at resting membrane potential (Figure 3.11A), with reduced PF 2 out of 5 (40%) patched cells were active, while with DAPT 3 out of 6 (50%) patched cells were able to produce spontaneous action potentials (Figure 3.11A).

The ability to respond to current by evoking action potentials was also assessed in both conditions (see methods section 2.6.3 for a detailed protocol). Both DAPT and reduced PF conditions produced evoked action potentials, however again time was a significant factor. There was a significant increase in the number of action potentials fired from day 35 to day 42, as well as a significant increase with current increase from -10mV to 40mV. However, no significant difference in the number of action potentials was observed between reduced PF and DAPT cultured cells (Standard three-way ANOVA: Current injection $p = <0.0001$, Time $p = 0.0003$, DAPT vs Reduced PF $p = \text{ns}$, all other interactions $p = \text{ns}$, N=2 Control lines).

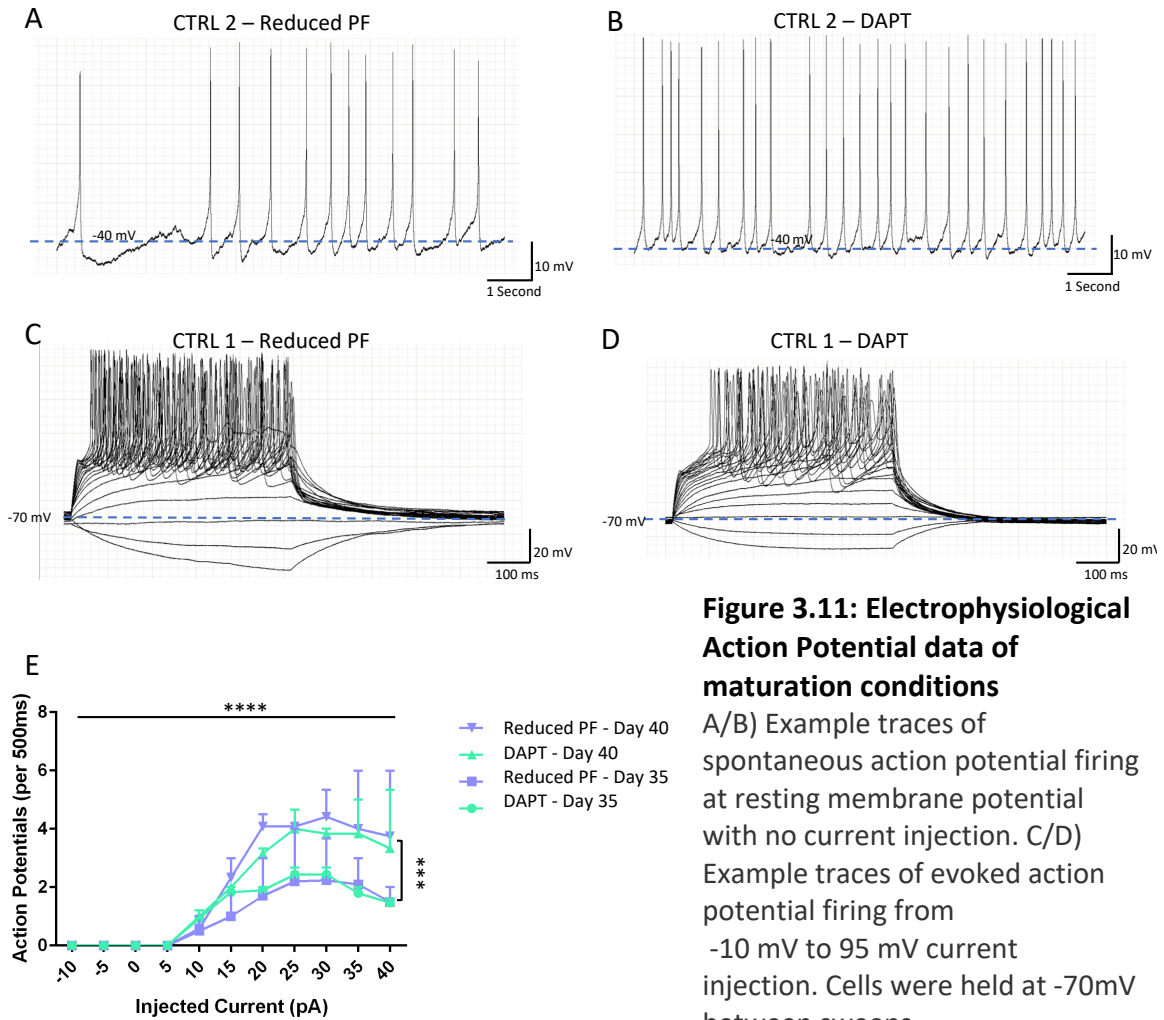


Figure 3.11: Electrophysiological Action Potential data of maturation conditions

A/B) Example traces of spontaneous action potential firing at resting membrane potential with no current injection. C/D) Example traces of evoked action potential firing from -10 mV to 95 mV current injection. Cells were held at -70mV between sweeps.

E) Average action potential firing per current injection step (Three-way ANOVA , N=2 control lines, mean $\pm$ sem)

3.5 Discussion

This chapter aimed to develop an iPSC-differentiation protocol able to generate mature motor neurons, suitable for whole cell patch clamp electrophysiology and calcium studies.

Initially the Qu *et al.*, 2014 protocol optimised by Dr R. Dafinca to yield 30% motor neurons, was trialled to assess its suitability for electrophysiology studies. However, differentiations produced dense monolayers of cells with ChAT staining indistinguishable from background staining. The combination of dense cells and low motor neuron efficiency is unfavourable for electrophysiology studies. This would increase the difficulty in patching the cell type of interest, as the morphology of individual cells is masked by the dense monolayer. Furthermore, the likelihood of patching a non-motor neuron is high as over 70% are non-motor neurons. Therefore, alternative differentiation protocols were explored with the aim of producing cultures with higher motor neuron percentages and less dense cultures.

The Du *et al.*, 2015 protocol was selected for trial as it was reported to produce over 90% ChAT positive and functionally mature motor neurons. Functionality was demonstrated by a combination of electrophysiology showing evoked action potentials and the ability of neurons to form neuromuscular junctions when co-cultured with mouse C2C12 myotubes. Advantageously, Du *et al.* also reported successful bulking and freezing of progenitor cells, allowing the banking of 1×10^4 motor neuron progenitors from 1 starting progenitor cell or 5×10^5 motor neurons from 1 stem cell.

Initial trials of this protocol confirmed the presence of mature motor neuron markers by ICC and RT-qPCR. ICC showed the presence of SMI-32, ChAT and ISL1 at day 28. RT-qPCR was used to determine the expression of *ISL1*, *HB9*, *SMI-32* and *ChAT* from day 0 to day 28. Interestingly, the two controls showed slightly different patterns of expression across the time course. As anticipated from in vivo studies (Thor et al., 1991; Ericson et al., 1992; Arber et al., 1999), CTRL 1 showed transient *HB9* and *ISL1* expression, peaking at day 18. Thereafter and throughout the maturation stage, *HB9* expression decreased. CTRL 2 showed an increase in both *HB9* and *ISL1* over time, which could indicate this line was maturing at a slower rate than CTRL 1 as the switch to repression on *HB9* and *ISL1* was not seen. This was noted and considered when using the line for future trials to ensure it did not deviate in function from the other controls. *SMI-32* did not show high enrichment compared to day 0 for CTRL 1 whereas CTRL 2 did show the expected increase in expression over time for a mature marker. However, CTRL 1 does show high *ChAT* enrichment over time which suggests the lack of *SMI-32* could be a technical issue. Although still high enrichment compared to day 0, both CTRL 1 and 2 show a slight decrease in *ChAT* expression at day 28 compared to day 18. This could be due to the proliferation of other cell types in the culture diluting the relative expression of *ChAT*. This pattern of reduction is not seen for the other mature marker *SMI-32*. This is not surprising as, although SMI-32 protein is restricted to MNs, *SMI-32* mRNA (not protein) is expressed in other cell types including iPSCs as evidenced in this study. Therefore, the expression of *SMI-32* mRNA will continue to increase with the other cell types. Following initial characterisation of the Du et al protocol, four optimisations to the protocols were trialled in an attempt to increase the maturation of the motor neurons for future patient vs controls studies. The four conditions included:

- Extending the standard Du protocol past day 28 referred to as 'Standard'.
- Removal of patterning factors after day 28 and again extending the time in culture as above, referred to as 'Reduced PF'. Patterning factors such as compound E were removed as some studies have shown prolonged use of gamma secretase inhibitors can lead to detrimental effects on neurons and in some differentiation protocols is now removed after short usage (Shen, 2014; Kemp *et al.*, 2016).
- The same conditions as above but Compound E exchanged for the more commonly used DAPT, referred to as 'DAPT' to assess efficiency in motor neuron protocols. DAPT and compound E are both gamma secretase inhibitors often used in differentiation protocols to induce maturation of the neural progenitors by forced cell cycle exit (Borghese *et al.*, 2010). Although DAPT is widely used, some studies have reported Compound E to be more effective in particular differentiation protocols such those used to generate dopaminergic neurons (Tieng *et al.*, 2014). Therefore, for this study it was of interest to determine which compound was most effective.
- Synaptojuice (applied from day 18 until day 40) with subsequent BrainPhys culture referred to as 'Synaptojuice'. This protocol is designed to produce active, mature neurons when applied to the neuronal progenitor stage of differentiation.

Characterisation of the four protocols using flow cytometry, confirmed that all four protocols were able to produce highly enriched neuronal cultures – with over 70% TUJ1 positive cells. Synaptojuice producing significantly higher percentages of neurons >90%. The standard protocol produced ~ 20% SMI-32/TUJ1 positive cells whereas both the

DAPT and reduced PF protocols produced ~ 40%. This supports the hypothesis that prolonged gamma secretase inhibition can cause detrimental effects to motor neurons as this protocol produced a lower percentage of neurons than the protocols with reduced DAPT/Compound E exposure. Remarkably, although Synaptojuice produced the highest percentage of neurons, less than 10% of these were SMI-32 positive by flow cytometry. Further validation using ICC and RT-qPCR analysis showed motor neuron production at a comparable level to the reduced PF and DAPT conditions, indicating a technical issue with the Synaptojuice flow cytometry analysis. There are several reasons as to why the flow cytometry profiling may not reflect the true population observed via ICC and RT-qPCR. It is possible that the morphology of the culture could have impacted the flow cytometry. Rather than the aggregated culture observed for the other conditions, Synaptojuice produced a monolayer with multiple neuronal projections. As SMI-32 is a neurofilament it primarily localises to neurites rather than the neuronal soma. Furthermore, during flow cytometry processing, cells are dissociated and neuronal projections mechanically sheared from the somas. Together, this might explain the poor somatic SMI-32 signal in Synaptojuice cultures. Saturating concentrations of SMI-32 antibody would need to be tested to confirm this hypothesis.

The Synaptojuice protocol was not ultimately used due to other issues with cell peeling during electrophysiology. However, Synaptojuice cultures formed as a monolayer with few clumps and other published protocols have shown remarkably active neurons following the two phase media addition (Telezhkin et al., 2016). Therefore, it could be interesting to optimise this protocol further.

Overall the *reduced PF* protocol was selected for future studies as this condition gave increased motor neuron percentages as compared to the Standard protocol.

Furthermore, cells cultured with reduced PF were suitable for patch clamp electrophysiology as evidenced by the ability to record intrinsic properties such as cell capacitance, membrane resistance, spontaneous action potentials and evoked action potentials. As discussed in Chapter 3.1.1.2 further recordings of resting membrane potential, cell capacitance, repetitive action potential firing, peak voltage gated sodium and potassium channel current and neurotransmitter response over time would be required to fully assess the maturation of the different media conditions.

Of note the DAPT condition in this study, appeared to be as effective as the use of Compound E in directing cells towards a mature state yielding a similar percentage and action potential activity of mature motor neurons.

Chapter 4:

***C9ORF72* iPSC patient derived motor neurons display functional dysregulation**

4.1 Introduction

The maturation study Chapter 3, determined that the optimised 'Reduced Patterning factor (PF)' modification of the Du motor neuron (MN) protocol was most suitable for downstream electrophysiological studies. This thesis chapter exploits this optimised protocol to generate iPSC-derived MN from patients harbouring the large hexanucleotide repeat expansion in the *C9ORF72* gene and from non-repeat carriers. These cells are then characterised to determine the effect of the hexanucleotide expansion on electrophysiological properties and cellular dysfunction.

4.1.1 Expanded *C9ORF72* functional phenotypes

As discussed in the Introduction (Chapter 1) the function of *C9ORF72* protein is still largely unknown and the disease-causing hypothesis is divided between three non-mutually exclusive areas: haploinsufficiency, toxic RNA foci and toxic protein. Although it is not known which mechanism, or combination of mechanisms are causative of the disease, there is consensus that RNA-processing is critically affected in ALS. RNA processing is important for overall cellular functioning but particularly in neurons for synapse function and strength (Liu-Yesucevitz *et al.*, 2011; Iacoangeli and Tiedge, 2013). Several functional phenotypes have been observed in neurons including excitotoxicity and calcium dysfunction (as discussed in the introduction Chapter 1.5) and the resulting excitability -linked phenotypes described below.

4.1.1.1 Published excitability phenotypes in *C9ORF72* gene expansion motor neurons.

The excitability of the neuron describes its ability and frequency of action potential firing. As described in Chapter 3, the ability of cells to fire trains of action potentials is an indication of maturity, but a change in the frequency, as observed for ALS, leads to cellular dysfunction.

Several studies have examined the excitability of iPSC-derived MNs harbouring the *C9ORF72* gene expansion and have found differing results. A study by Sareen *et al* in 2013 showed a decrease in patient iPSC-derived MN action potential firing compared to controls. While a study by Wainger *et al* in 2014 reported hyperexcitability in *C9ORF72* gene expansion, *SOD1*^{+/A4V} and *FUS* (MGH5b, frameshift mutation at residue 511) iPSC-derived motor neurons (Wainger *et al.*, 2014) . These contradictory findings may be explained by the differences in the differentiation protocols used or by the fact that the MNs tested by (Sareen *et al.*, 2013) were much older. The patients in the study by Sareen *et al*, also displayed a more depolarised resting membrane potential than the controls, which could have resulted in a higher percentage of inactivated sodium channels and so reduced ability to fire action potentials. Further to these studies another group have used a third method of iPSC-derived MN differentiation analysing the excitability of MNs neurons over time. This method appears to reconcile the previous studies and shows a shift in excitability from hyper- to hypo- excitability as the cells age in culture (Devlin *et al.*, 2015). In support if this, the study also reported a reduction in both voltage-gated sodium and voltage-gated potassium ion current with time in culture.

However, another recent study has shown no differences in the excitability of neurons between patient and control cells. This study used a motor neuron differentiation

protocol which produced negligible numbers of glial cells in comparison to the above discussed studies (Selvaraj *et al.*, 2018). As discussed in Chapter 1.5, the interplay between neurons and glia appears to play a large role in synapse dysfunction, modulating both excitability and excitotoxicity.

Overall the MN dysfunction story is still emerging in respect to excitotoxicity and its related calcium and excitability phenotypes. Therefore, this study aims to thoroughly characterise patient and control iPSC-derived motor neuron cultures to allow robust interpretation of any phenotypic differences between the groups. A thorough characterisation of the cells produced will allow for detailed comparison against previously published work, and will add weight to the hypotheses of either hypo- or hyper- excitability if observed. Combining internal calcium release data with excitability studies adds another angle of exploration to the mechanism behind hyper- or hypo- excitability.

4.1.2 Aims of Chapter

This chapter will focus on the characterisation of patient vs control iPSC derived motor neurons using the 'Reduced PF' protocol and any dysfunctional phenotypes observed between the groups. This chapter aims to:

- Select three patient iPSC lines with the *C9ORF72* gene repeat expansion and three control iPSC lines without the expansion and validate expansion presence/absence.
- Differentiate three patient iPSC lines and three control lines using the optimised 'Reduced PF' Du motor neuron protocol.
- Compare MN marker protein expression between patient and controls to assess whether the presence of the *C9ORF72* gene expansion impacts the final population of motor neurons.
- Compare MN marker mRNA expression between patient and controls to further assess whether the presence of the *C9ORF72* gene expansion impacts the differentiation capability at different time points.
- Compare the electrophysiological intrinsic properties and excitability properties of patient vs control iPSC-derived motor neurons.
- Compare the intracellular calcium release properties of patient vs control iPSC-derived motor neurons using pharmacological stimulation.

4.2 Induced pluripotent stem cell line selection and validation

To analyse the effect of the hexanucleotide repeat expansion in the *C9ORF72* gene, three patient iPSC lines and three control iPSC lines were selected for differentiation (Figure 4.1). All iPSC lines were generated by Sendai Virus reprogramming in Dr Sally Cowley's laboratory, in collaboration with Professor Kevin Talbot's laboratory that provided the published patient lines (Dafinca *et al.*, 2016).

It should be noted that based on their clinical symptoms, two patients' (ALS 2 and ALS 3) were diagnosed as ALS with FTD while the third patient (ALS 1) had a diagnosis of ALS with no FTD. As alluded to in the introduction Chapter 1.1.2, the *C9ORF72* gene repeat expansion form of both ALS and FTD are closely linked. 50% of ALS patients show cognitive impairment and 10-15% are diagnosed with FTD, causing ALS and FTD to often be considered a continuum disease (Ji *et al.*, 2017). Twin studies have also highlighted the uncertain nature of the *C9ORF72* gene expansion and whether it will cause ALS, FTD or ALS-FTD. Studies have reported cases where one twin presented clinical ALS (no cognitive impairment) while the other twin presented only cognitive impairment but no ALS symptomology (Xi *et al.*, 2014). Therefore, considering the iPSC lines available for this thesis, the presence or absence of the *C9ORF72* gene expansion was the deciding factor for patient selection rather than a pure ALS clinical presentation (Figure 4.1).

Label	Identification Code	Method of Reprogramming	Age	Gender	Genotype
CTRL 1	856-03-04	Cytotune (SeV)	78	F	Control
CTRL 2	AH017-3	Sendai virus (SeV)	67	F	Control
CTRL 3	065-03-03	Cytotune 2 (SeV)	65	M	Control
ALS 1	OXC9-04-01	Cytotune (SeV)	39	M	ALS
ALS 2	OXC9-01-06	Cytotune (SeV)	72	M	ALS-FTD
ALS 3	OXC9-02-02	Cytotune (SeV)	62	F	ALS-FTD

Figure 4.1: iPSC lines selection

Selection of three patient and three control iPSC lines and the corresponding donor information including: age of donor when sample taken, gender, method to reprogram cells and clinical disease classification. Key: F = Female, M = Male, SeV = Sendai virus

Following patient and control selection, the presence of the *C9ORF72* gene expansion in patient iPSC lines and the absence in control iPSC lines was checked using repeat primed PCR. Due to the nature of the repeat primed PCR protocol, cells positive for the expansion show a stuttered pattern in the fragment analysis. This is a result of the higher PCR reaction efficiency at amplifying shorter fragments and decreasing amplification efficiency with longer fragments. This presents as a gradient on the resulting fragment analysis from high relative fluorescent unit readings at low basepairs to low relative fluorescence units at high basepairs. Conventional PCR amplification is not efficient enough to detect this stuttered pattern, repeat primed PCR includes an additional third *anchor* primer that binds to the forward primer to help amplify repeat fragments. This technique is used to detect the presence or absence of the repeat expansion, it cannot be used to calculate the size of the expansion due to the limited detection of larger repeat fragments. To determine the size of the *C9ORF72* repeat expansion the Southern Blotting technique would need to be used.

Repeat primed PCR was optimised in this study for use on iPSC and iPSC-derived MNs, based on the published protocol by Hantash *et al.*, 2010 and DeJesus-Hernandez *et al.*, 2011 to validate the presence or absence of the *C9ORF72* gene expansion before and after MN differentiation (reduced PF Du differentiation) (Figure 4.2).

At the iPSC stage and day 47 none of the controls showed the presence of a gene expansion, however, all patients showed the amplification pattern typical of highly repetitive sequences (Figure 4.2).

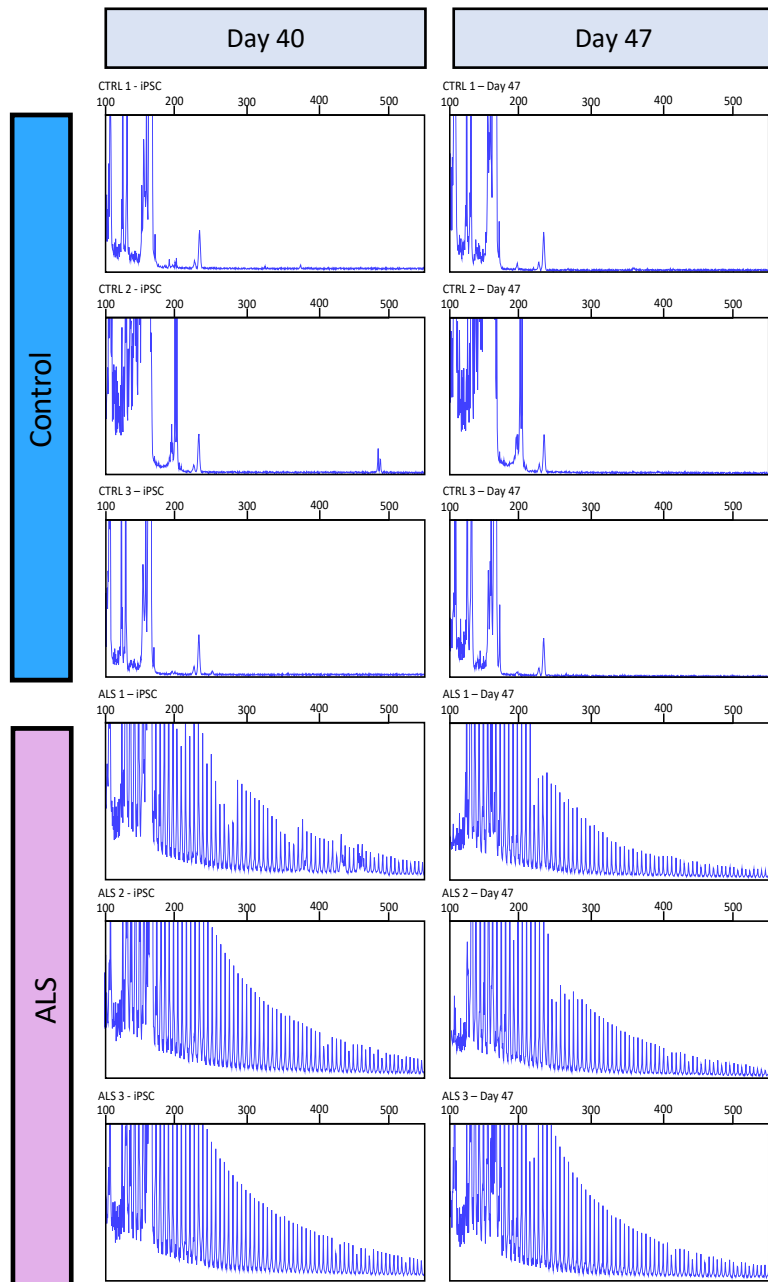


Figure 4.2: Presence/absence *C9ORF72* gene repeat expansion
 The presence of the *C9ORF72* gene repeat expansion in patient cells and lack of expansion in control cells was checked using repeat primed PCR. Y-axis zoomed to 2000 relative fluorescent units. X-axis in base pairs

4.3 ALS vs CTRL iPSC-derived MNs show comparable morphology but differing MN marker protein expression

Following validation of genotype, comparison of cellular morphology and MN marker expression were assessed using phase contrast microscopy, flow cytometry and immunocytochemistry (ICC).

As mentioned above, following the maturation study the 'reduced PF' optimised Du MN protocol was selected for electrophysiological characterisation (Figure 4.3A). To promote neuronal maturation, the final maturation stage was extended to include day 40 and day 47 in culture. For logistical reasons, all experiments were performed within ± 2 days of a given timepoint.

To document changes in morphology both between lines and between differentiations and to assess whether any changes in morphology correlate to phenotypical changes, cells were imaged along each differentiation for every line. Throughout the differentiation, the morphology of patient and control cells was indistinguishable as shown for CTRL 1 and ALS 1 (Figure 4.3B-C). Patient and control cells also behave in a similar manner during the colony passaging, neurosphere formation and single cell replating.

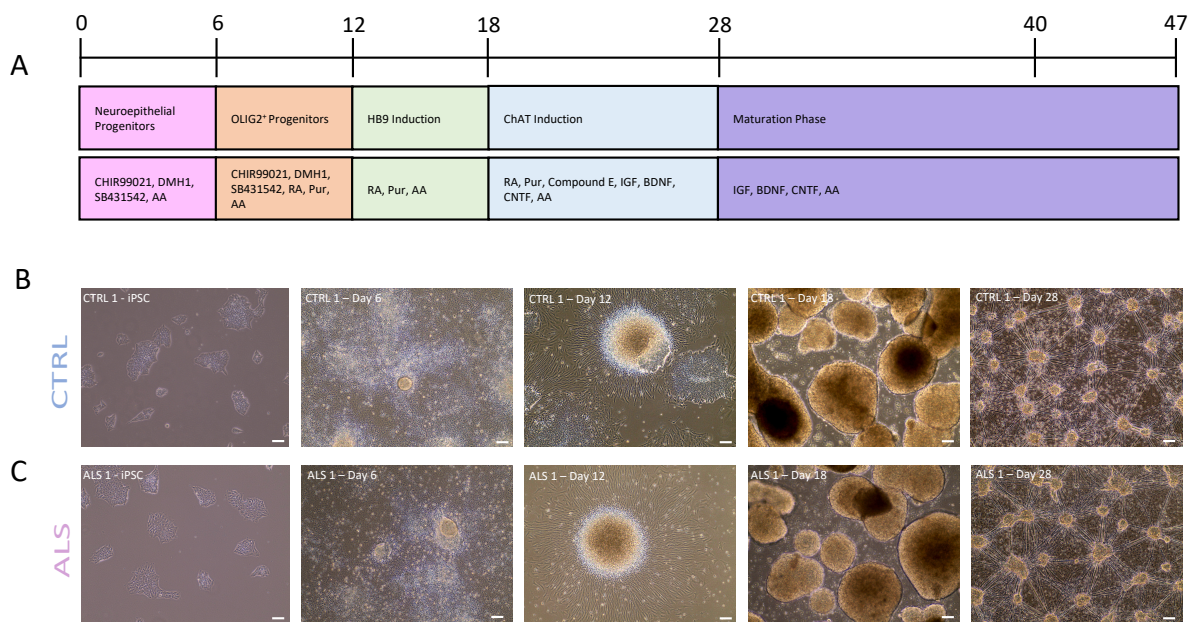


Figure 4.3: Optimised 'Reduced PF' Du motor neuron protocol.

A) Representation of the optimised 'Reduced PF' Du motor neuron protocol. Cells are colony passaged every 6 days until day 18 when the cells are dissociated and plated as single cells for maturation. B/C) Phase contrast images of one representative control iPSC line (CTRL 1) and one patient line (ALS 1) throughout the optimised 'Reduced PF' Du motor neuron protocol. Scale bars = 100 μ m.

As the morphology of the cells was indistinguishable, MN marker protein expression was assessed between patient and control lines to determine whether there were differences in motor neuron formation. As used in the maturation study, flow cytometry was completed on cells at day 40 and 47 to determine the percentage of neurons (TUJ1 positive cells) and motor neurons (SMI-32/TUJ1 positive cells). As before, cells were gated using an unstained control and then each line gated using the relevant IgG control before detection of SMI-32 and TUJ1 positive cells.

Combining the three patient (ALS 1, ALS 2 and ALS 3) and three control (CTRL 1, CTRL 2, CTRL 3) iPSC-derived neurons (each line differentiated 3 times) indicated both genotypes produced over 80% TUJ1 positive neurons at day 40 and 47 (Day 40, N=3, mean $\pm$ SEM: CTRL 88.23% $\pm$ 0.87, ALS 87.55% $\pm$ 0.18 and day 47, N=3, mean $\pm$ SEM: CTRL 82.66% $\pm$

2.07, ALS $87.38\% \pm 1.33$) with no significant differences between genotype or time (Two-way RM ANOVA) (Figure 4.4A).

Both controls and patient iPSC-derived MNs produced over 50% of TUJ1 positive neurons co-staining for MN marker SMI-32 (Day 40, N=3, mean $\pm$ SEM: CTRL $50.66\% \pm 0.73$, ALS $66.18\% \pm 3.51$ and day 47, N=3, mean $\pm$ SEM: CTRL $50.57\% \pm 3.41$, ALS $58.56\% \pm 2.40$) (Figure 4.4B). A significant difference was detected for genotype, specifically at day 40 but again no significant difference in percentage of SMI-32 positive cells over time was apparent (Two-way RM ANOVA: genotype $p = 0.0233$, time= ns, interaction= ns with Sidak's multiple comparisons, Day 40 CTRL $50.66\% \pm 0.73$ vs ALS $66.18\% \pm 3.51$, $p = 0.0080$, N= 3).

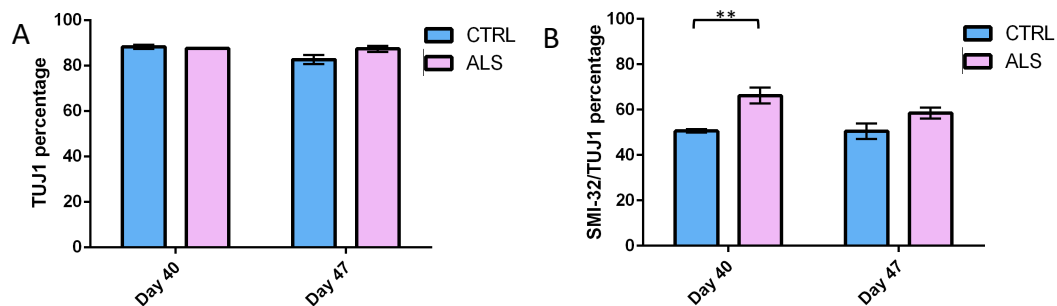


Figure 4.4: Flow cytometry analysis of patient vs control optimised Du differentiated MNs.

Flow cytometry completed using cells stained with IgG control antibodies and cells stained with the neuronal marker TUJ1 and the motor neuron marker SMI-32. A/B) Show data pooled from three patient and three control lines differentiated 3 times. (Two-way RM ANOVA with Sidak's post hoc analysis)

To validate the results from flow cytometry and to establish whether other MN markers were expressed, immunocytochemistry (ICC) was performed on all iPSC patient (ALS 1, ALS 2, ALS 3) and control lines (CTRL 1, CTRL 2, CTRL 3) at both day 40 (Figure 4.5) and 47 (Figure 4.6). ICC was performed at the end of each differentiation to check the presence of MN markers as well as astrocyte markers.

Cells positive for MN markers ISL1 and SMI-32, as well as general neuronal marker TUJ1 were seen for all lines at both day 40 (Figure 4.5) and 47 (Figure 4.6). SMI-32 staining shows a high percentage of double positive SMI-32/TUJ1 neurons, therefore validating the results reported from the flow cytometry. ICC images also suggest a potential decline in the percentage of ISL1 positive cells from day 40 to day 47. However, due to the 'clumpy' nature of the culture, accurate quantification using ICC is challenging but could be of interest for future flow cytometry analysis.

Glial fibrillary acidic protein (GFAP), a marker of astrocytes (Sofroniew and Vinters, 2010), was observed again at day 40 and 47 in culture (Figure 4.5/4.6). This marker was tested as a result of the flow cytometry which estimated ~20% of cells in the culture were not motor neurons (It should be noted that the flow cytometry cells are gated based on an unstained control, meaning extremely large or small cells are excluded from analysis). It is clear from this ICC that astrocytes are present in the culture but again need further quantification possibly by flow cytometry.

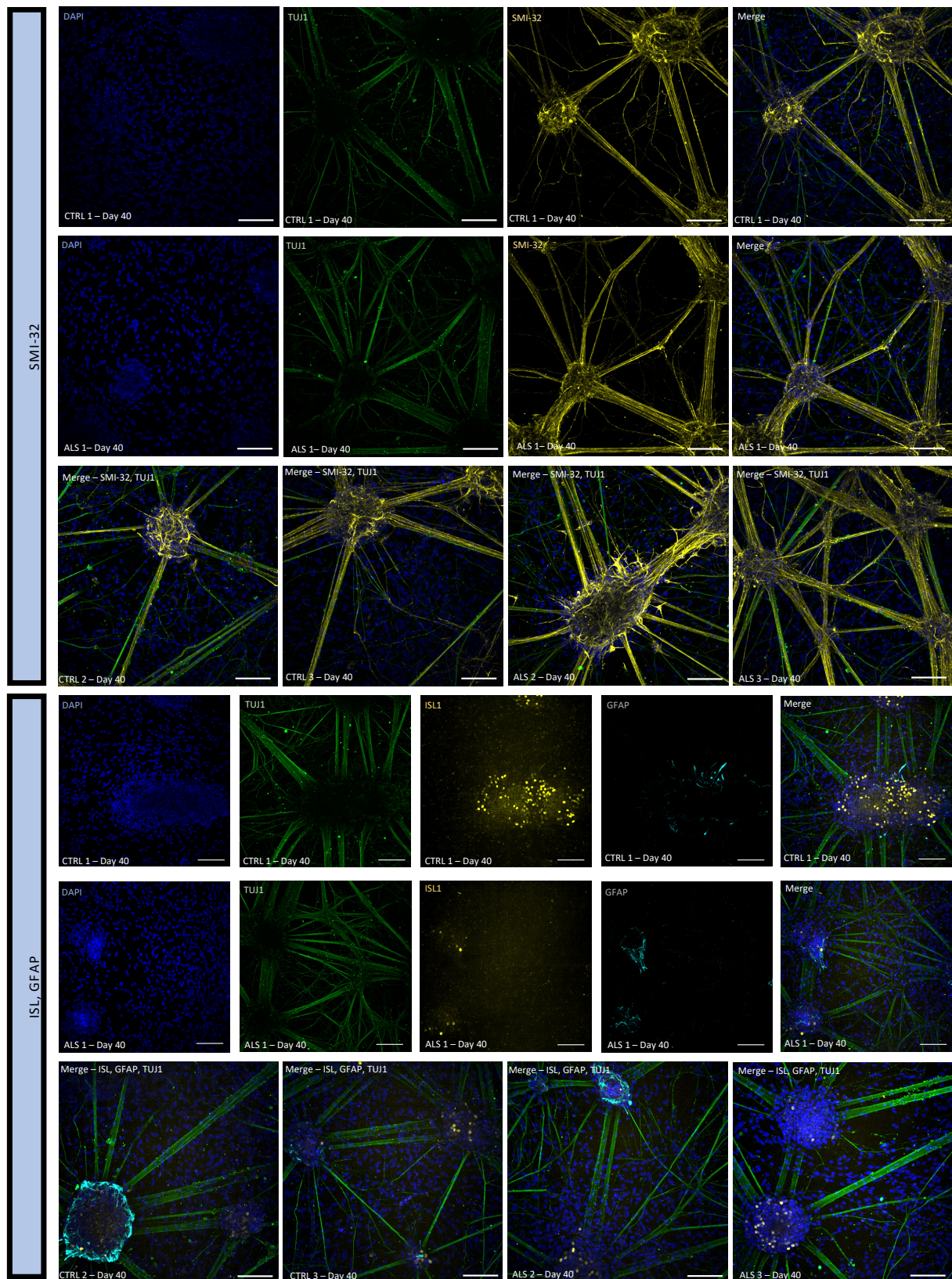


Figure 4.5: Immunocytochemistry at day 40 on iPSC-derived motor neurons

Spinning disk confocal images of iPSC-derived motor neurons immunostained for SMI-32 and TUJ1 in the top panel. Individual channels are shown for CTRL 1 and ALS 1, only the final colour merge is shown for the remaining lines. Bottom panel shows spinning disk confocal images of iPSC-derived motor neurons immunostained for ISL1, GFAP and TUJ1. CTRL 1 and ALS 1 are again displayed with each channel separate, remaining lines are shown as the merge of these channels.

Scale bars = 100 μm

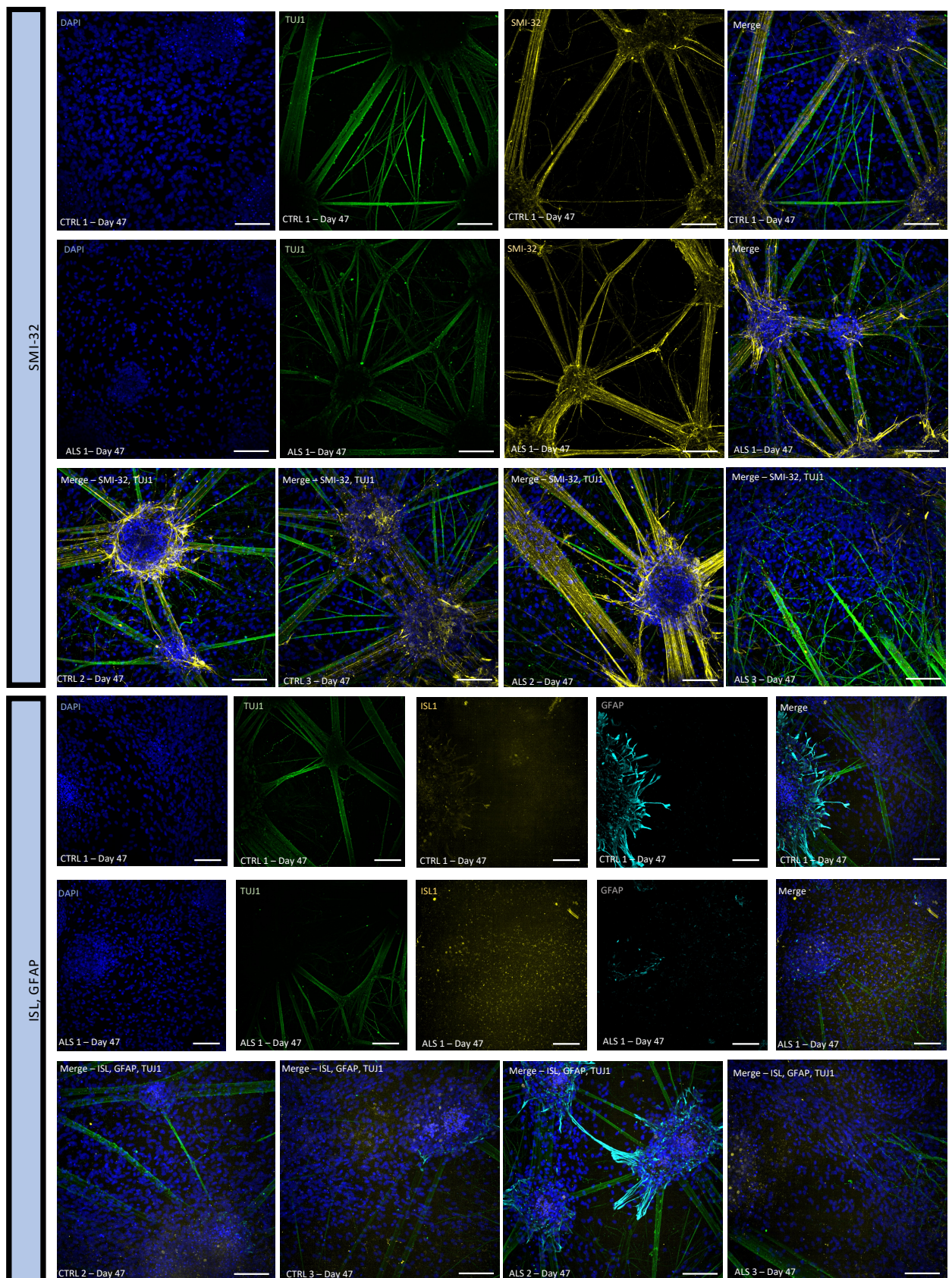


Figure 4.6: Immunocytochemistry at day 47 on iPSC-derived motor neurons

Spinning disk confocal images of iPSC-derived motor neurons immunostained for SMI-32 and TUJ1 in the top panel. Individual channels are shown for CTRL 1 and ALS 1, only the final colour merge is shown for the remaining lines. Bottom panel shows spinning disk confocal images of iPSC derived motor neurons immunostained for ISL1, GFAP and TUJ1. CTRL 1 and ALS 1 are again displayed with each channel separate, remaining lines shown as the merge of these channels. Scale bars =100 μm

4.4 Motor Neuron Marker mRNA Expression

In addition to protein expression, the mRNA expression was analysed at day 0, 6, 12, 18, 40 and 47 throughout the differentiation to investigate whether maturation markers were differentially expressed in *C9ORF72* expansion carriers during differentiation.

HB9, *ChAT*, *ISL* and *SMI-32* were all assessed at all 6 time points using the dCT method of analysis. The Livak method was not used for this analysis to compare time points against iPSC levels, as it was important to keep the variation for this time point to assess whether there were any initial differences in expression.

Pooling data from the three patient samples and three control sample results for each time point showed no difference between patient and control samples for any of the motor neuron markers analysed (*HB9*, *ChAT*, *ISL* and *SMI-32*) (Figure 4.7 A-D, Two-way RM ANOVA). However, as anticipated from the maturation study the expression of all four markers was significantly affected by time in culture (Two-way RM ANOVA, *ISL1*: Time $p = <0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$, *HB9*: Time $p = <0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$, *ChAT*: Time $p = <0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$, *SMI-32*: Time $p = <0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$, with Sidak's multiple comparison Table 4.1, Figure 4.7A-D). As seen in the maturation study for CTRL 1, this study also shows a significant increase in *HB9* and *ISL1* expression until day 18 and after day 18 a significant decrease is then observed (Figure 4.7A-B, Table 4.1). This pattern of increased then reduced expression is observed for both genotypes (expanded vs non-expanded iPSC-derived motor neuron lines) and is expected considering the transient expression in vivo of *HB9* and *ISL1*. *ChAT* also shows the same pattern of expression with a significant

increase in expression for both genotypes until day 18 followed by a significant decrease after day 18 (Figure 4.7C, Table 4.1). Reasons for this decrease were considered in the discussion section of the maturation Chapter 3.

SMI-32 showed a different expression pattern in line with that observed for CTRL 2 in the maturation chapter. As seen in (Figure 4.7D, Table 4.1), *SMI-32* expression increases significantly over time, peaking by day 40. This is consistent with increased MN maturation over this period.

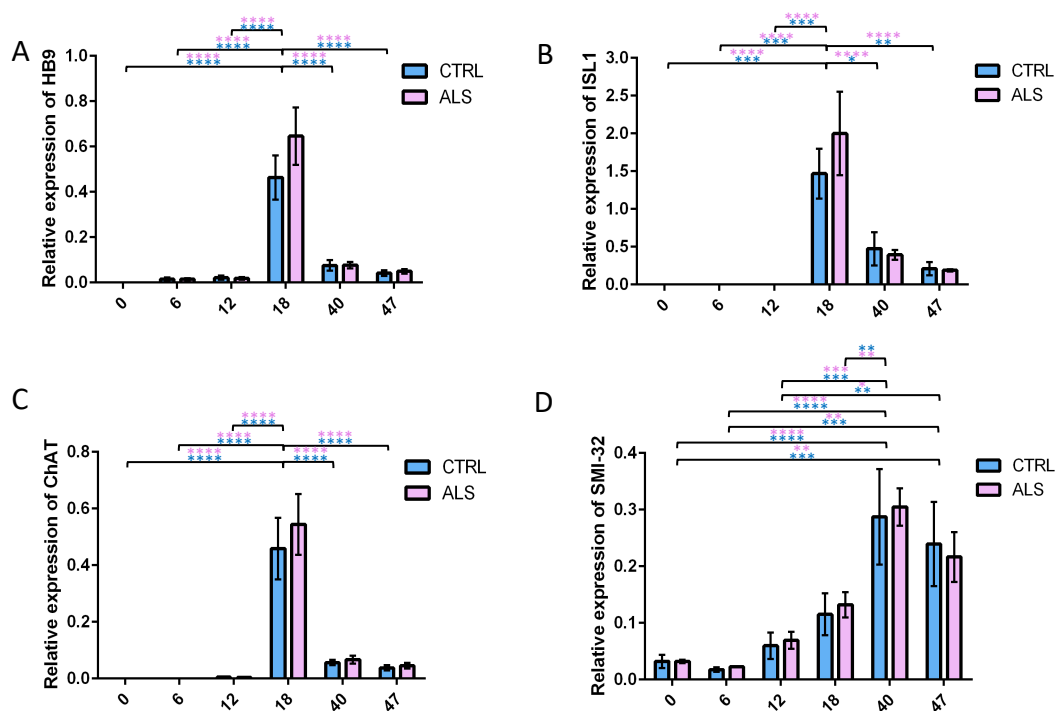


Figure 4.7: RT-qPCR of motor neurons marker expression throughout Du differentiation

SYBR green reagent was used to run RT-qPCRS for HB9, ISL1, ChAT and SMI-32. A-D) Pooled data of three patient and three control lines using the dCT method of analysis. N = 3 (each patient/ control differentiated twice) (Two-way RM ANOVA with Sidaks posthoc analysis)

<i>ISL1</i>	Time	CTRL (mean $\pm$ SEM) N=3 CTRL lines	CTRL <i>p</i> -value	ALS (mean $\pm$ SEM) N=3 ALS lines	ALS <i>p</i> -value
	0 vs 18	0.0006 $\pm$ 0.0002 vs 1.4679 $\pm$ 0.3314	0.0003	0.001 $\pm$ 0.0002 vs 1.998 $\pm$ 0.5533	<0.0001
	6 vs 18	0.000025 $\pm$ 0.000007 vs 1.4679 $\pm$ 0.3314	0.0003	0.00002 $\pm$ 0.0000018 vs 1.998 $\pm$ 0.5533	<0.0001
	12 vs 18	0.0010 $\pm$ 0.0006 vs 1.4679 $\pm$ 0.3314	0.0003	0.001 $\pm$ 0.0002 vs 1.998 $\pm$ 0.5533	<0.0001
	18 vs 40	1.4679 $\pm$ 0.3314 vs 0.4732 $\pm$ 0.2208	0.0190	1.998 $\pm$ 0.5533 vs 0.394 $\pm$ 0.0654	<0.0001
	18 vs 47	1.4679 $\pm$ 0.3314 vs 0.2092 $\pm$ 0.0861	0.0019	1.998 $\pm$ 0.5533 vs 0.188 $\pm$ 0.0147	<0.0001
<i>HB9</i>	0 vs 18	0.0002 $\pm$ 0.00003 vs 0.4635 $\pm$ 0.09686	<0.0001	0.0002 $\pm$ 0.00004 vs 0.6465 $\pm$ 0.12658	<0.0001
	6 vs 18	0.0145 $\pm$ 0.007 0.4635 $\pm$ 0.09686	<0.0001	0.0142 $\pm$ 0.00541 vs 0.6465 $\pm$ 0.12658	<0.0001
	12 vs 18	0.0213 $\pm$ 0.00935 vs 0.4635 $\pm$ 0.09686	<0.0001	0.0186 $\pm$ 0.00632 vs 0.6465 $\pm$ 0.12658	<0.0001
	18 vs 40	0.4635 $\pm$ 0.09686 vs 0.0756 $\pm$ 0.02299	<0.0001	0.6465 $\pm$ 0.12658 vs 0.0773 $\pm$ 0.01419	<0.0001
	18 vs 47	0.4635 $\pm$ 0.09686 vs 0.0413 $\pm$ 0.01206	<0.0001	0.6465 $\pm$ 0.12658 vs 0.0493 $\pm$ 0.01048	<0.0001
<i>ChAT</i>	0 vs 18	0.00001 $\pm$ 0.000004 vs 0.45899 $\pm$ 0.108532	<0.0001	0.00001 $\pm$ 0.000002 vs 0.54439 $\pm$ 0.107100	<0.0001
	6 vs 18	0.00005 $\pm$ 0.000013 vs 0.45899 $\pm$ 0.108532	<0.0001	0.00012 $\pm$ 0.000024 vs 0.54439 $\pm$ 0.107100	<0.0001
	12 vs 18	0.00509 $\pm$ 0.001568 vs 0.45899 $\pm$ 0.108532	<0.0001	0.00419 $\pm$ 0.001539 vs 0.54439 $\pm$ 0.107100	<0.0001
	18 vs 40	0.45899 $\pm$ 0.108532 vs 0.05660 $\pm$ 0.008771	<0.0001	0.54439 $\pm$ 0.107100 vs 0.06699 $\pm$ 0.014211	<0.0001
	18 vs 47	0.45899 $\pm$ 0.108532 vs 0.03781 $\pm$ 0.0089	<0.0001	0.54439 $\pm$ 0.107100 vs 0.04551 $\pm$ 0.009864	<0.0001
<i>SMI-32</i>	0 vs 40	0.032 $\pm$ 0.012 vs 0.287 $\pm$ 0.084	<0.0001	0.032 $\pm$ 0.003 vs 0.305 $\pm$ 0.033	<0.0001
	0 vs 47	0.032 $\pm$ 0.012 vs 0.239 $\pm$ 0.074	0.0007	0.032 $\pm$ 0.003 vs 0.217 $\pm$ 0.044	0.0024
	6 vs 40	0.018 $\pm$ 0.004 vs 0.287 $\pm$ 0.084	<0.0001	0.023 $\pm$ 0.001 vs 0.305 $\pm$ 0.033	<0.0001
	6 vs 47	0.018 $\pm$ 0.004 vs 0.239 $\pm$ 0.074	0.0003	0.023 $\pm$ 0.001 vs 0.217 $\pm$ 0.044	0.0014
	12 vs 40	0.06 $\pm$ 0.023 vs 0.287 $\pm$ 0.084	0.0002	0.069 $\pm$ 0.015 vs 0.305 $\pm$ 0.033	0.0001
	12 vs 47	0.06 $\pm$ 0.023 vs 0.239 $\pm$ 0.074	0.0033	0.069 $\pm$ 0.015 vs 0.217 $\pm$ 0.044	0.0213
	18 vs 40	0.115 $\pm$ 0.037 vs 0.287 $\pm$ 0.084	0.0050	0.132 $\pm$ 0.022 vs 0.305 $\pm$ 0.033	0.0049

Table 4.1: Two- way RM ANOVA posthoc Sidak's multiple comparisons results

4.5 iPSC-derived *C9ORF72* gene expansion Motor

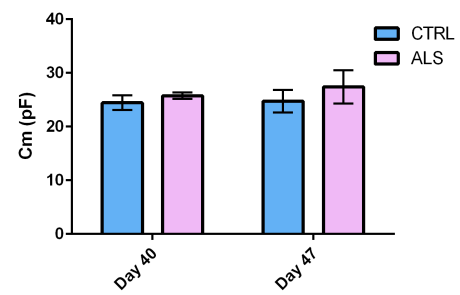
Neurons show Hyperexcitability

Following from the above characterisation of the three patient and control iPSC-derived motor neuron lines, electrophysiological analysis was performed to characterise neuronal excitability and intrinsic electrophysiological properties. For all electrophysiology the three ALS and CTRL lines were independently differentiated twice, with the exception of CTRL 1 in which the second differentiation was excluded from analysis as it contained fewer than 30% MNs. 2-6 cells were 'patched' for each ALS or CTRL line.

At day 40 and 47 there is no significant difference between patient and control lines for membrane capacitance (Two-Way RM ANOVA, pooled data, Figure 4.8A), membrane resistance (Two-Way RM ANOVA, pooled data, Figure 4.8B) or resting membrane potential (Two-Way RM ANOVA, pooled data, Figure 4.8C). Suggesting that *C9ORF72* gene expansion iPSC-derived MNs and non-expanded MNs have comparable sizes, ion channel numbers and resting membrane potential.

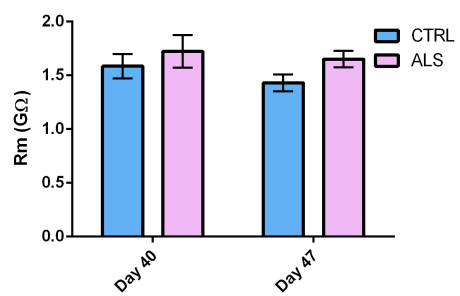
A

Genotype	Cm Day 40		Cm Day 47	
	Mean (pF)	SEM	Mean (pF)	SEM
CTRL 1	22.23	-	28.84	-
CTRL 2	26.92	4.22	22.13	1.43
CTRL 3	24.18	0.47	23.20	1.21
ALS 1	26.91	4.23	33.18	7.06
ALS 2	25.29	2.25	22.73	0.47
ALS 3	25.07	2.17	26.29	2.68



B

Genotype	Rm Day 40		Rm Day 47	
	Mean ($G\Omega$)	SEM	Mean ($G\Omega$)	SEM
CTRL 1	1.80	-	1.31	-
CTRL 2	1.42	0.45	1.58	0.15
CTRL 3	1.53	0.33	1.40	0.43
ALS 1	1.42	0.28	1.50	0.17
ALS 2	1.91	0.37	1.7	0.10
ALS 3	1.83	0.16	1.75	0.15



C

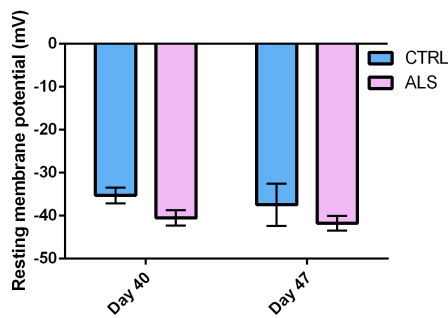


Figure 4.8: Intrinsic properties of motor neurons at day 40 and 47.

Pooled data includes ALS 1, 2 and 3 and CTRL lines 1, 2 and 3, each line differentiated twice (2-6 cells were recorded.) A) Membrane capacitance of neurons separately and pooled. B) Membrane resistance of neurons separately for each line and pooled. C) Resting membrane potential in $I=0$.

4.5.1 Spontaneous action potential firing shows a trend towards hyperexcitability in C9ORF72 gene expansion carriers.

As there is no significant difference between patient and control iPSC-derived motor neuron intrinsic electrophysiological properties, further exploration of neuronal excitability can be completed without any confounding affects due to cell size or initial resting membrane potential between the groups.

Spontaneous action potential firing in all six iPSC-derived motor neuron lines at day 40 and 47 was recorded (Figure 4.9). Each line was independently differentiated twice, as discussed above, with the exception of CTRL 1 in which the second differentiation was excluded from analysis as it contained fewer than 30% motor neurons. Both the percentage of active cells (those firing at least one AP with an amplitude over 40 mV) and the number of APs (per 30 seconds), show a trend towards increased excitability in the patient vs control neurons at day 40 and day 47. However, this trend did not reach statistical significance (Two-way RM ANOVA), which may be due to the low number of cells fitting the acceptable criteria per line per differentiation, as outlined in the methods.

The percentage of active cells for both iPSC-derived MNs harbouring the *C9ORF72* gene expansion and controls significantly increased from day 40 to day 47. Again, consistent with an ongoing maturation over the time course (Two-way RM ANOVA: Time $p=0.0039$, Genotype $p=ns$, Interaction $p=ns$ with Sidak's multiple comparison: CTRL day 40 16.667 ± 16.667 vs CTRL day 47 44.444 ± 12.108 , $p=0.0220$, $N=3$ ALS/, ALS day 40 45.833 ± 10.486 vs ALS day 47 70.833 ± 14.633 , $p=0.0314$, $N=3$ ALS/CTRL lines). Furthermore, significant increase in the number of action potentials fired (per 30 seconds) was

observed from day 40 to 47 for both patient and control groups (Figure 4.9C) implying in increase in maturity for both groups (Two-way ANOVA RM: Time $p=0.0421$, genotype $p=$ ns, interaction $p=$ ns Sidak's multiple comparison test).

Following the observation of a trend towards increased excitability, the number of APs fired per 30 seconds was plotted individually for each patient and control to assess whether the patient and controls were behaving consistently. Figure 4.9D shows a clear variation among the patients' spontaneous action potential firing. ALS 1 and ALS 2 appear to consistently have increased action potential firing compared to controls whereas ALS 3 appears to show a firing rate in line with controls.

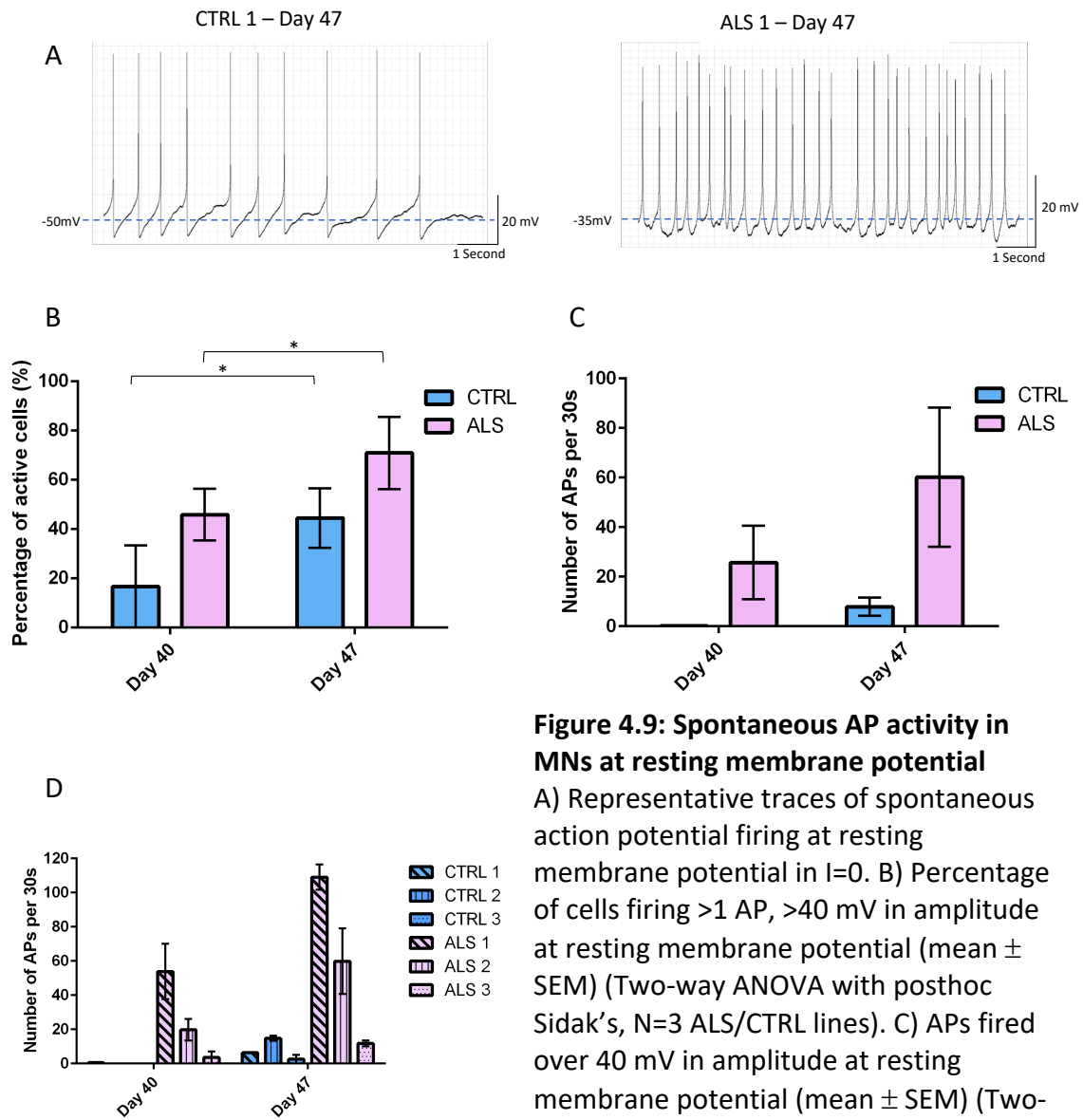


Figure 4.9: Spontaneous AP activity in MNs at resting membrane potential

A) Representative traces of spontaneous action potential firing at resting membrane potential in $I=0$. B) Percentage of cells firing >1 AP, >40 mV in amplitude at resting membrane potential (mean $\pm$ SEM) (Two-way ANOVA with posthoc Sidak's, $N=3$ ALS/CTRL lines). C) APs fired over 40 mV in amplitude at resting membrane potential (mean $\pm$ SEM) (Two-way ANOVA with posthoc Sidak's, ns, $N=3$ ALS/CTRL lines). D) APs fired over 40 mV in amplitude at resting membrane potential for each iPSC-derived motor neuron line (mean $\pm$ SEM) $N=2$ differentiations (except CTRL 1 $N=1$)

4.5.2 Evoked action potential firing hyperexcitability in C9ORF72 expansion carriers

Considering the trend in spontaneous action potential firing, evoked action potential firing was subsequently studied (Figure 4.10A-B). A biased current was applied to manually lower the resting membrane potential of each cell to -70 mV between sweeps, then neurons were injected with current in 5 pA steps to elicit action potentials. As expected for functional neurons, there is a significant correlation in AP firing with current injection at both day 40 and 47 (Two-way ANOVA: Day 40: Current injection $p = <0.0001$, Genotype $p = ns$, Interaction $p = 0.0048$ N=3, Day 47: Current injection $p = <0.0001$, genotype $p = ns$, interaction $p = ns$ N=3 ALS/CTRL lines)

Comparing day 40 and day 47, it is apparent that increased action potential firing can be observed in patients compared to controls at day 40 only (Figure 4.10B-C). Two-way ANOVA, showed no overall difference between patients and controls (likely as low current injection AP firing is very similar between patients and controls), however, Sidak's posthoc analysis at day 40 (Figure 4.10C) showed a significant increase in action potential firing at higher current injection of 30, 35 and 40 mV injection (Two-way ANOVA: Sidak's multiple comparisons CTRL 1.667 ± 0.096 vs ALS 3.333 ± 0.0347 30mV $p = 0.0308$ N=3, CTRL 1.833 ± 0.192 vs ALS 3.583 ± 0.488 35mV $p = 0.0198$ N=3, CTRL 1.556 ± 0.294 vs ALS 3.514 ± 0.725 40mV $p = 0.0063$ N=3 ALS/CTRL lines). Two-way ANOVA as shown above also reported a significant interaction ($p = 0.0048$), again suggesting a difference in how the patients and controls respond to current injection.

Finally, rheobase at both day 40 and day 47 (Figure 4.10C-D) was not significantly different between the patient and control groups (Standard T-test). This indicates that the minimal current required to generate an action potential for both patient and

control iPSC-derived motor neurons is not significantly different. In addition, there is no difference between the rheobase at day 40 and day 47 (Two-way RM ANOVA).

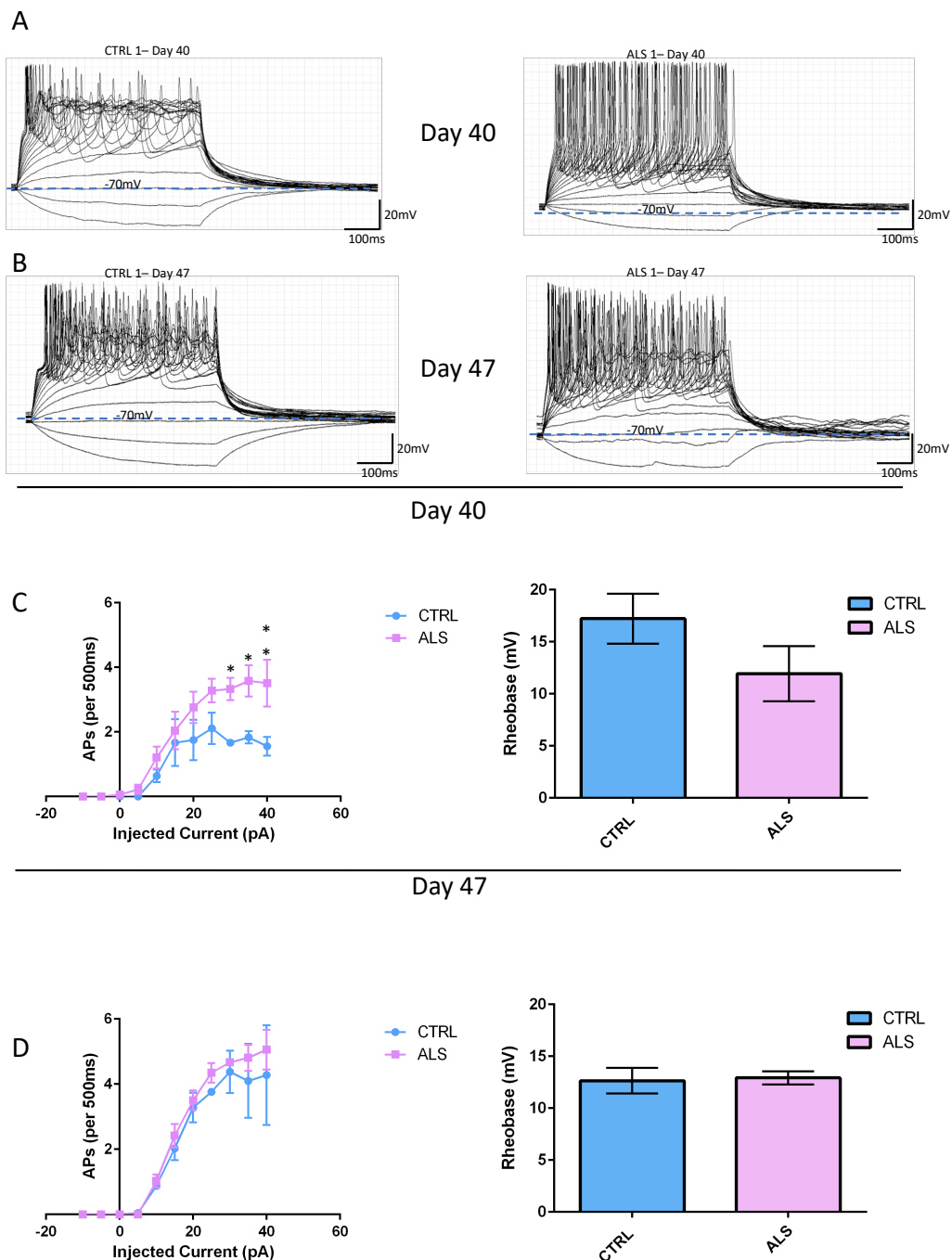


Figure 4.10: Evoked Action Potential Firing at day 40 to day 47.

A)/B Representative traces of evoked action potential firing from -10 mV to 95 mV, with a holding potential of -70 mV (biased current) between sweeps. C) Average action potential firing per 500 ms for each current step from -70 mV (biased current) (mean $\pm$ SEM) N=3 ALS/CTRL lines. Rheobase of action potential firing at day 40, (Mean $\pm$ SEM) N=3 ALS/CTRL lines. D) Average action potential firing per 500 ms for each current step from -70 mV holding (mean $\pm$ SEM) N=3 ALS/CTRL lines. Rheobase of action potential firing at day 40, (Mean $\pm$ SEM) N=3 ALS/CTRL lines.

4.5.3 Sodium and Potassium Channel currents are unaffected by C9ORF72 expansion

Considering the trend in action potential firing toward hyperexcitability, the mechanism behind this was investigated. As explained in the introduction Chapter 3.1.1.2, Na_v channels play a large role in the upstroke of an action potential. While A-type and delayed rectifier K_v are involved in the repolarisation downstroke of the action potential. Therefore, both Na_v and K_v ion currents were analysed at day 40 and 47 to check their current flow. Both channels were measured in the same recording without the use of channel blockers, this may impact the peak Na_v and A-type K_v current but should not affect any differences between patients and controls. Figure 4.11 shows representative Na_v and K_v ion currents and the parameters (orange dashed lines) used to differentiate Na_v , A-type K_v ion currents and delayed rectifier K_v ion currents. It should also be noted that currents referred to as A-type K_v ion currents are a combination of both A-type and delayed rectifier current.

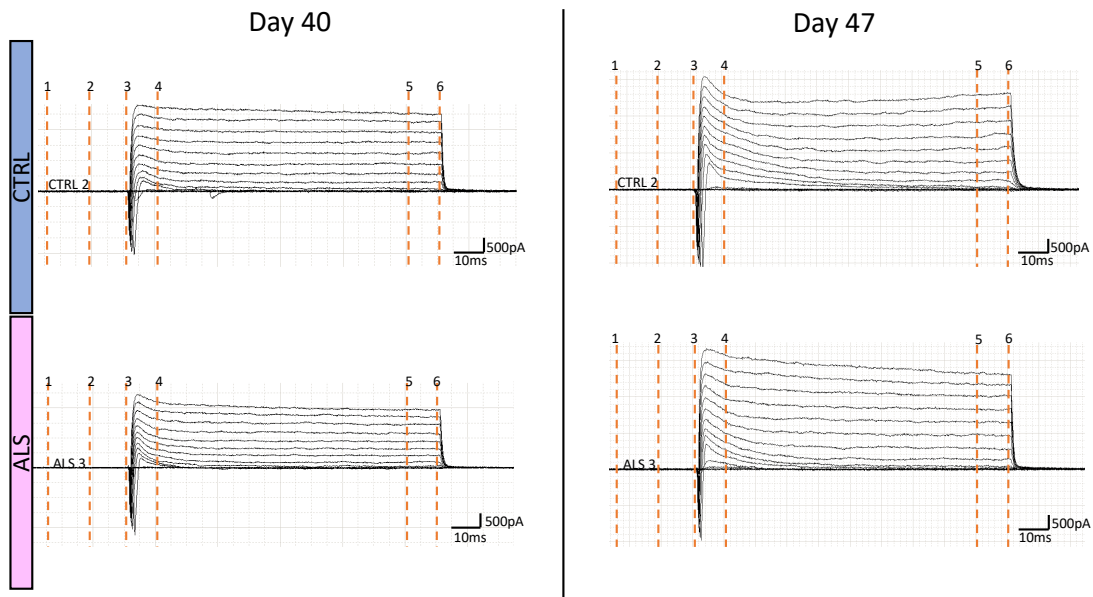


Figure 4.11: Voltage-gated channel current at day 40 and 47.

Representative voltage-gated channel current measured in voltage clamp mode. Neurons were held at -70 mV between sweeps and were stepped from -70 to +70 mV in 10 mV increments. A baseline gating was applied between orange dashed lines 1 and 2. To measure the upward peaks of A-type potassium current at each voltage step and downward sodium current compared to baseline, a 10 ms gating was applied between orange dashed lines 3 and 4. A 10 ms gating was applied to measure the average delayed rectifier potassium current at each voltage step compared to baseline between orange dashed lines 5 and 6.

As expected for functional neurons, a significant correlation in Na_v channel current was observed with changes in voltage at both day 40 and 47 (Figure 4.12A/B) (Two-way RM ANOVA: day 40, Voltage $p = <0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$, $N=3$ ALS/CTRL lines, Day 47, Voltage $p = <0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$ $N=3$ ALS/CTRL lines). At day 40 and 47 there was no significant difference in current at any voltage between iPSC-derived MNs from patients and controls (Figure 4.12A/B) (Two-way RM ANOVA above). Furthermore, no difference was observed in peak Na_v channel current at day 40 and 47 between patients and controls. However, a significant increase in peak current was detected from day 40 to 47 (Figure 4.12C) (Two-way RM ANOVA: Interaction $p = \text{ns}$, Time $p = 0.0145$, genotype $p = \text{ns}$, $N=3$ ALS/CTRL lines with Sidak's multiple comparison ns).

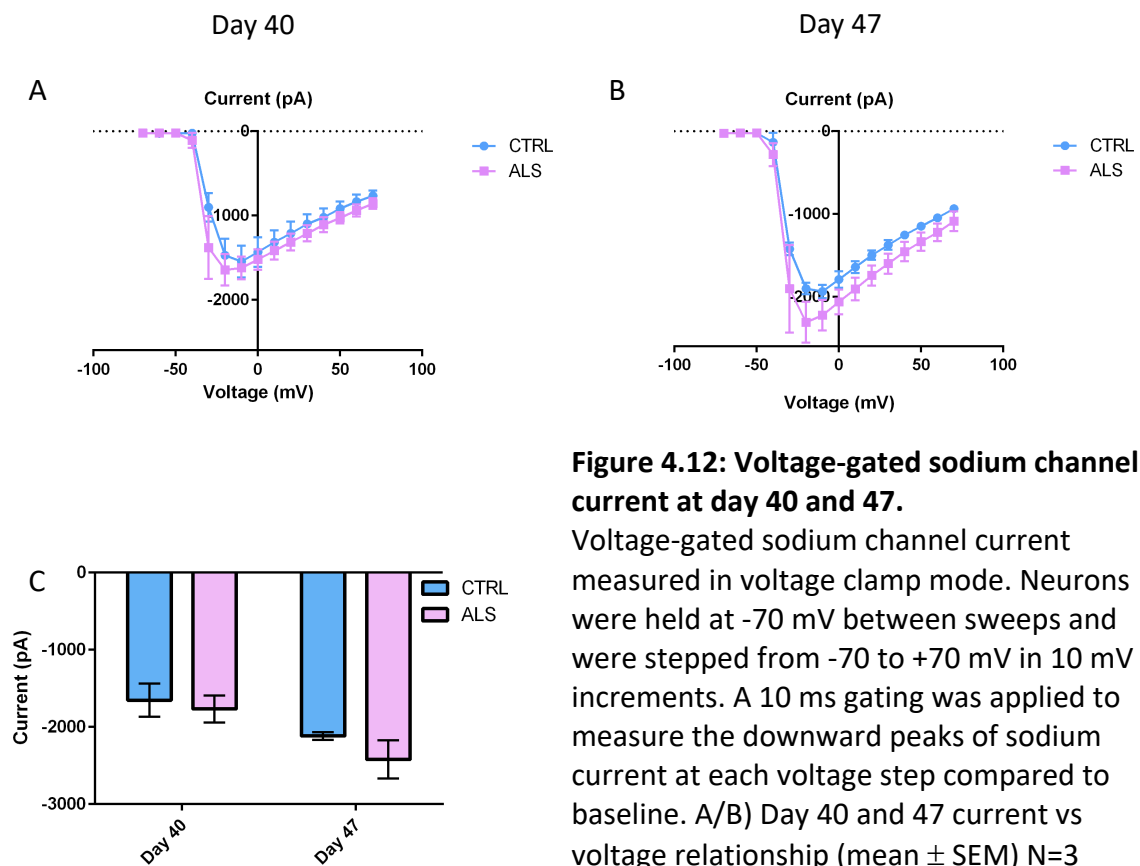


Figure 4.12: Voltage-gated sodium channel current at day 40 and 47.

Voltage-gated sodium channel current measured in voltage clamp mode. Neurons were held at -70 mV between sweeps and were stepped from -70 to +70 mV in 10 mV increments. A 10 ms gating was applied to measure the downward peaks of sodium current at each voltage step compared to baseline. A/B) Day 40 and 47 current vs voltage relationship (mean $\pm$ SEM) N=3 ALS/CTRL lines.

C) Peak voltage-gated sodium channel current at day 40 and day 47 (mean $\pm$ SEM) N=3 ALS/CTRL lines.

K_v current was analysed at day 40 and 47 to establish whether there was dysfunction in the repolarisation of the iPSC-derived motor neurons in patients vs controls. Both delayed rectifier and A-type potassium channels were analysed. The first 10 ms peak in current was measured and is referred to here as A-type potassium channels however, it should be noted that this measurement is a combination of both A-type and delayed rectifier current. Delayed rectifier current in the absence of A-type current was then measured in the last 10 ms of the trace (Figure 4.11).

As expected for functional potassium channels, there is a significant correlation in current for both A-type potassium channels and delayed rectifier channels with voltage at both day 40 and 47 (Figure 4.13, Two-way RM ANOVA, A-type: Day 40 Voltage $p < 0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$, N=3 ALS/CTRL lines, Day 47 Voltage $p < 0.0001$, Genotype $p = \text{ns}$, Interaction $p = 0.02$, N=3 ALS/CTRL lines Delayed rectifier: Day 40 Voltage $p < 0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$ N=3, Day 47 Voltage $p < 0.0001$, Genotype $p = \text{ns}$, Interaction $p = \text{ns}$, N=3 ALS/CTRL lines).

At day 40 (Figure 4.13A) and 47 (Figure 4.13B) no significant differences were detected between patient and control A-type potassium channel current at any voltage (Two-way RM ANOVA above). Interestingly, there was a significant interaction between the response to voltage and genotype in A-type potassium channels ($p = 0.02$) at day 47. This suggests that the patients and controls respond overall significantly differently to voltage. Reasons for this will be discussed chapter 4.7.

No difference in peak current between patients and controls was detected at either day 40 or 47, however, a significant correlation in peak current was detected over time (Figure 4.13C, Two-way RM ANOVA: Interaction $p = \text{ns}$, Time $p = 0.0282$, Genotype $p = \text{ns}$,

N=3 ALS/CTRL lines, (posthoc analysis=ns)). Finally, there is no significant difference in average delayed rectifier current at day 40 or 47 (Figure 4.13E-F, Two-way RM ANOVA). Overall, these results suggest little involvement of potassium and sodium channels in the hyperexcitability phenotype, however, this will be discussed further chapter 4.7.

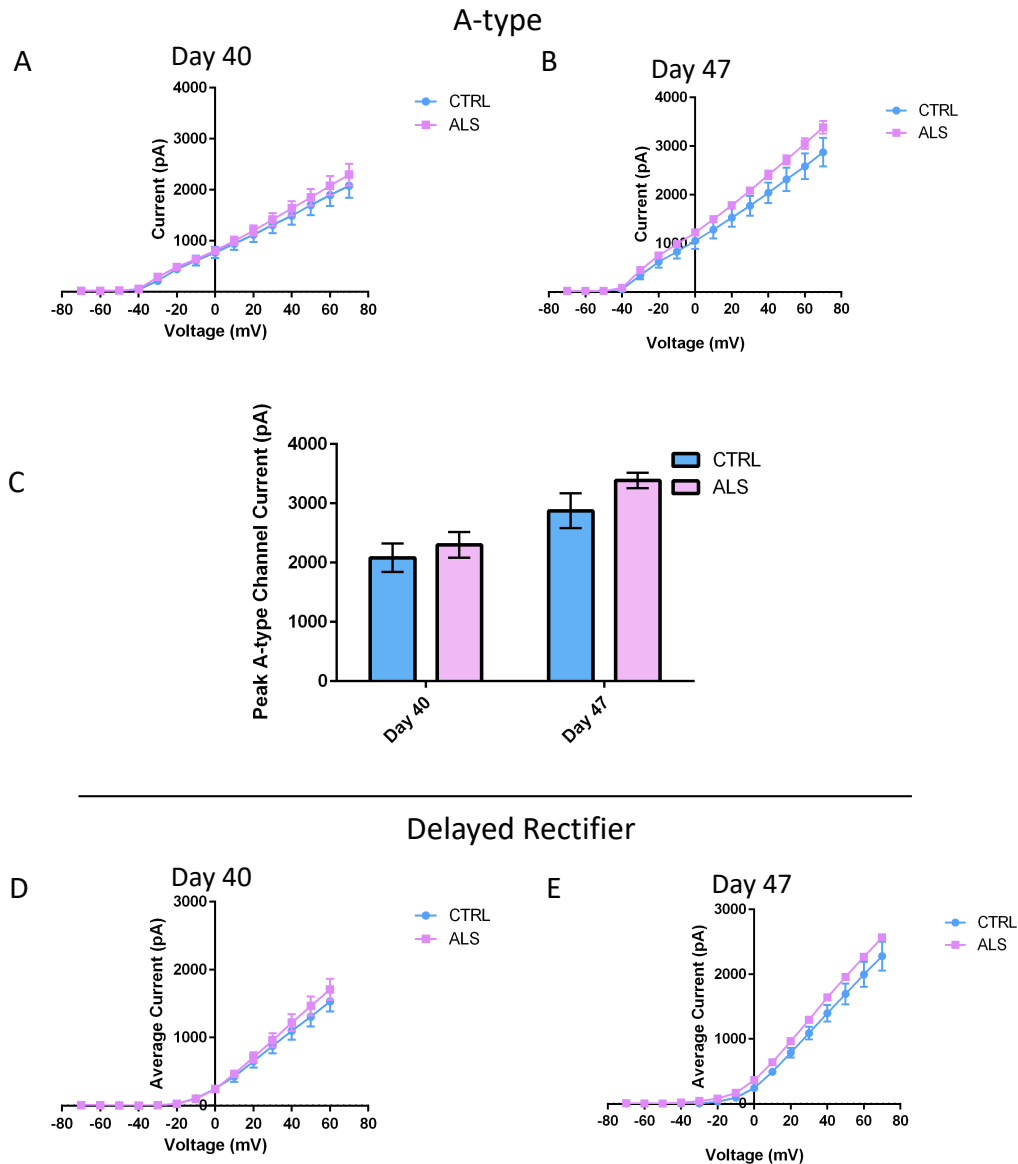


Figure 4.13: Voltage-gated A-type and delayed rectifier Potassium current at day 40 to day 47.

Voltage-gated potassium channel current measured in voltage clamp mode. Neurons were held at -70 mV between sweeps and were stepped from -70 to +70 mV in 10 mV increments. A 10 ms gating was applied to measure the upward peaks of A-type potassium current at each voltage step compared to baseline (it should be noted that this measurement is a combination of both A-type and delayed rectifier current). A 10 ms gating was applied to measure the average delayed rectifier potassium current at each voltage step compared to baseline. A/B) Day 40 and 47 A-type potassium channel current vs voltage relationship (mean $\pm$ SEM) N=3 ALS/CTRL lines. B) Peak voltage-gated potassium channel current at day 40 and 47 (mean $\pm$ SEM) N=3 ALS/CTRL lines. D/E) Day 40 and 47 delayed rectifier current vs voltage relationship (mean $\pm$ SEM) N= 3 ALS/CTRL lines patient/control lines.

4.6 Calcium Dyshomeostasis is shown in *C9ORF72* gene expansion iPSC-derived motor neurons

As discussed in Chapter 1.5, as well as excitotoxicity and excitability, calcium (Ca^{2+}) phenotypes are also observed in *C9ORF72* gene expansion patient iPSC-derived motor neurons.

Fura-2 AM is a dye that binds to free intracellular calcium and acts as a fluorescent indicator. In this chapter it is used to study calcium release from internal stores in response to Ionomycin and Carbonyl cyanide-p trifluoromethoxyphenylhydrazone (FCCP). The Fura-2 AM assay is a ratiometric dye which is excited at both 340 and 380 nm, the ratio of emission from these excitation wavelengths correlates to the presence of internal free calcium. Using a ratiometric dye negates issues with uneven dye loading or cell density per well.

Ionomycin binds to Ca^{2+} in a 1:1 stoichiometry and is commonly used to examine Ca^{2+} dynamics in cells (Chao-min and Theron, 1978). Ionomycin often shows a biphasic response, although this is both cell type and concentration dependent. The biphasic response is due to initial release of Ca^{2+} from intracellular stores followed store operated Ca^{2+} entry (SOCE) (Chao-min and Theron, 1978).

For the calcium assays performed here, the three patient (ALS 1, ALS 2 and ALS 3) and control (CTRL 1, CTRL 2 and CTRL 3) lines were differentiated multiple times. At day 40, ALS 2, ALS 3, CTRL 1 and CTRL 3 were differentiated three independent times and ALS1 and CTRL 2 two independent differentiations. For day 47, ALS 2, ALS 3, CTRL 1, CTRL 2

and CTRL 3 include three independent differentiations and ALS 1 includes 2 independent differentiations. Lines with fewer than 30% MNs were excluded from analysis.

Figure 4.14A shows the ratiometric Fura-2 AM calcium assay with baseline recording for the first 30 seconds prior to ionomycin injection at day 40 and 47. Averaging the first seven time points before ionomycin injection for each of the three patient and control lines showed no difference for day 40 or 47 (Figure 4.14A, Unpaired two-tail T-test: Day 40: CTRL 1.058 ± 0.01427 vs ALS 1.015 ± 0.01338 , $p = \text{ns}$, $N = 3$ ALS/CTRL lines, Day 47: CTRL 0.9858 ± 0.03561 vs ALS 0.9113 ± 0.02378 , $p = \text{ns}$, $N = 3$ ALS/CTRL lines). This indicates no difference in basal internal calcium levels.

After correcting for baseline reading to account for any slight baseline differences, the expected biphasic calcium response was observed at both day 40 and 47 (Figure 4.14B). Interestingly, an interaction effect was observed and posthoc analysis confirmed a significant difference between timepoints 45 seconds to 70 seconds during the peak of calcium release at day 40 between patients and controls (Figure 4.14B) but not Day 47 (Figure 4.14B)(Two-way RM ANOVA: Day 40: interaction $p = <0.0001$, Time $p = <0.0001$, genotype $p = \text{ns}$, $N = 3$ ALS/CTRL lines Day 47: interaction $p = \text{ns}$, Time $p = <0.0001$, genotype $p = \text{ns}$, $N = 3$ ALS/CTRL lines).

Observing the patients and controls individually at day 40 and day 47 (Figure 4.14C) it is clear that at day 40 there is a greater separation between the patients and controls, whereas at day 47 both genotypes show overlapping calcium release profiles.

Analysing specifically the delta of peak release in pooled data of patient vs controls using (Figure 4.14D) two-way RM ANOVA reported a significant decrease in calcium release over time specifically in patient cells as well as a posthoc significant difference between patient and control cells at day 40 (Two-way RM ANOVA: Interaction $p = \text{ns}$,

Time $p=0.0069$, Genotype $p=ns$ Day 40 CTRL 0.7022 ± 0.05304 vs ALS 0.8947 ± 0.02879 ,
 $p=0.0267$, N=3 ALS/CTRL lines, ALS day 40 0.8947 ± 0.02879 vs ALS day 47 0.703 ± 0.052 ,
 $p=0.0141$, N=3 ALS/CTRL lines).

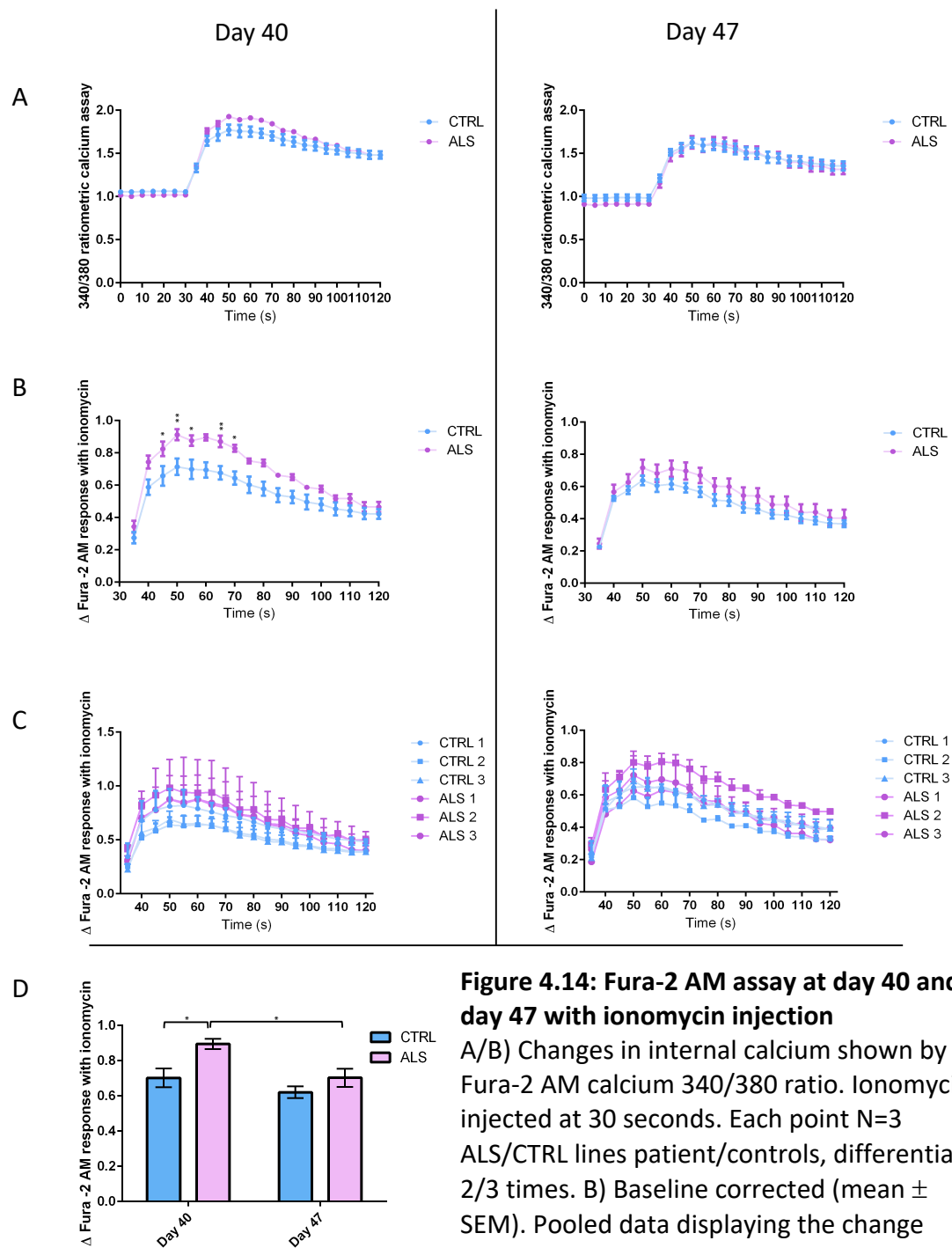


Figure 4.14: Fura-2 AM assay at day 40 and day 47 with ionomycin injection
 A/B) Changes in internal calcium shown by Fura-2 AM calcium 340/380 ratio. Ionomycin injected at 30 seconds. Each point N=3 ALS/CTRL lines patient/controls, differentiated 2/3 times. B) Baseline corrected (mean $\pm$ SEM). Pooled data displaying the change (delta) compared to baseline. C) Patient and control lines baseline corrected for each time point displayed individually N=2/3 differentiations. D) Delta at peak calcium release due to ionomycin N=3 ALS/CTRL lines.

FCCP was used in addition to Fura-2 AM, to explore the calcium release specifically from the mitochondrial stores. Again, averaging the first seven time points (baseline) before FCCP injection for each of the three patient and control lines showed no difference at day 40 or day 47 (Unpaired T-test: Day 40: CTRL 1.057 ± 0.01807 vs ALS 1.012 ± 0.005365 , $p = \text{ns}$, $N = 3$ ALS/CTRL lines, Day 47: CTRL 0.9808 ± 0.02835 vs ALS 0.9265 ± 0.03619 , $p = \text{ns}$, $N = 3$ ALS/CTRL lines). This again indicates no difference in basal internal calcium levels.

After correcting for baseline readings to account for any differences in baseline (Figure 4.15B), a significant difference over time is observed at day 40 and 47, as well a significant interaction at day 47. The significant interaction suggests there is a difference in genotype response to FCCP over time (Two-way RM ANOVA: Day 40: interaction $p = \text{ns}$, Time $p = <0.0001$, genotype $p = \text{ns}$, $N = 3$ ALS/CTRL lines Day 47: interaction $p = 0.0072$, Time $p = <0.0001$, genotype $p = \text{ns}$, $N = 3$ ALS/CTRL lines). In agreement with the pooled data, no clear differences are observed when patients and controls are analysed individually at day 40 and day 47 (Figure 4.15C). Furthermore, no difference is observed in the delta from baseline between patients and controls or overtime at day 40 or day 47 (Figure 4.15D, Two-way RM ANOVA).

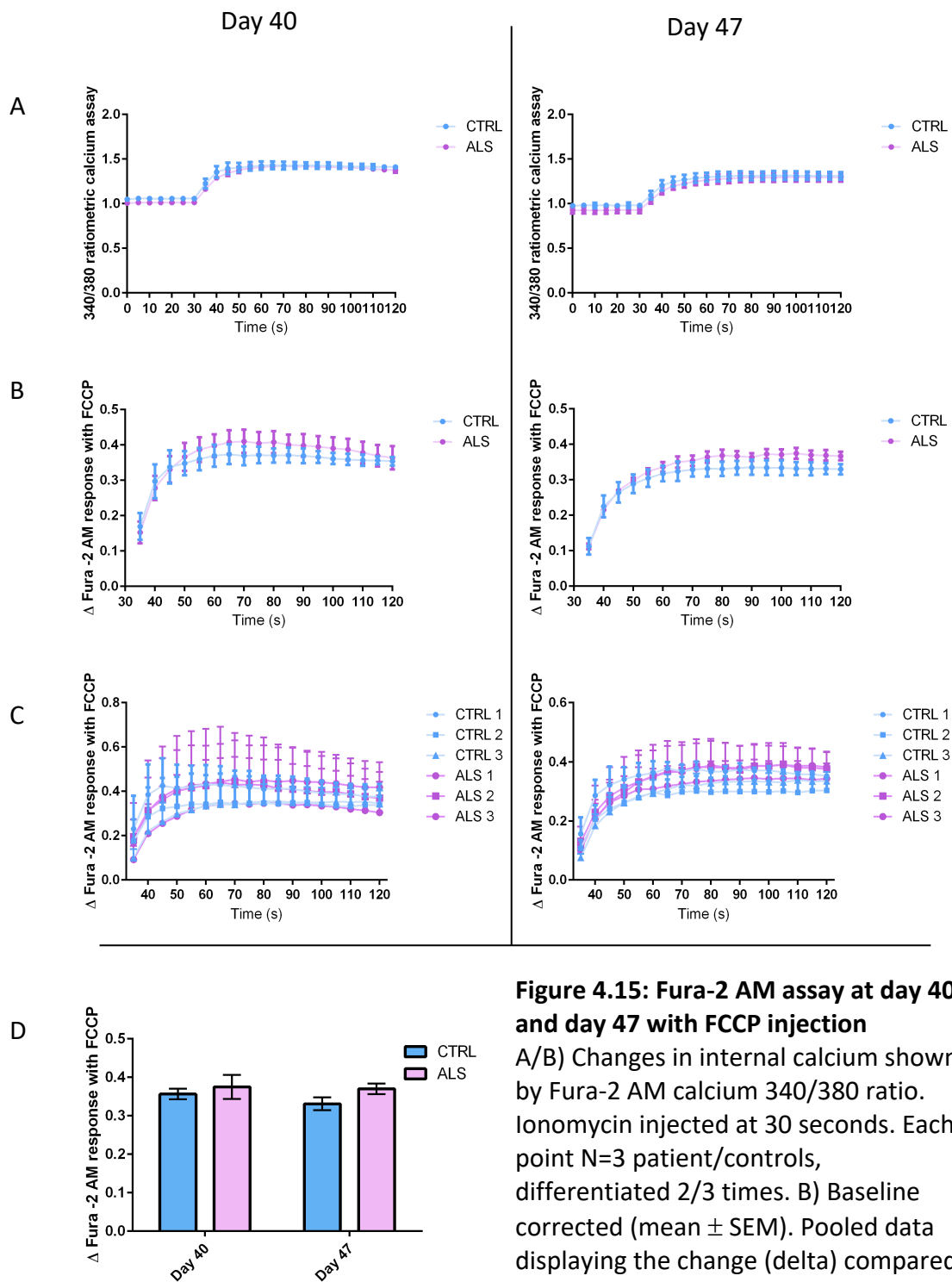


Figure 4.15: Fura-2 AM assay at day 40 and day 47 with FCCP injection

A/B) Changes in internal calcium shown by Fura-2 AM calcium 340/380 ratio. Ionomycin injected at 30 seconds. Each point N=3 patient/controls, differentiated 2/3 times. B) Baseline corrected (mean $\pm$ SEM). Pooled data displaying the change (delta) compared to baseline. C) Patient and control lines baseline corrected for each time point displayed individually N=2/3 differentiations. D) Delta at peak calcium release due to ionomycin N=3 ALS/CTRL lines.

4.7 Discussion

The overall aim of this chapter was to characterise three patient and control iPSC-derived motor neuron lines and analyse their excitability and calcium phenotypes using whole cell patch clamp electrophysiology and the ratiometric Fura-2 AM assay.

The lines were characterised using a combination of repeat primed PCR, motor neuron markers for maturity and basic electrophysiological properties.

As expected, repeat primed PCR indicated all three patients harboured the *C9ORF72* gene expansion, while all three controls lacked the expansion. However, the length of the expansion cannot be accurately determined from the repeat primed PCR electropherogram. Southern blot would be required to establish this, which was tested but unfortunately not successful. However, for future studies it may be of interest to optimise the southern blot protocol, as it is currently still debated whether repeat length affects disease progression and phenotypes observed (reviewed: Van Mossevelde *et al.*, 2017). This study reported differences between patient lines in motor neuron marker expression and excitability (as discussed below), it would be interesting to analyse whether these are correlated to repeat length.

Characterisation of the neurons using flow cytometry, indicated at day 40 and 47, patient and control lines produced over an 80% neuronal population with no significant difference between the groups (again as discussed chapter 3 it should be noted larger cells >40 μm are filtered and gated out of the population of interest). Over 50% of both the patient and control neuronal populations was positive for SMI-32 at day 40 and 47. Patients showed a significant increase in the percentage of positive SMI-32/TUJ1 positive

cells at day 40 compared to controls which was not present at day 47. Interestingly, from day 40 to 47 the percentage of control SMI-32 cells remained consistent however, this percentage seemed to decrease in patient cells. This requires further investigation using cell death assays such as the ToxiLight adenylate kinase (Lonza: LT17-217) assay but it suggests declining health in the motor neuron population of *C9ORF72* carriers.

Flow cytometry profiling, was validated with ICC which confirmed that over 50% of the neuronal population - TUJ1 positive cells – expressed the mature motor neuron marker SMI-32. Although not quantified, ICC did show both ISL1 and GFAP were present at both day 40 and day 47. The presence of ISL1 confirms in combination with SMI-32 a motor neuron population is present whereas the presence of GFAP indicates the presence of astrocytes. It would be of interest to quantify these using flow cytometry to gain a more accurate characterisation of the culture. A recently published study highlights the importance of culture characterisation in regards to phenotypes observed (Selvaraj *et al.*, 2018). They previously reported hyper- and hypo- excitability phenotypes in their mixed culture, however, changing to a more enriched motor neuron protocol producing less astrocytes, these excitability phenotypes were no longer detected (Devlin *et al.*, 2015; Selvaraj *et al.*, 2018). This is in-line with previous reports of non-cell autonomous disease mechanisms in *SOD1*- ALS (Di Giorgio *et al.*, 2007)

RT-qPCR was used to further validate the presence of motor neurons and confirm that the *C9ORF72* gene expansion did not affect motor neuron marker expression. As expected, following the results from ICC, expression of *HB9*, *ISL1*, *ChAT* and *SMI-32* are enriched in mature neurons day 40 and 47 compared to iPSC levels. In-line with previous reports (Sances *et al.*, 2018), both *ISL1* and *HB9* to show transient expression that

declines during the maturation stage. *SMI-32* showed the same general trend of increase over the time-course and *ChAT* a decrease as observed and discussed in Chapter 3.

Following characterisation of the iPSC-derived motor neurons lines, preliminary functional characterisation was performed - including, resting membrane potential, channel permeability (as measured by membrane resistance) and cell capacitance - to check for intrinsic differences between patients and controls. Overall no differences were observed at day 40 or 47 for cell capacitance, membrane resistance or resting membrane potential suggesting that functional maturation occurs in both genotypes at the same rate. It was important to consider intrinsic electrophysiological properties in light of previous studies looking into the excitability of motor neurons carrying the *C9ORF72* expansion. Sareen *et al.*, 2013 reported hypoexcitability of the motor neurons however, the resting membrane potential between the two groups differed. The patient resting membrane potential was hypopolarized compared to controls during the evoked action potential recordings. This could increase the likelihood of sodium ion channel inactivation and therefore reduce the firing rate in *C9ORF72*-expansion carriers compared to controls. Therefore, for this study where spontaneous action potentials from resting membrane potential were analysed it was important to rule out any difference in resting membrane potential.

Overall there was no significant difference in spontaneous action potential firing or percentage of active cells at day 40 or 47 between patients and controls. Interestingly, there was a trend towards increased firing and increased percentage of active cells in the patient lines. However, higher cell numbers and validation in other patient lines would be required to confirm a true difference in motor neuron activity. Additionally, both the number of action potentials and the percentage of active cells for both patients and

controls increased from day 40 to 47. This could be due to ongoing maturation during the time-course analysed and/or to increased network interconnectivity. However, neuronal maturation is typically associated with increasing hyperpolarisation of the resting membrane potential and this was not seen in the cultures.

Interestingly, evoked action potentials at day 40 and day 47 show no significant difference in rheobase between patients and controls or over time. This implies the same level of current is required to evoke an action potential over 40 mV in amplitude. However, there was a significant increase in action potentials fired at day 40 in patients compared to controls at higher current injection, as well as a significant interaction between current injection and genotype. This suggests that with increasing stimulation patient cells fire disproportionately to controls. This was not observed at day 47.

As discussed in the introduction to this chapter voltage gated sodium and potassium channels play a large role in the correct functioning of action potentials. As a trend towards hyperexcitability was observed for both spontaneous and intrinsic action potential firing, it was of interest to look further at sodium and potassium channels.

Voltage gated sodium channels showed no difference between patient and control recordings at day 40 and day 47 and no differences were observed in potassium current levels between patient and control samples. However, an interaction between genotype and voltage at day 47 for A-type potassium channels was observed suggesting that the patients and controls respond overall differently to changes in voltage.

Overall, these results suggest that voltage gated sodium channels show little involvement in the hyperexcitability phenotypes seen here whereas A-type potassium channels may play a role at day 47 when intrinsic hyperexcitability is no longer observed. Further investigations are required to elucidate the mechanisms behind the increased

excitability seen here. It would be interesting to further investigate the hypothesis of excitotoxicity as a cause for the increase in patient spontaneous action potential firing. Several studies have shown an increase in the calcium permeable GluR1 glutamate receptor in *C9ORF72* gene expansion iPSC-derived motor neurons resulting in glutamate induced increased action potential firing (Selvaraj *et al.*, 2018; Shi *et al.*, 2018). Although it should be considered that other mechanisms could cause/contribute this increase in activity such as decreased inhibitory signalling from surrounding cells such as interneurons or altered glial function as glia are important for maintaining neurotransmitter concentrations (Harvey and MacIntosh, 1940).

The increase in intrinsic evoked action potential firing at day 40 in patients could be caused by an alternate mechanism such as an increase in voltage gated calcium channel current or as observed in mutant SOD1 mice could be due to glial non-cell autonomous mechanisms of unknown toxic factor release (Nagai *et al.*, 2007; Fritz *et al.*, 2013; Chang and Martin, 2016).

Considering the strong links between calcium and action potential firing (Harvey and MacIntosh, 1940), calcium dysfunction was also investigated. Calcium studies using ionomycin showed an increase in total calcium released from patient internal stores compared to controls at day 40 but not seen at day 47. At day 47 although not overall significant between patient and control cells, there was a significant interaction between Fura2-AM response and genotype in response to FCCP which causes calcium release from the mitochondria.

These data suggest at day 40 the mitochondria do not show internal calcium accumulation, rather the calcium is accumulated in an alternate internal store such as the ER. Whereas by day 47 there is a significant difference in mitochondrial release in

patients compared to controls. This data supports previous studies which hypothesise that motor neuron death is due to a toxic shift of calcium from the ER to the mitochondria (Berridge, 2002; Grosskreutz *et al.*, 2007; Grosskreutz, Van Den Bosch and Keller, 2010). Lack of calcium in the ER then leads to accumulated mis-folded proteins. Calcium is usually shuttled from the ER to mitochondria in response synaptic excitatory input, therefore changes in excitability of the neurons and calcium buffering are highly associated (Grosskreutz, Van Den Bosch and Keller, 2010). Therefore, it would again be of interest to measure cell death at day 47 as overloaded mitochondria typically induces mitochondrial swelling, leading to rupture and release of apoptotic factors (Cai, Yang and Jones, 1998; Brustovetsky *et al.*, 2002)

In summary, this study shows significantly increased evoked action potential firing at day 40 without sodium or potassium channel phenotypes and a trend towards increased spontaneous action potential firing. It is hypothesised that excitotoxicity with increased calcium permeable AMPA receptors could cause spontaneous action potential firing, with evoked action potential firing caused by an alternate mechanism such as increased voltage gated calcium channel current. Further studies would be of interest to establish whether the mechanisms of intrinsic hyperexcitability and excitotoxicity causing calcium influx through AMPA receptors may converge in signalling pathways leading to increased intracellular calcium as observed at day 40. As discussed above motor neurons are known to have a low calcium buffering capacity and this proposed change in calcium storage could be an indication of a toxic shift of calcium from the ER to the mitochondria leading to an accumulation of misfolded proteins and potential rupture of mitochondria (Cai, Yang and Jones, 1998; Berridge, 2002; Brustovetsky *et al.*, 2002; Lewinski and Keller, 2005; Grosskreutz *et al.*, 2007; Grosskreutz, Van Den Bosch and Keller, 2010).

For future studies it may be of interest to extend the time in culture to assess how the phenotype changes over a longer time-course as the cells continue to mature. Evidence of increasing potassium and sodium channel current over time suggests the neuronal population is still maturing. This study also highlights acute changes that can occur in cultures over a short time frame such as the shifts in hyperexcitability and calcium phenotypes observed.

Chapter 5:

Dual Cre/Lox Optogenetic Systems

are Functional in Mature Motor

Neurons

5.1 Introduction

As discussed in the general introduction there are many advantages to the use of optogenetics in neuroscience. A combined optogenetic tool and reporter gene will allow the control of MNs for electrophysiological studies but also the tracking of MNs throughout differentiation. Here I generated and tested a fused ChR2-mCherry cassette for the purpose of optogenetics and motor neuron tracking.

5.1.1 Channelrhodopsin for optogenetic experiments in iPSC derived motor neurons

5.1.1.1 ChR2 Structure

ChR2 (Figure 5.1) is one of the most widely-used optogenetic tools for use in vivo and in vitro. It has a rapid on-rate and slower moderate closing rate (Nagel *et al.*, 2003; Ishizuka *et al.*, 2006). Channelrhodopsins (ChRs) were discovered in fresh water unicellular green alga *Chlamydomonas reinhardtii* and are involved in flagellar motion (Suzuki *et al.*, 2003; Wong, Abilez and Kuhl, 2012). ChRs are composed of 7 trans-membrane helices which form the ion channel and a very long C-terminal extension of unknown function (Müller *et al.*, 2011)

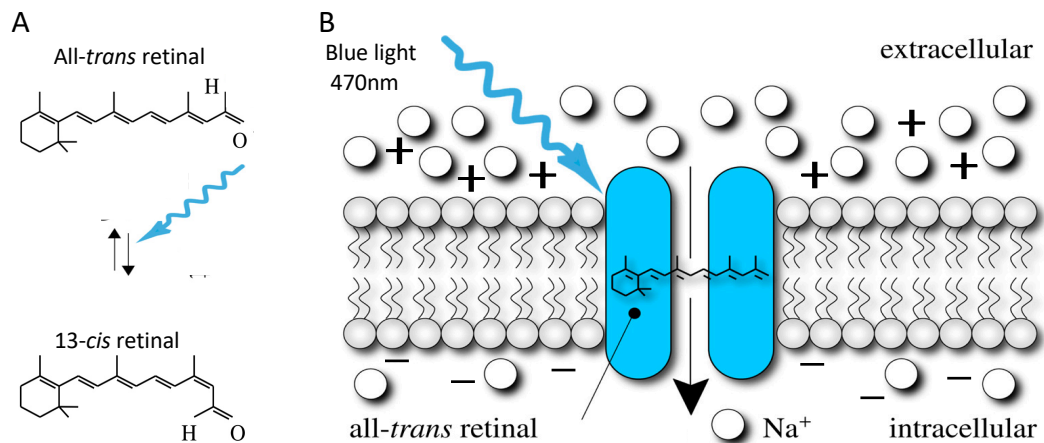


Figure 5.1: Channelrhodopsin opening in response to blue light.

A) Activation of channelrhodopsin causes photoisomerization of all-*trans* retinal into 13-*cis* retinal causing opening of the channel. B) Blue light gated channelrhodopsin is a cation channel positioned in the plasma membrane. Adapted from Wong, Abilez and Kuhl, 2012

Retinal, a derivative of vitamin A, is positioned within the 7 transmembrane helices linked to a lysine residue on the 7th helix. Upon photon absorption retinal isomerizes from all-*trans* to 13-*cis* retinal (Figure 5.1A) causing a conformational change within the 7 transmembrane domains, leading to a side-flip of a helix, opening the ion channel pore to allow the movement of cations (Figure 5.1B). Re-isomerisation of retinal when exposure to photons ceases causes the channel to return to its original conformation closing the pore (Kuhne *et al.*, 2015).

5.1.1.2 ChR2 Iterations

Since the first use of channelrhodopsins, alterations have been applied to the structure to increase its utility in vivo and in vitro systems.

The first improvement was to the size of the channel. As discussed above it was known that the long C-terminal domain did not perform an essential function to the channel, which led to studies truncating the C-terminal end so reducing the protein from 737 amino acids to only 320 amino acids (Nagel *et al.*, 2002; Nagel, Szellas, *et al.*, 2005). An important improvement to ChR2 particularly for iPSC derived motor neuron model systems, was to codon optimize the protein to improve expression in human cells. However, excessive halorhodopsin (an optogenetic chloride ion pump) expression can cause endoplasmic reticulum (ER) blebbing (Gradinaru, Thompson and Deisseroth, 2008; Zhao *et al.*, 2008), therefore many opsins now include C-terminal ER export signals to circumvent this issue. This is commonly the export signal from the vertebrate inward rectifier potassium channel 2.1 (Zhao *et al.*, 2008). Another commonly applied improvement to ChR2 sequences was the H134R substitution which enables increased light sensitivity and slower channel closing, which gives larger photocurrents although less precision (Nagel, Brauner, *et al.*, 2005). Since these first mutations, many further modifications have been created to alter the channel closing properties, ion permeability or wavelength of light it responds to (for review (Lin, 2011)).

Optogenetics, as discussed in the General Introduction 1.6 has opened more options for the study of motor neurons in model systems by allowing researchers to control the activation, inactivation and signalling pathways in these cells in a precise and controlled way.

5.1.2 Motor Neuron Reporter Genes

In addition to optogenetic tools, the need for reporter systems in electrophysiology studies should not be overlooked. The fluorescent identification of motor neurons in a mixed iPSC derived culture is of great advantage to electrophysiology as it allows these cells to be patched directly. Without a reporter system, neurons are patched and then filled with biocytin for post-hoc staining to establish cell type. Post-hoc staining leads to increased experimental duration, not only due to the additional staining required, but also due the extra number of cells needing to be patched as some will be discarded after staining if found to be non-motor neurons.

5.1.2.1 *HB9 Promoter*

As discussed in Chapter 4, HB9 is considered one of the most specific motor neuron transcription factors and its promoter is one of the most widely used and validated for reporter systems (Arber *et al.*, 1999). The optogenetic tool/reporter designed and generated here does not employ the full length *HB9* promoter but rather a truncated mouse 3.6 kb version which allows for easier lentiviral packaging (Figure 5.2) (Peviani *et al.*, 2012). In vivo studies initially demonstrated that a 9 kb fragment, 5' of the mouse *Hb9* gene was enough to drive mouse spinal motor neuron expression (Arber *et al.*, 1999; Wichterle *et al.*, 2002). However, for viral packaging, 9 kb promoters are not optimal due to the packaging size constraints of many viruses. Therefore, several groups

have tried to reduce the size of the promoter region (Nakano *et al.*, 2005; Peviani *et al.*, 2012).

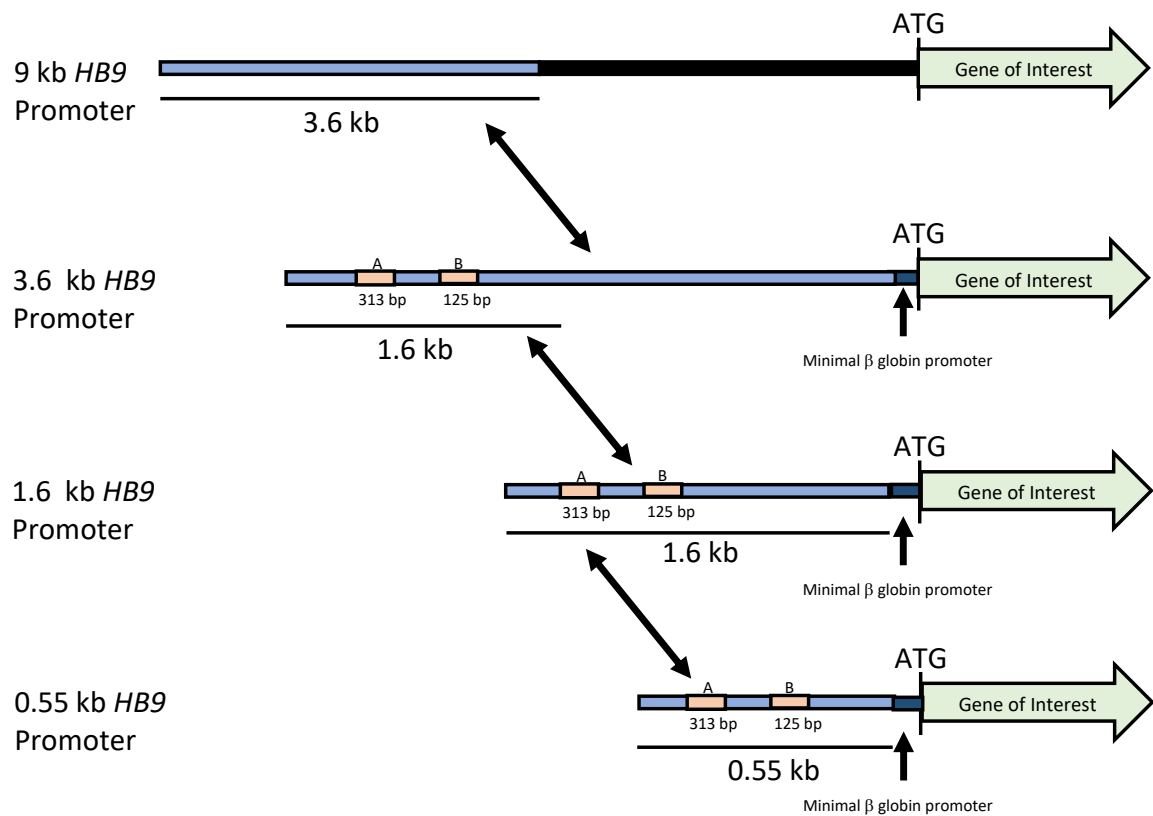


Figure 5.2: HB9 promoter optimisations/truncations

Several adaptations were made to the original mouse *Hb9* promoter, shortening its length from 9 kb to 0.55 kb.

Adapted from (Peviani *et al.*, 2012)

Studies continued to compare human and mouse upstream noncoding sequences of the *Hb9* gene with the aim of finding conserved regions which may function as regulatory regions (Müller, Blader and Strähle, 2002; Nakano *et al.*, 2005). Five regions of homology were observed, two in the 5.4 kb region directly 5' of the *Hb9* gene and three in an adjacent 3.6 kb region. The 3.6 kb fragment as a potential enhancer with a beta globin minimal promoter and the 5.4 kb fragment with no minimal promoter were assessed in mice in vivo. In vivo the 3.6 kb enhancer with beta globin minimal promoter performed

similarly to the 9 kb previously established full promoter (Arber *et al.*, 1999; Nakano *et al.*, 2005). Further sequence homology comparisons between the mouse 3.6 kb region with rat, human and pufferfish identified 313 bp (region A) and 125 bp (region B) regions of homology (Figure 5.2). The 125 bp region is the core enhancer element with additional specificity information provided by the 313 bp region (Nakano *et al.*, 2005). Pevani *et al* reduced the size of the 3.6 kb promoter testing 1.6 kb and 550 bp promoters driving GFP expression in vivo (Figure 5.2). The 1.6 kb promoter showed good MN specificity whereas the shorter 550bp promoter showed unspecific GFP expression in glial cells, indicating the spacer between the 125 bp and 313 bp regions to the minimal beta globin is of importance (Peviani *et al.*, 2012). The mouse 3.6 kb truncated version is used in this human study as it shares high homology (>86%) with the human *HB9* gene promoter and is more widely used and validated than the further truncated 1.6 kb promoters (Nakano *et al.*, 2005).

5.1.2.2 Cre/Lox systems

In addition to the chosen promoter the method used to generate fluorescence in reporter systems, should be considered carefully. Reporter systems can be in direct control of fluorescence i.e. the gene specific promoter directly drives the expression of the fluorescent tag or they can indirectly drive fluorescence. As used in the second generation optogenetic/reporter constructs in this study, the Cre/Lox system or similar Flp/FRT are examples of indirect expression fluorescence regulation.

Cre and Flp both function as DNA recombinases and are part of the λ integrase family of 'site specific recombinases' (Broach, Guarascio and Jayaram, 1982; Abremski and Hoess,

1984). The Cre and Flp specific binding sites have relatively similar structures and both contain two 13 basepair palindromic sequences separated by an 8 basepair core (Hoess, Ziese and Sternberg, 1982). The orientation of these binding sites in relation to each other determines the recombination outcome. Two repeated sites results in excision of the sequence between the sites whereas (Figure 5.3A), two inverted sites will lead to inversion of the intervening sequence (Figure 5.3B) (Branda and Dymecki, 2004)

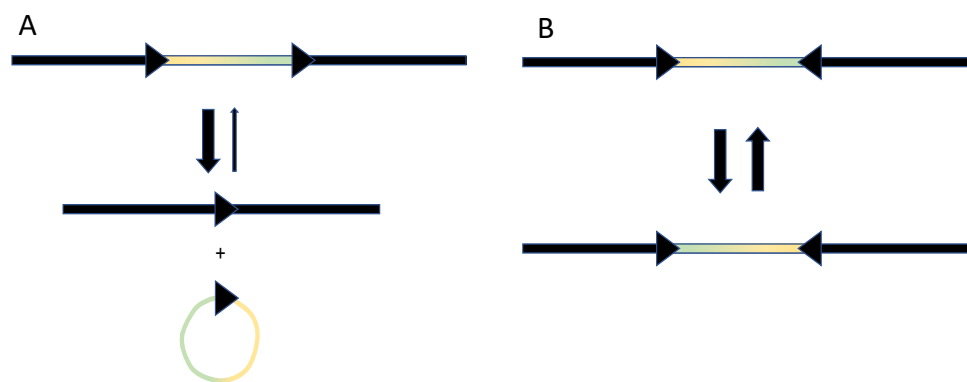


Figure 5.3: Recombination of CRE at lox sites

Lox sites shown as black triangles, direction of triangle depicts direction of lox sequence.

Indirect expression using the Cre/Lox system (or the similar Flp/Frt) is often used when the gene specific promoter is weak. In this instance Cre is driven by a generally weak gene specific promoter as only small concentrations of Cre are required for its action. Cre expression then causes DNA re-arrangement at the Lox sites (often removal of a STOP site) leading to expression of the fluorescent tag from a strong constitutively active promoter.

5.1.3 Gene Delivery

There are several methods to transfer reporters and optogenetic vectors into cells.

Vectors can be transduced virally or transfected. There are several methods of non-viral transfection including the use of calcium phosphate, lipid complexes or protein complexes. Physical transfection can also be used including: electroporation, micro-injections, aerosol or gene guns (Mulherkar, 2001; Sharma *et al.*, 2004; Mali, 2013).

Finally, there are several methods of viral transduction some of which are non-integrating and some integrating. Integrative viruses include: retroviruses, lentiviruses (sub-type of retrovirus) and adeno-associated viruses (AAV) whereas non integrative viruses include: adenoviruses and Sendai virus (Mali, 2013; Park *et al.*, 2016).

5.1.3.1 Lentiviral delivery

Lentiviruses are usually based on human immunodeficiency virus 1 (HIV-1) and have the great advantage, particularly for this project, to infect both dividing and non-dividing cells, allowing flexibility in timing of cell transduction (Bukrinsky *et al.*, 1993). Reviewed extensively by Frankel and Young, 1998 and Ramezani and Hawley, 2002, the molecular biology of HIV-1 comprises the 9.3 kb viral RNA genome with 9 open reading frames (ORFs), three of which encode Gag, Pol and Env polyproteins which are cleaved into further common viral elements. Gag forms the proteins: Matrix (MA), capsid (CA), nucleocapsid (NC), P6, P2 and P1. The Pol polyprotein is cleaved to form a protease (PR), reverse transcriptase (RT) and integrase (IN) which function as replication proteins.

Finally, Env is cleaved forming surface and transmembrane glycoproteins for binding and viral invasion into cells. Four of the remaining six proteins are not essential to viral replication whereas Tat (transactivator of transcription) is required to increase viral mRNA transcription rates and Rev (regulator of expression of virion proteins) controls mRNA splicing by binding to RRE sites (Frankel and Young, 1998; Ramezani and Hawley, 2002).

For research purposes several generations of lentiviral vector systems adapting the polyproteins described above have been developed with continued improvements to the safety of their use and decreasing the risk of replication-competent viruses. Three generations of lentivirus have been developed and commonly used, with a 4th generation also recently described in 2017 (Vink *et al.*, 2017). This study is based on the third-generation system which was first described in 1998 (Dull *et al.*, 1998). The third generation virus systems consist of the main viral plasmid with the gene of interest (GOI) inserted and three 'helper' plasmids based on Gag-Pol, Rev and Env to create viruses which are self-inactivating/replication incompetent (Dull *et al.*, 1998). This generation has all four unessential accessory proteins removed as well as Tat (Kim *et al.*, 1998). Tat function is no longer required as newer third generation systems replace the viral U3 promoter in the 5' long tandem repeats (LTRs) of the viral genome with stronger promoters such as Rous Sarcoma Virus promoter which are still able to produce high viral titers in the range 10^7 /ml (Dull *et al.*, 1998; Kim *et al.*, 1998). Viral titers and lentiviral constructs will be discussed throughout the chapter.

5.1.4 Aims of Chapter

The overall focus of this chapter was to create a dual optogenetic and MN reporter lentiviral construct. For this thesis the generation of the reporter element of the construct is of most benefit to enable identification of live motor neurons in a mixed culture. These labelled MNs can then be 'patched' and excitability measured using the technique of electrophysiology. The optogenetic element of the construct (ChR2 - allowing activation of transduced neurons using blue light) will be beneficial for future studies requiring precise manipulation of activation events or paired neuronal recordings which are beyond the scope of this thesis. Overall this chapter aims:

- To design a dual optogenetic and MN reporter construct for use in iPSC derived MN differentiations. The designed third-generation lentiviral construct should be able to transduce iPSC cells and maintain expression during MN differentiation.
- To apply the dual construct at the iPSC stage and assess its functionality at the mature motor neuron stage of iPSC differentiation. Functionality of the construct can be assessed using ICC to check for colocalization of MN markers (e.g. SMI-32 and ChAT) with the transduced fluorescent MNs to show specific expression of the construct. The optogenetic element (ChR2) can be assessed by flashing blue light on transduced motor neurons and recording using electrophysiology whether a depolarisation response is observed.
- To use the reporter element of the dual construct to enable the live identification of iPSC-derived MNs for patient vs control electrophysiology studies.

5.2 F1 Optogenetic Tools

Two versions of the first generation (F1) optogenetic tools were designed and cloned, one containing the ChR2 optogenetic channel driven by the mouse 3.6Kb *Hb9* promoter (Figure 5.4A) and a dual reporter control construct (Figure 5.4B). The optogenetic construct (Figure 5.4A) labelled pHb9-ChR2mCherry when transduced into cells will give fluorescent mCherry fused ChR2 (ChR2mCherry) when the mouse *Hb9* promoter is active.

The control construct pHb9-mCherry-EF1-GFP contains an mCherry driven by the 3.6 kb *Hb9* promoter and the constitutive EF1 α promoter driving eGFP expression (Figure 5.4B). The pHb9-mCherry-EF1-GFP was designed for validation purposes. All pHb9-mCherry-EF1-GFP transduced cells will express eGFP, while only cells of the Hb9 lineage express mCherry. This enables a transduction percentage to be calculated for the number of viral particles applied per cell (MOI). This transduction efficiency can then be extrapolated to the optogenetic virus which does not have the EF1 GFP feature.

Both pHb9-ChR2mCherry and pHb9-mCherry-EF1-GFP were cloned using Gibson assembly (NEB). The Gibson assembly kit, originally developed by Daniel Gibson et al, at the Venter Institute contains a 5' exonuclease, DNA polymerase and DNA ligase (Gibson *et al.*, 2009; NEB, 2013). The exonuclease removes nucleotides from the 5' ends of fragments creating 3' overhangs. The 3' overhangs act as sticky ends facilitating the binding of fragments with complementary ends. The DNA polymerase fills in gaps in the overhangs which the DNA ligase then seals (Gibson *et al.*, 2009; NEB, 2013). The kit works optimally with fragment's that share an overlap of 15-20 bp.

Briefly, to clone pHb9-ChR2mCherry, the *Hb9* promoter was PCR amplified from the Addgene Hb9-RFP plasmid, then gel purified (Figure 5.4C). The same process of PCR followed by gel purification was completed for the ChR2mCherry fragment from the pLenti-CAMKIIa-hChR2(h134R)-mCherry parent plasmid (Figure 5.4D). The lentiviral backbone pRRL-cmv-eGFP-WPRE was prepared for fragment assembly by restriction digestion using *SpeI* and *Clal* then gel purified (Figure 5.4E). Gibson assembly was then used to join the backbone, Hb9 and ChR2mCherry fragments before transformation into chemically competent *Escherichia coli* (*E. coli*) bacteria (NEB Top 10 bacteria). Bacterial colonies were picked, grown overnight, the DNA extracted by mini prep, then restriction digested to assess whether the plasmid contained the desired fragments (Figure 5.4F). Correct colonies were further sequenced to check for any point mutations. Both pHb9-ChR2mCherry and pHb9-mCherry-EF1-GFP were successfully cloned into lentiviral backbones using the above workflow.

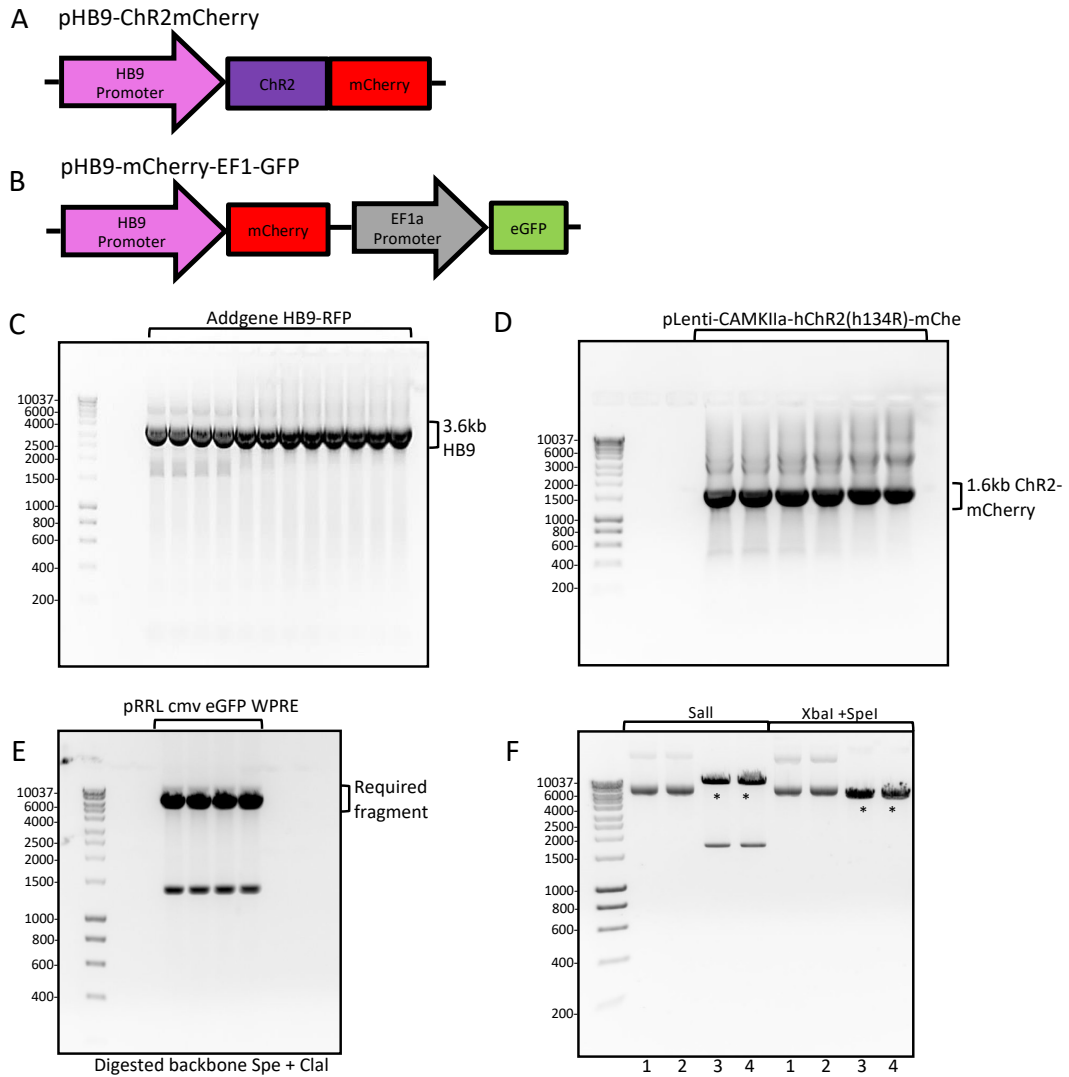


Figure 5.4: Generation of first generation optogenetic tools

A+B) Versions of the first-generation tools created. A) Optogenetic version. B) Control construct to test transduction efficiency and effectiveness of the Hb9 promoter. C) PCR amplify the Hb9 promoter using the ROCHE long expand kit from the Addgene RFP construct (20ng), a gradient from 45-65°C left to right for 6 lanes then repeated. D) PCR amplify ChR2mCherry using the KAPA HiFi kit from pLenti-CAMKIIa-hChR2(h134R)-mCherry (20ng) again using a gradient from 52 to 68 °C left to right. E) Lentiviral backbone pRRL cmv eGFP WPRE (10µg across 4 lanes) was digested and prepared for Gibson assembly to insert the Chr2mCherry and HB9 fragments. F) After Gibson assembly, colonies were picked and mini prepped then digested to check if they contained the desired inserts.* = correct sequence mini preps.

In addition to pHb9-ChR2mCherry and pHb9-mCherry-EF1-GFP, a set of vectors with an 'internal ribosome entry site' (IRES) and puromycin (PURO) resistance were designed and cloned (Figure 5.5A-B). These constructs were created with the purpose of downstream selection, the plasmids could be applied early in the differentiation, then after *HB9* expression, non-*HB9* expressing cells could be killed with Puromycin selection. The IRES directs translation from its site allowing the PURO to be driven by the *Hb9* promoter but not fused to the mCherry (Figure 5.5A-B). These constructs were made as a secondary option as the IRES and PURO add to the size of the plasmid which may hinder the lentiviral packaging (12.6 kb for pHb9-ChR2-mCherry-IRES-PURO and 13.7 kb for pHb9-mCherry-IRES-PURO-EF1-GFP).

Construction was based on the original pHb9-ChR2mCherry and pHb9-mCherry-EF1-GFP ligating IRES and PURO after the mCherry in both (Figure 5.5A-B). IRES + PURO was PCR amplified from the parent plasmid pEF1-GFP-IRES-PURO then gel purified (Figure 5.5C). Before final purification, the IRES + PURO fragment was digested with *SpeI* and *BmtI*, the same enzymes used to digest the parent lentiviral backbone pHb9-ChR2mCherry (Figure 5.5D). Once the IRES-PURO cassette was ligated, the plasmid was transformed into chemically competent *E. coli*. Again, colonies were picked, grown overnight and DNA harvested. Colony DNA was again digested to check the presence of the expected insert and then correct colonies sequenced (Figure 5.5E-F). The same workflow was completed for pHb9-mCherry-IRES-PURO-EF1-GFP.

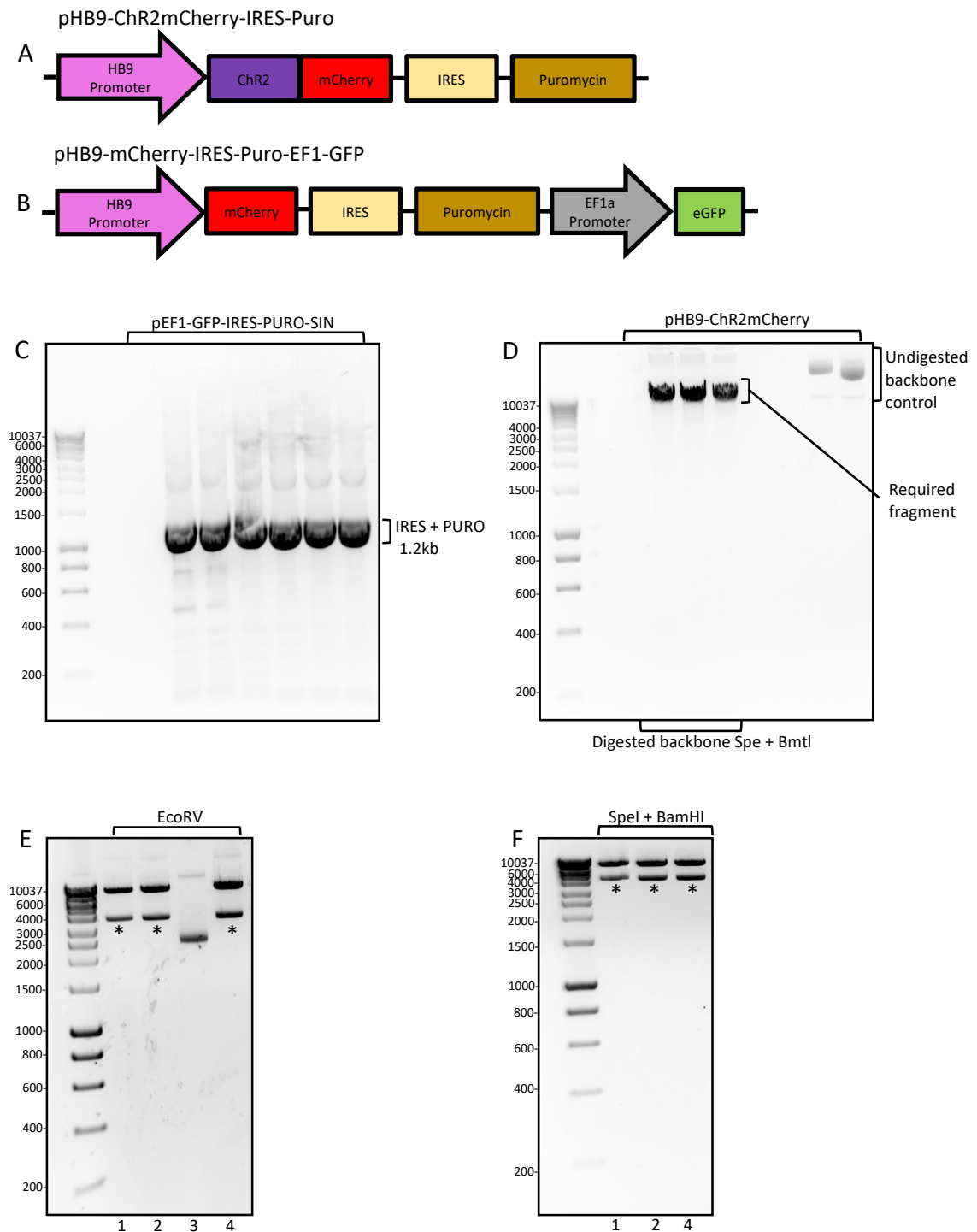


Figure 5.5: Generation of first generation optogenetic tools

A+B) Versions of the first-generation tools created with IRES and PURO included. A) Optogenetic version. B) Control construct to test transduction efficiency and effectiveness of the Hb9 promoter. C) PCR amplify IRES+PURO using KAPA HiFi kit from pEF1-GFP-IRES-PURO-SIN (20ng) using a gradient from 60 to 75 °C left to right. E) Lentiviral backbone pHB9-ChR2mCherry (5µg across 3 Lanes) was digested and prepared for ligation to insert IRES+PURO. F) After ligation, colonies were picked and mini prepped then digested to check if they contained the desired inserts.* = correct sequence mini preps.

5.3 F1 Vector Validation

As reviewed chapter 5.1.3 the mode of plasmid delivery is important and the use of lentiviral transduction is particular of use for this study.

However, before lentiviral packaging the fusion of mCherry to Chr2 was validated in Human embryonic kidney (HEK) 293T cells. Cells were transfected with either the pHb9-ChR2mCherry or pHb9-mCherry-EF1-GFP plasmid using lipofectamine LTX. Transfection with pHb9-ChR2mCherry was used for the fusion validation whereas pHb9-mCherry-EF1-GFP was used as the control to compare normal unfused mCherry molecular weight (MW). Although theoretically HEK cells should not express mCherry or fused Chr2mCherry as their expression should be driven by the *Hb9* promoter, it is widely known that HEK cells do express unexpected genes. mCherry expression was detected for both transfected plasmids (Figure 5.6A-B). To confirm the expression of the Chr2-mCherry fusion, immunoblotting for mCherry was performed on HEK293T lysates. Fused Chr2mCherry is expected to have a higher molecular weight than standard mCherry. Figure 5.6C shows a ~ 29kDa band following transfection with pHb9-mCherry-EF1-GFP corresponding to mCherry and a new ~65 kDa band in pHb9-ChR2mCherry transfected cells that corresponds to fused Chr2-mCherry.

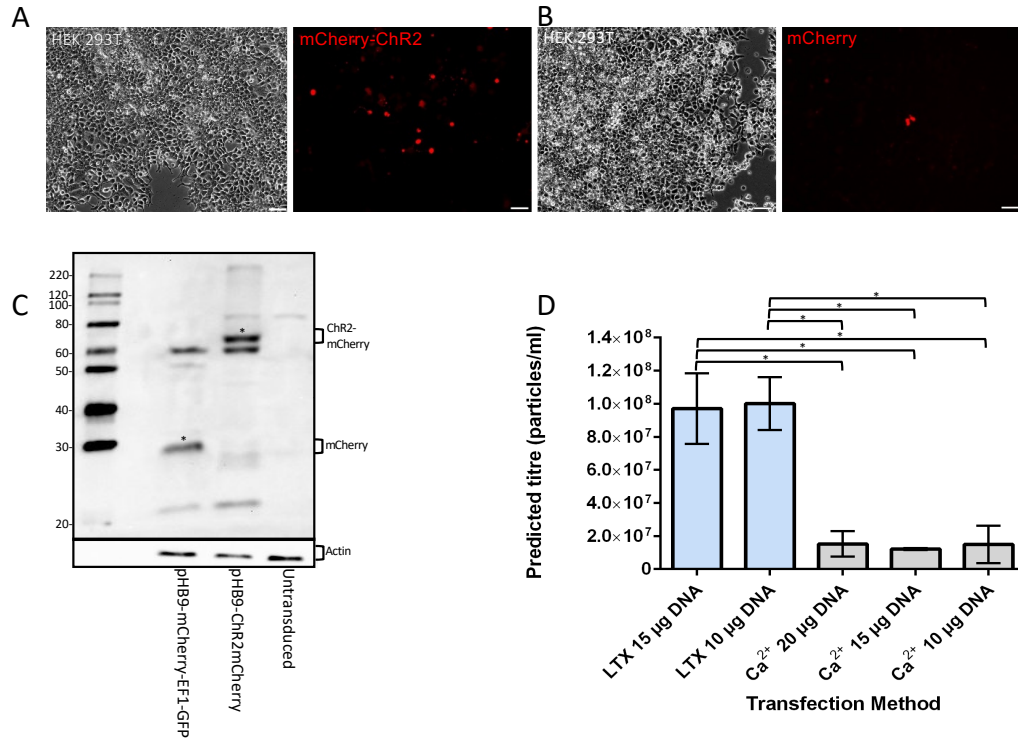


Figure 5.6: Initial test of optogenetic/reporter in HEK 293T cells

A-B) Native fluorescence of mCherry and ChR2-mCherry fusion following transfection of HEK293T cells with (A) pHb9-ChR2mCherry and (B) pHb9-mCherry-EF1-GFP respectively. Scale bars= 70 µm. C) Western blot against mCherry following lentiviral vector transduction to assess ChR2 fusion, lane 1: pHb9-mCherry-EF1-GFP, lane 2: pHb9-ChR2mCherry. Stars indicate mCherry in lane 1 and fused Chr2mCherry in lane 2. D) Optimisation of lentiviral vector packaging using calcium phosphate or lipofectamine LTX transduction and different micrograms of DNA. Mean $\pm$ SEM (n=2), One-Way ANOVA with post-hoc Tukey multiple comparisons, * $p < 0.05$.

5.4 F1-Lentiviral Packaging Optimisation

Once the expression of the fusion protein was confirmed the optogenetic/reporter vectors could be virally packaged for use. The theoretical lentiviral packaging capacity of pro-viral RNA that can be effectively encapsidated is 10 kb. Increased insert length can produce virus in excess of 12 kb but at reduced viral titer (Kumar *et al.*, 2001).

Considering the titer of the control constructs was likely to be lower due to the size of the plasmids e.g. 12.5 kb for pHb9-mCherry-EF1-GFP the packaging protocol was tested and optimised prior to full scale lentiviral packaging. A crucial stage in the 3rd generation lentiviral packaging process is transfection of the chosen plasmid and three packaging plasmids (Rev, Gag-Pol, Env) into the packaging line. HEK293T cells were selected here as the packaging line. HEK293T cells were selected as they contain the SV40 T-antigen. This has been shown to increase packaging yields and no detectable deterioration in viral titer with increased passage, when compared to standard HEK293 (Gama-Norton *et al.*, 2011; Ausubel *et al.*, 2012).

Two transfection methods at varying DNA concentrations were tested in single 15 cm plates (Figure 5.6D). Calcium phosphate with 10 µg, 15 µg and 20 µg of DNA and Lipofectamine LTX with 10 µg and 15 µg of DNA was evaluated (20 µg of DNA was not assessed as the corresponding amount of lipofectamine required for a full-scale lentiviral production would not be cost effective).

Supernatant from transfected plates was used to transduce titrating plates and then the number of GFP positive cells quantified by flow cytometry. This number was then used to estimate the theoretical titer of full-scale packaging.

The full-scale theoretical titer obtained from these tests showed Lipofectamine LTX to be much more effective than calcium phosphate for HB9-mCherry-EF1-GFP packaging.

Theoretical titers with both 10 µg and 15 µg lipofectamine LTX conditions were significantly higher than any of the calcium phosphate conditions (One-way ANOVA: $p=0.009$ with (posthoc) Tukey's multiple comparison (LTX15 $9.7 \times 10^7 \pm 2.134 \times 10^7$ vs $\text{Ca}_3(\text{PO}_4)_2$ 20 $1.528 \times 10^7 \pm 7.7 \times 10^6$, $p=0.0376$, LTX15 $9.7 \times 10^7 \pm 2.134 \times 10^7$ vs $\text{Ca}_3(\text{PO}_4)_2$ 15 $1.2 \times 10^7 \pm 5.5 \times 10^5$, $p=0.0325$, LTX15 $9.7 \times 10^7 \pm 2.134 \times 10^7$ vs $\text{Ca}_3(\text{PO}_4)_2$ 10 $1.494 \times 10^7 \pm 1.122 \times 10^7$, $p=0.0370$, LTX10 $1 \times 10^8 \pm 1.599 \times 10^7$ vs $\text{Ca}_3(\text{PO}_4)_2$ 20 $1.528 \times 10^7 \pm 7.7 \times 10^6$, $p=0.0327$, LTX10 $1 \times 10^8 \pm 1.599 \times 10^7$ vs $\text{Ca}_3(\text{PO}_4)_2$ 15 $1.2 \times 10^7 \pm 5.5 \times 10^5$, $p=0.0283$, LTX10 $1 \times 10^8 \pm 1.599 \times 10^7$ vs $\text{Ca}_3(\text{PO}_4)_2$ 10 $1.494 \times 10^7 \pm 1.122 \times 10^7$, $p=0.0321$, $N=2$) (Figure 5.6D).

Increasing the amount of transfected DNA:lipofectamine did not result in increased titers (LTX10 $1 \times 10^8 \pm 1.599 \times 10^7$ vs LTX15 $9.7 \times 10^7 \pm 2.134 \times 10^7$, $p=\text{ns}$ $N=2$), therefore

Lipofectamine LTX 10 µg was selected for full scale production. Following this trial pHB9-mCherry-EF1-GFP was packaged and achieved a titer of 1.8×10^8 .

5.5 F1 transduction of iPSC derived Motor Neurons

The control construct pHb9-mCherry-EF1-GFP was initially tested in the (Qu *et al.*, 2014) differentiation to assess whether the virus was able to transduce cells and remain active throughout the differentiation protocol. As part of this protocol, cells are dissociated and counted at day 14. This day was chosen for viral transductions as the cell number is required to calculate the number of viral particles required for a given multiplicity of infection (MOI). MOI represents the number of viral particles added per target cell and in this initial study MOIs of 1, 5 and 10 were tested (Figure 5.7). As expected, increasing GFP expression was detected with increased MOI 3 days after transduction with the pHb9-mCherry-EF1-GFP vector. However, there was a marked decrease in GFP expression at day 27, for all MOIs tested (Figure 5.7). Which could be due to silencing of the construct. No Hb9-driven-mCherry expression was detected in any of these conditions.

To confirm the absence of mCherry and diminishing GFP expression was not a differentiation protocol dependent observation, the pHb9-mCherry-EF1-GFP virus as well as a control virus expressing GFP driven by EF1 α (labelled as pRRL U6:shCTRL2eF1a:Egfp-iPSC) previously designed and used by the Wade-Martins' group was tested in the Du et al motor neuron differentiation protocol at stage the iPSC stage (Figure 5.8)

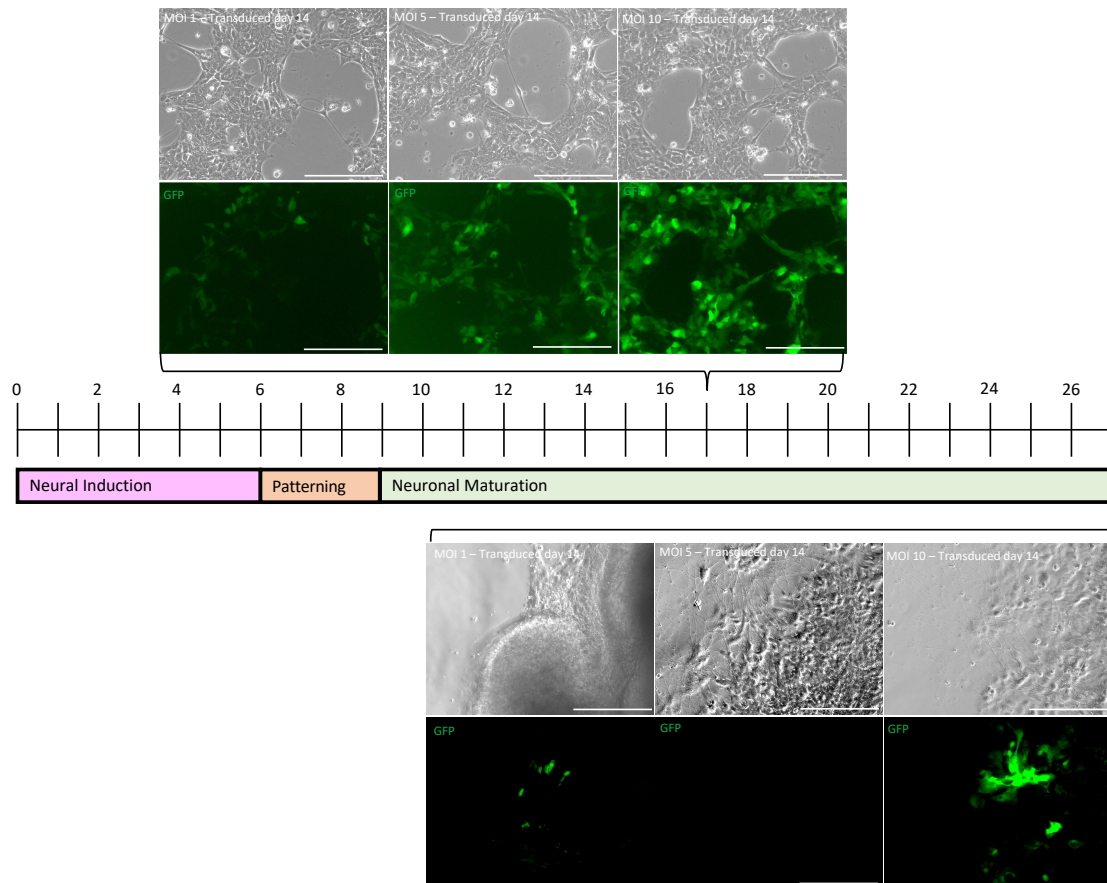


Figure 5.7: Lentiviral transduction of iPSC derived motor neurons using the Qu protocol

Cells were transduced at day 14. An MOI of 1, 5 and 10 were tested, then cells imaged at day 17 and day 27. Scale bars = 200 μm . MOI is the number of lentiviral particles applied. 1= the same number of particles as cell number, MOI 5 is 5 times more than the number of cells.

The pHb9-mCherry-EF1-GFP virus was applied MOI 5 on the iPSC stage and on day 6 and day 18 during the Du differentiation (Figure 5.8). The cells were counted at both the iPSC stage and day 18 allowing for accurate calculation of MOIs. Cells were passaged in clumps on day 6 so actual cell numbers are an indirect estimation and MOIs were inferred. The control pRRL U6:shCTRL2eF1a:Egfp-iPSC was applied at the iPSC stage, as unpublished data from the Wade-Martins Laboratory suggests this vector remains active in dopaminergic differentiations for many weeks, therefore would be anticipated to remain active for the 28 time course of this differentiation.

The iPSC stage would be the ideal time point to add virus as at this stage the cells are still rapidly dividing. Therefore, a reduced amount of virus can be added to a small number of cells that can then be expanded prior to the differentiation.

Transduction at the iPSC stage quickly demonstrated the control pRRL U6:shCTRL2eF1a:Egfp-iPSC showed higher GFP fluorescence than pHb9-mCherry-EF1-GFP (Figure 5.8). Transduction of pHb9-mCherry-EF1-GFP at day 6 and a separate well at day 18 gave detectable GFP expression by fluorescent microscopy, but again not at the level seen for pRRL U6:shCTRL2eF1a:Egfp-iPSC added at the iPSC stage. Fluorescent microscopy at day 28 following transductions showed pHb9-mCherry-EF1-GFP transduced at the iPSC stage no longer expressed visible GFP, however, the pRRL U6:shCTRL2eF1a:Egfp-iPSC transduced cells were still expressing GFP although considerably dimmer. No GFP was detected at day 28 for pHb9-mCherry-EF1-GFP transduced at day 6. Transduction at day 18, however, showed increased GFP expression at day 28, most likely (based on morphological observations) due to viral uptake by non-motor neurons at day 18 which then continue to proliferate. More importantly, similarly

to the results obtained with the Qu differentiation no detectable mCherry expression was observed in any of the conditions tested

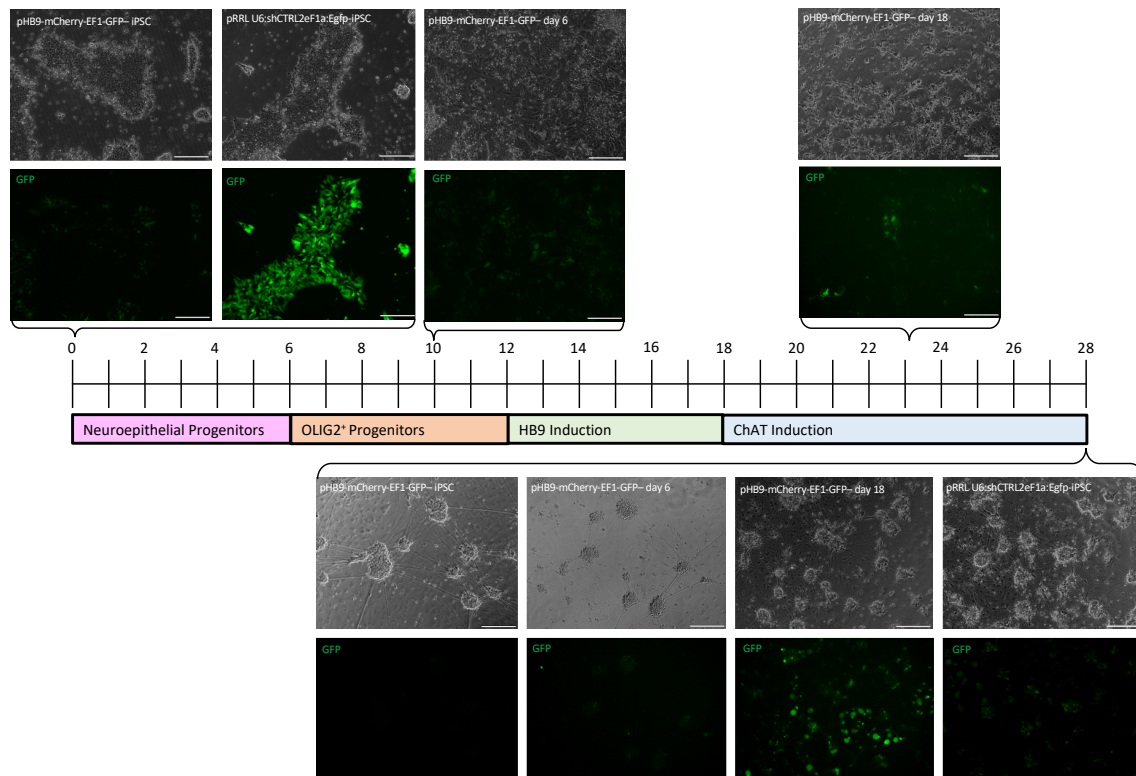


Figure 5.8: Lentiviral transduction of iPSC derived motor neurons using the optimised Du protocol

Cells were transduced using MOI 5 at the iPSC stage, day 6 and day 18 using pHb9-mCherry-EF1-GFP. An EF1 α control plasmid (pRRLL U6:shCTRL2 ef1a:eGFP) was also assessed at the iPSC stage using an MOI 5. Cells were images 4/5 days after transduction indicated by arrows on timeline, then again at day 28. Scale bar= 200 μ m

5.6 F2 Optogenetic Tools

The lack of success of the first-generation constructs in sustained long-term GFP expression and absence of mCherry expression together with the emerging evidence of *HB9* down-regulation in mature motor (Arber *et al.*, 1999; Sances *et al.*, 2016) led to the generation of a second generation (F2) of optogenetic/reporter tools. Through testing the pHb9-mCherry-EF1-GFP virus it became apparent that the inclusion of EF1 α driving GFP was of great benefit. The EF1 α driving GFP allows for fluorescent titering of the virus by flow cytometry as well as assessment of the transduction efficiency when applied during MN differentiation. Without EF1-GFP it would not be known what percentage of the cells were transduced and so unknown whether a subsequent low *Hb9* driven mCherry expression was due to low overall *Hb9* expression or due to low viral transduction. The second generation optogenetic/reporter tool (Figure 5.9) is based on the Cre/Lox system using a dual promoter and dual non-compatible pairs of lox sites (LoxP and Lox2272) described 5.1.2.2 .

EF1 α drives a floxed eGFP adjacent to a floxed inverted ChR2-mCherry cassette (inverted to prevent expression) followed by the viral WPRE element. In front of the viral Cppt and EF1 α promoter is the 3.6 kb *Hb9* promoter driving Cre. Expression of the *Hb9* promoter during differentiation will lead to the production of Cre recombinase which will cause inversion of the ChR2 cassette and stable excision of eGFP as the remaining LoxP and Lox2272 single sites cannot interreact (Figure 5.9). At the end of the differentiation cells expressing or having expressed *Hb9* will be ChR2mCherry positive, GFP negative. Any transduced cells not of the *Hb9* lineage will remain GFP positive.

Low GFP expression and potential silencing issues of the first-generation constructs might have been due to mutations introduced during the cloning, affecting essential viral elements. Furthermore, a difference in the EF1 α sequence between both vectors was also discovered (Figure 5.8). To address these issues, all second generation vectors were cloned into the parental pRRL U6:shCTRL2eF1a:Egfp-iPSC lentiviral backbone. Additionally, it should be noted that the *Hb9* enhancer elements in the original first-generation constructs are upstream of the viral minimal CMV promoter rather than the minimal beta globin promoter used by (Nakano *et al.*, 2005; Peviani *et al.*, 2012). This could also contribute to poor viral vector expression as viral sequences are more likely to be silenced by the host cell (Brooks *et al.*, 2004) Therefore, the second generation construct has the minimal beta globin promoter as used in the original construct (Nakano *et al.*, 2005; Peviani *et al.*, 2012)

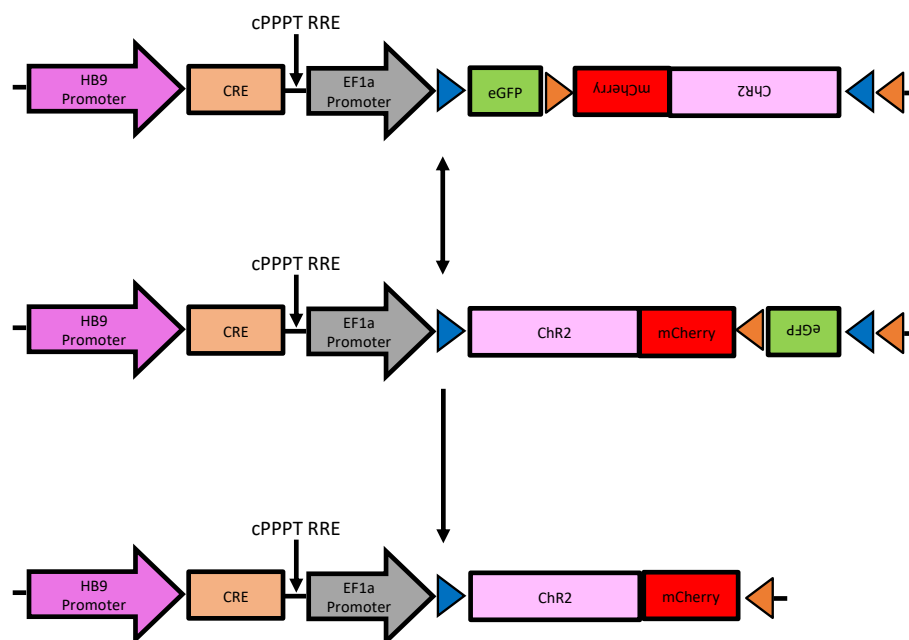


Figure 5.9: CRE/LOX second generation optogenetic tool/reporter

Mechanism of inversion and excision of cassettes in new second generation vectors. Lox sites = red or blue triangles, red and blue represent different sites. The blue lox sites will interact and cause the inversion of the Chr2 cassette, the red lox sites will now be in the same orientation and so will interact to excise a blue lox site as well as the GFP. The red and blue sites can then no longer interact and the cassette is stable. The same sequence of events can also occur beginning with the alternate Lox pair.

The second-generation virus was cloned using a similar workflow to the first-generation tools. Briefly, the virus was cloned into the lentiviral backbone in two halves. The *Hb9* driving Cre was inserted upstream of viral central polypurine tract (Cppt) element (Importance of which is discussed in 5.7) and second the EF1 α -GFP cassette was inserted between the RRE and WPRE.

The backbone pRRL U6:shCTRL2eF1a:Egfp-iPSC was digested with XbaI and XhoI (Figure 5.10A) to remove the U6 SH and to open for insertion of *Hb9* and Cre. The beta globin minimal promoter fragment to replace CMV was generated using short overlapping primers and PCR amplified together (Figure 5.10B). *Hb9* was amplified from the first generation pHb9-ChR2mCherry and Cre from pDbx1-Hsp68-CRE (and kind gift from Garcia-Moreno Group). To circumvent issues in assembly due to the large difference in fragment sizes between the *Hb9* promoter (3.6kb) and beta globin minimal promoter (120bp), the beta globin and Cre fragments were amplified together by overlap extension PCR (Figure 5.10E-F). This was possible as both fragments had overlaps in sequence designed for Gibson assembly. I used the improved Gibson assembly mix known NEBuilder HiFi to successfully include the fragments into the plasmid.

Unfortunately, introduction of the second half EF1-floxGFP-floxChR2mCherry resulted in rearrangement of the Lox sites during the bacterial amplification of the plasmid.

The sequence of the maxiprep construct confirmed the excision of GFP and inversion of ChR2mCherry. This occurred as WT Cre was used which bacteria are able to express regardless of the promoter placed upstream. This phenomenon has been previously described and prevented with the use of modified Cre called Cre-M (Kaczmarczyk and Green, 2001). Cre-M contains an artificial intron, based on the β -actin intron, in the

coding region of Cre. Therefore, bacteria are unable to process the intron but mammalian cells can remove the intron and produce active Cre.

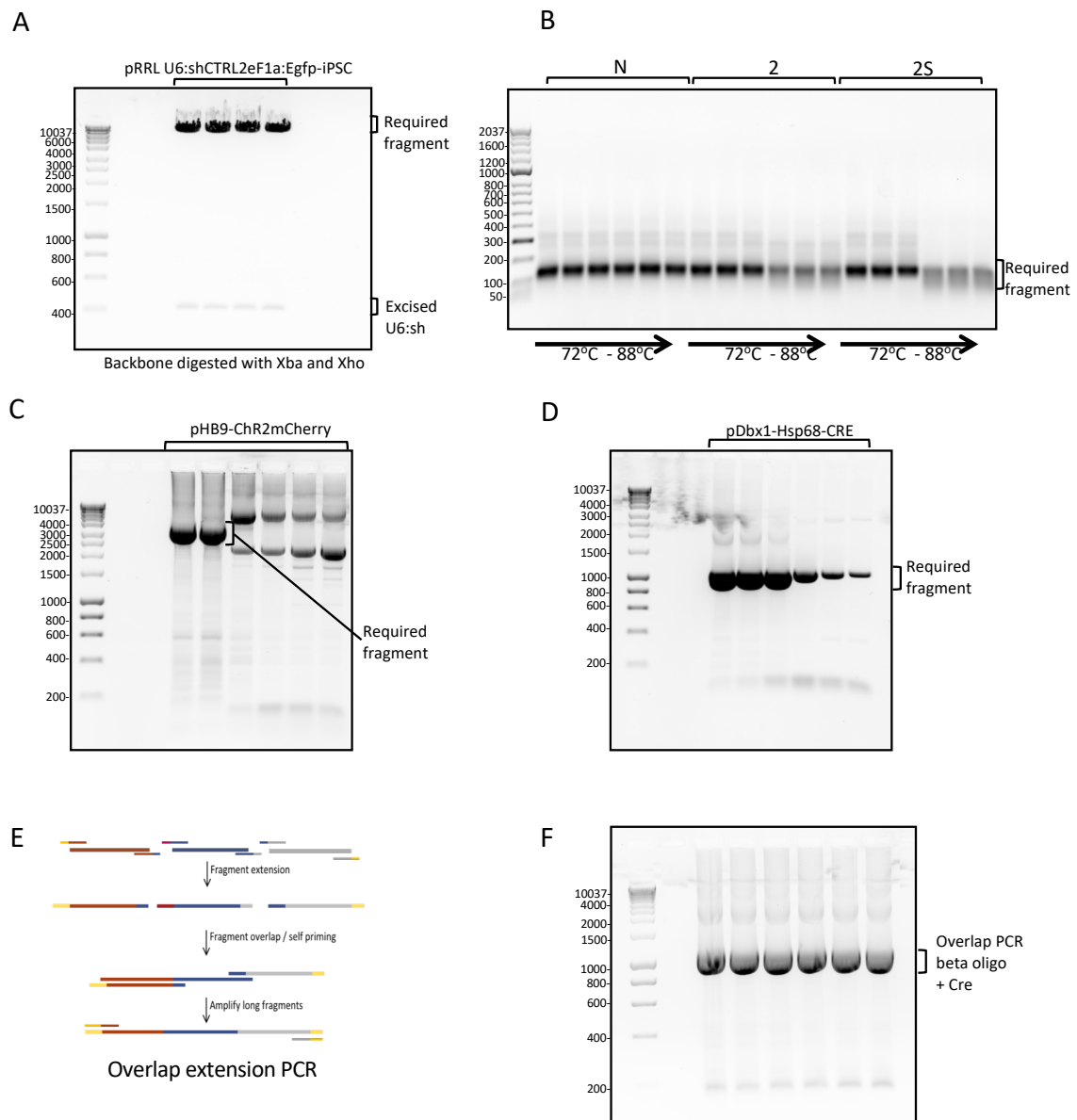


Figure 5.10: Generation of second generation optogenetic tools – insertion of HB9 CRE.

A) Lentiviral backbone pRRL U6:shCTRL2eF1a:Egfp-iPSC (10 μ g across 4 Lanes) was digested and prepared for HiFi Assembly. B) Primer only PCR to generate beta globin minimal promoter from overlapping primers, N, 2, and 2S denote the cycling conditions used described in further details in the methods chapter 2. C) PCR amplification of *Hb9* using KAPA HiFi kit (20ng) using an annealing gradient from 60 to 75 °C left to right. D) PCR amplification of Cre using KAPA HiFi kit (20ng) using an annealing gradient from 60 to 75 °C left to right. E) Schematic of the overlap extension PCR strategy. F) Overlap extension PCR of beta to Cre using an annealing gradient from 60 to 75 °C left to right.

The second-generation plasmid was successfully completed once the Cre was replaced by Cre-M. Briefly, this plasmid was generated using a similar work flow to above. The second half was inserted into the backbone first followed by the *Hb9* driving Cre-M. The backbone pRRL U6:shCTRL2eF1a:Egfp-iPSC required additional cloning steps. First, the U6 SH was removed, then the sticky ends blunted with mung bean nuclease and re-ligated (Figure 5.11A). Secondly, the re-ligated backbone was reopened just before the viral Cppt with HpaI and SalI restriction enzymes (Figure 5.11B). Thirdly, GFP was PCR amplified, and gel purified from parent plasmid pRRL U6:shCTRL2eF1a:Egfp-iPSC (Figure 5.11C). Modified overlap extension PCR (detailed further in methods) was used to join the double Lox sites to the Chr2mCherry cassette (from parent plasmid pHb9-Chr2mCherry) while Cppt was joined to the EF1 α fragment (both from parent plasmid pRRL U6:shCTRL2eF1a:Egfp-iPSC) (Figure 5.11D-E). This strategy involves additional PCR and therefore increases the risk of mutations; however, it reduces the number of fragments to be assembled, improving the HiFi assembly rate. These fragments were inserted successfully and correct colonies sequenced (Figure 5.11F). Finally, the *Hb9* driving Cre-M cassette was successfully inserted and sequenced before the viral Cppt without recombination of the lox sites using the same cloning strategy as previously used (Figure 5.12A-D).

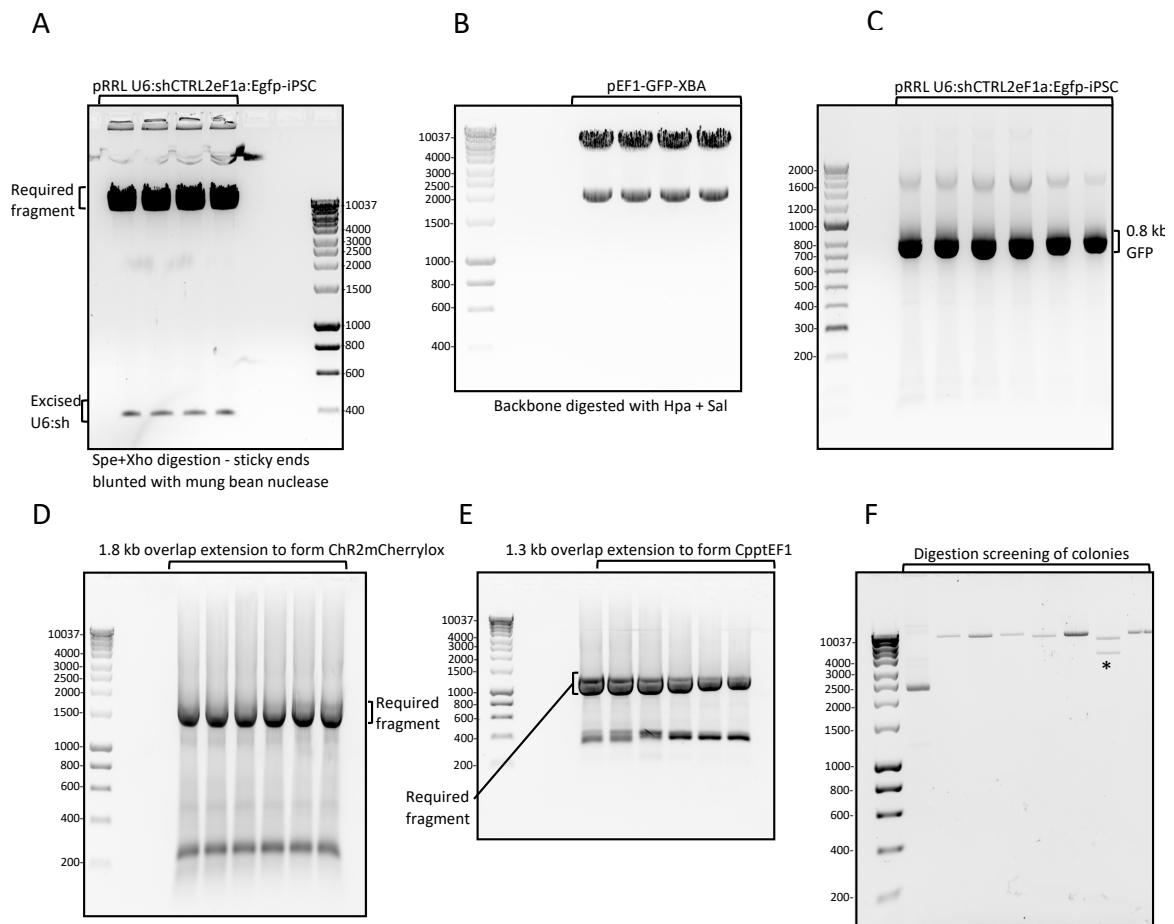


Figure 5.11: Generation of second generation optogenetic tools – insertion of EF1 cassette.

A) Lentiviral backbone pRRL U6:shCTRL2eF1a:Egfp-iPSC (10 μ g across 4 Lanes) was digested to remove U6 SH, then treated with mung bean nuclease and re-ligated. B) Re-ligated backbone digested in preparation of fragment insertion. C) PCR amplification of GFP using KAPA HiFi kit (20ng) using an annealing gradient from 60 to 75 $^{\circ}$ C left to right. D +E) Overlap extension PCR of lox to Chr2mCherry and Cppt to EF1 α using an annealing gradient from 60 to 75 $^{\circ}$ C left to right. F) Screening digestion to check for correct colonies. * indicated correct fragments expected.

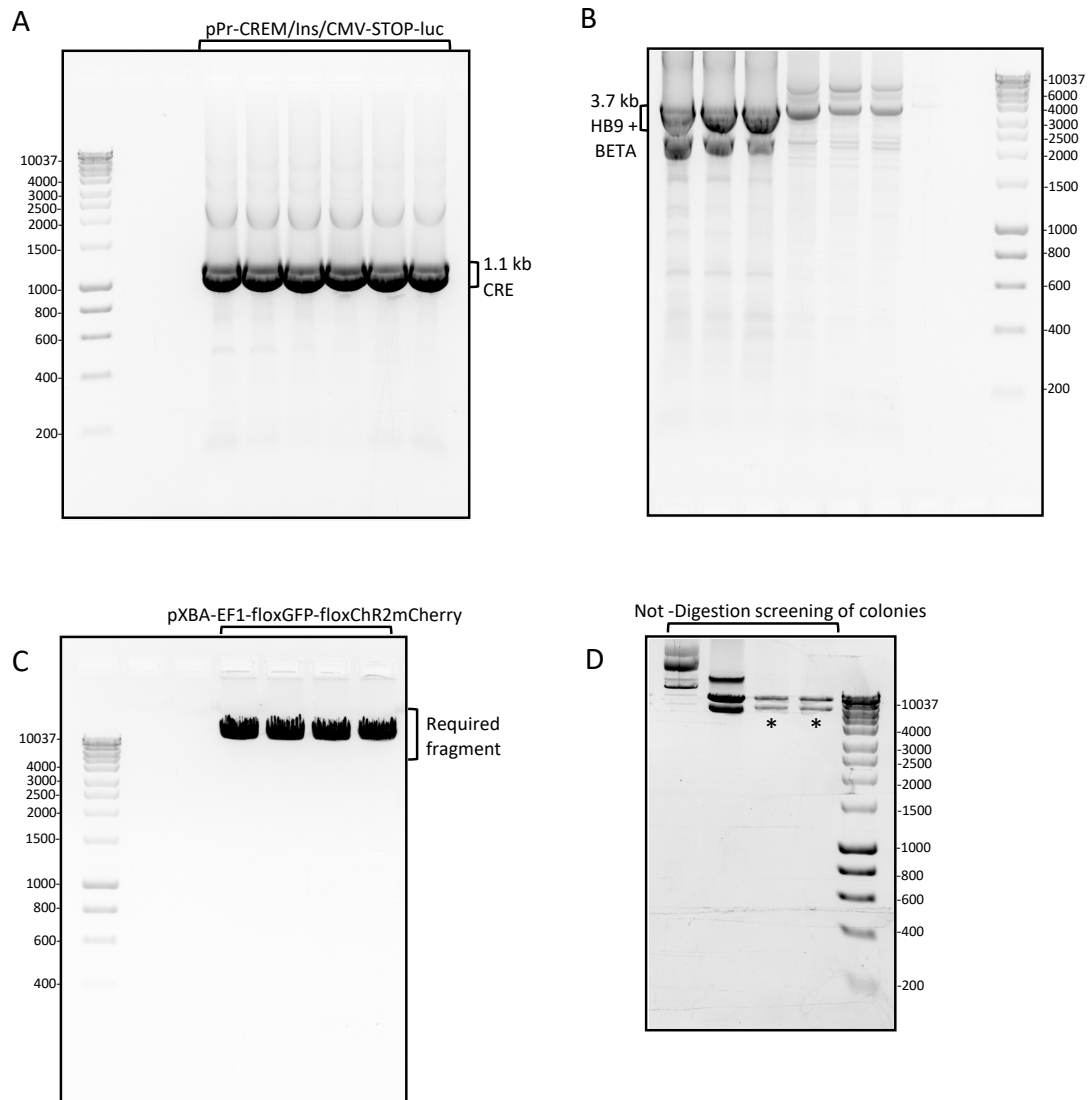


Figure 5.12: Generation of second generation optogenetic tools – insertion of Hb9 CRE-M.

A) PCR amplification of Cre-M using KAPA HiFi kit (20ng) using an annealing gradient from 60 to 75 °C left to right. B) PCR amplification of Hb9 joined to beta globin from failed initial second generation plasmid using KAPA HiFi kit (20ng) using an annealing gradient from 60 to 75 °C left to right.) Lentiviral backbone pXBA-EF1-floxGFP-floxChr2mCherry (10μg across 4 Lanes) was digested and prepared for HiFi Assembly. D) Screening digestion to check for correct colonies. * indicated correct fragments expected.

5.7 Second Generation Lentiviral iPSC derived

Motor Neuron Transduction

Following successful generation of the second generation optogenetic/reporter tool pHb9-CRE-EF1-floxGFP-floxChR2mCherry, the virus was packaged using the optimised 10 µg Lipofectamine LTX for large vectors (Figure 5.6D). Although a low titer was achieved the vector was successfully packaged. To increase viral yield, the vector was packaged three times and titered, achieving titers of: 9.6×10^6 , 1.0×10^7 and 2.6×10^6 particles per ml. These titers are suitable for in vitro experiments if applied at the iPSC stage.

Initially the virus was applied at the iPSC stage to a control line and one patient line using an MOI of 4 in a reduced media volume to facilitate infection. However, the control line did not survive (reasons for which will be discussed section 5.7) and the patient line (ALS 3) showed a poor granular morphology. Another control iPSC line (CTRL 1) was transduced with a lower MOI (1.2) to reduce toxicity to the cells and again in reduced volume to maximise infection. Virus was additionally added at day 18 again to CTRL 1 and ALS 3 at MOI 4, however, again the morphology of these cells was poor 4 days after transduction. Finally, virus MOI ~ 1 was added at day 25 again to ALS 3 and CTRL 1 (Figure 5.13A). GFP expression followed through CTRL 1 during differentiation and was detectable throughout but fainter in the latter stages with again no visible mCherry expression (Figure 5.13B-D). To assess whether the lack of mCherry was due to a lack of protein or due to the dim fluoresce of the protein itself, an anti-mCherry antibody was used in both flow cytometry (Figure 5.14A) and immunofluorescence (Figure 5.14C-D) at

Day 47. Flow cytometry was performed on CTRL 1 iPSC transduced and un-transduced cells at day 47. Untransduced cells were analysed using SMI-32 to detect MNs and TUJ1 as a general neuronal marker. This differentiation produced a low percentage of MNs ~15% . Assuming that the virus infects MNs and non-MN cell equally, a percentage mCherry expression of 15% or lower would be anticipated for correct functioning of the reporter. 9% mCherry expression was observed suggesting the virus is functioning as expected although further validation is required (Figure 5.14A-B).

CTRL 1 iPSC and ALS 3 day 25 transduced cells were also fixed for immunostaining using anti-mCherry to confirm the mCherry expression and evaluate the morphology of mCherry expressing cells. Both showed mCherry expression by immunocytochemistry in cells with neuronal morphology (Unfortunately SMI-32 staining was unsuccessful). Both also showed GFP expression using the anti-GFP antibody again confirming the long-term persistence of the virus (Figure 5.13B-D).

Together, these results indicate that mCherry is present but is not natively visible, giving indication that the reporter element of the construct is functioning although further validation of Chr2 function would need to be conducted. Future plans for the construct and validation are discussed 5.7.

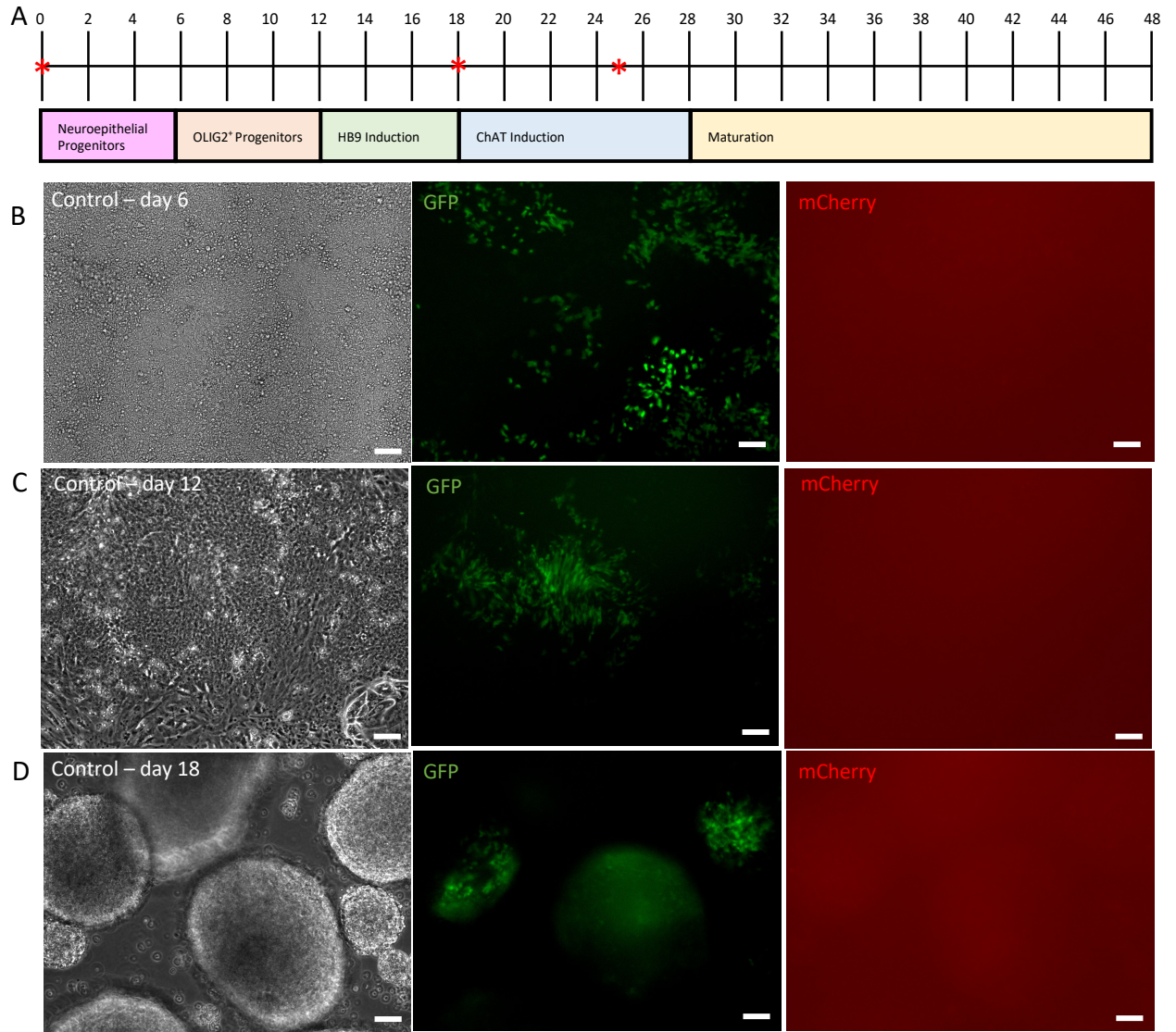
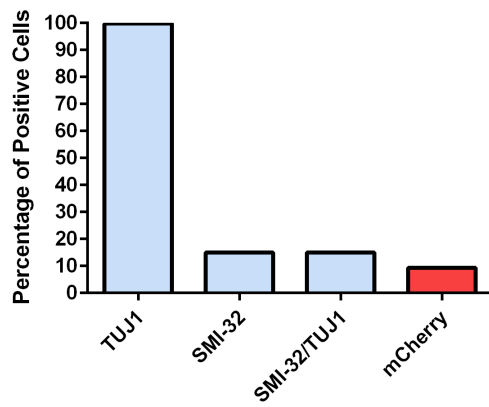


Figure 5.13: Second generation optogenetic tool/reporter lentiviral transduction into iPSCs.

A) Timepoints of lentiviral transduction represented by ‘*’. Cells were transduced using at the iPSC stage, day 18 and day 25 using pHb9-CRE-EF1-floxGFP-floxChR2mCherry. B,C,D) Native fluorescence of virally transduced cells. Control line 1 was transduced at the iPSC stage expanded and then differentiated to motor neurons using the optimised Du et al protocol. Scale bars = 70 μ m

A



Marker	% Positive Cells
TUJ1	99.8
SMI-32	15.0
SMI-32/TUJ1	15.1
mCherry	9.4

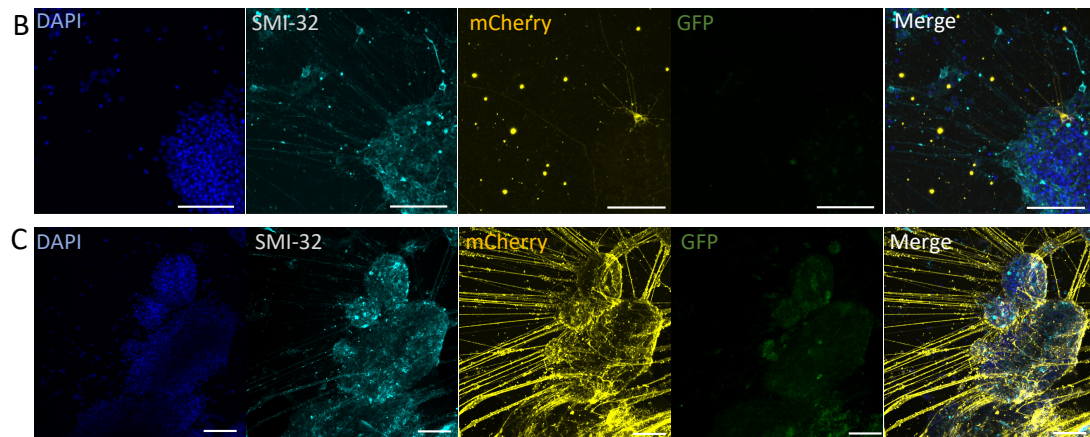


Figure 5.14: Validation of Second generation optogenetic tool/reporter expressing GFP and mCherry

A) Flow cytometry of un-transduced and transduced CTRL 1 iPSC derived motor neurons at day 47. Untransduced cells stained for TUJ1 and SMI-32, and transduced cell stained mCherry. Results also shown in table. B) iPSC transduced CTRL 1 stained for mCherry and SMI-32 at day 47. C) Day 25 transduced ALS 3 stained for GFP, mCherry and SMI-32 at day 47. Viral cells were mitoC treated. Scale bars= 100 μ m

5.8 Discussion

This chapter overall demonstrated the successful design and cloning of a dual optogenetic-reporter lentiviral vector. The initial aim of the chapter was to create a construct with a motor neuron specific promoter driving the optogenetic tool ChR2, fused to a fluorescent protein for visualisation. This construct was designed to allow both the visualisation of motor neurons in a mixed culture but also the specific control and activation of these neurons.

Particularly for electrophysiology studies, the inclusion of a reporter system able to label living cells is of great advantage, as it enables direct patching of motor neurons without the need for post-hoc staining. The inclusion of ChR2 also allows high temporal and spatial control of neurons (Deisseroth and Hegemann, 2017), giving greater flexibility in the electrophysiological experiments that can be performed. Single neurons as well as populations of neurons can be activated without the need for direct patching to analyse their role in neural networks.

Finally, the construct needed to be suitable for lentiviral packaging and delivery, as lentiviral delivery offers flexibility in transduction timing. Lentiviruses are able to infect both dividing and post-mitotic cells, meaning the virus could be used at both the actively dividing progenitor stage or at the post-mitotic neuronal stage (Bukrinsky *et al.*, 1993). Initially four first generation (F1) constructs were designed. An optogenetic-reporter construct was designed with the mouse 3.6 kb truncated *Hb9* promoter (Nakano *et al.*, 2005; Peviani *et al.*, 2012) with minimal CMV driving ChR2 fused mCherry (p*Hb9*-ChR2mCherry). A second test construct was designed with the 3.6 kb truncated

Hb9 promoter driving mCherry and the constitutive EF1 α promoter driving GFP (pHb9-mCherry-EF1-GFP). The final two constructs were adaptations of the above with IRES and puromycin inserted to allow for puromycin selection. The test construct, pHb9-mCherry-EF1-GFP was designed to assess transduction efficiency using the constitutively expressed GFP. However, when transduced into cells in both the (Qu *et al.*, 2014) and (Du *et al.*, 2015) protocols no GFP expression was detected throughout the protocol. As an unrelated construct which expressed GFP driven by EF1 α (pRRL U6:shCTRL2eF1a:Egfp-iPSC) was applied in parallel and was found to express GFP throughout the differentiation, it became apparent there was some dysfunction in the test construct.

In addition to the silencing of GFP, mCherry expression was not detectable throughout the differentiation. This was consistent with previous reports showing that the human *HB9* gene is only transiently expressed and downregulated in mature motor neurons (Arber *et al.*, 1999). Therefore, there was a need to redesign the optogenetic-reporter construct.

The second generation (F2) vector was designed using a dual Cre/Lox cassette (p*Hb9*-CREM-EF1-floxGFP-floxChR2mCherry), design not previously used in the iPSC-derived motor neuron field. This construct, again employed the mouse *Hb9* promoter with a minimal beta globin promoter to drive Cre-M. This was followed by the viral elements Cppt and RRE, then the EF1 α promoter driving a floxed GFP and inverted floxed ChR2-mCherry cassette. With this design, when the construct is first transduced into cells, these will express GFP, allowing the identification and percentage of transduced cells to be calculated. Following the activation of *Hb9*, Cre-M is expressed which will interact with the Lox P and Lox 2772 sites, causing an inversion of the ChR2-

mCherry cassette and excision of GFP. This conversion is irreversible as the final construct after conversion contains one Lox P and one Lox 2772 which cannot interact. Therefore, at the end of the differentiation cells actively expressing or cells which have previously expressed *Hb9* will express mCherry, cells not belonging to the motor-neuron lineage will remain expressing GFP.

The Cre-M version of Cre was selected after initial trials using standard Cre lead to recombination within the bacterial strains used to amplify the construct. Cre-M includes a small intron from beta globin within the sequence (Kaczmarczyk and Green, 2001), this intron is able to be excised in mammalian cells but not in bacteria. Therefore, the dual Cre/Lox construct can be amplified in bacteria successfully.

The minimal beta globin promoter was inserted in place of minimal CMV following the truncated mouse *Hb9* promoter, as minimal beta globin was used in the original published version (Nakano *et al.*, 2005; Peviani *et al.*, 2012). Minimal CMV is also a viral element derived from Cytomegalovirus which is known to be susceptible to silencing by methylation in mammalian cells, therefore it was beneficial to expression to remove this element (Teschendorf *et al.*, 2002; Brooks *et al.*, 2004).

As described, when testing the F2 construct there appeared to be some issues of toxicity with MOI 5 or above not seen with previous F1 viruses. This could be due to the low titer of the F2 virus. The F2 virus was packaged with titers of $< 1 \times 10^7$ particles/ml whereas the smaller F1 test virus (pHb9-mCherry-EF1-GFP) packaged with titers at 1.8×10^8 particles/ml. Therefore, to apply the same MOI between the two viruses a larger volume of F2 virus would need to be applied to the cells. As the virus is diluted in TSSM (20 mM Tromethamine, 100 mM NaCl, 10 g/l Sucrose and 10 g/l D-Mannitol) and applied at half media volume in a 12 well plate at the iPSC stage this could have affected

cellular viability. Particularly at the iPSC stage cells are very sensitive to changes in media components and single cell passaging. Therefore, single cell dissociation followed by addition of TSSM in the reduced media volume could lead to cellular stress and activation of cell death pathways (Bai *et al.*, 2015). A potential to solution to this issue could be to either concentrate the virus further by resuspending in a lower volume of TSSM in the production stage or to resuspend in a different buffer e.g. DMEM to reduce the toxicity to cells.

For final usage the virus does require minor alterations and validation to be fully functional in live neurons. As described in the results section 5.6, at present the virus can only be visualized with the use of antibody staining using anti-mCherry antibodies. The mCherry would need to be removed and substituted for an alternative fluorophore such as mKate2 or mScarlet (Bindels *et al.*, 2017)

Observing 9% mCherry positive cells compared to 15% positive SMI-32 cells by flow cytometry does suggest the virus is functioning effectively in labeling motor neurons.

However, due to technical issues the ICC was not successful in colocalizing mCherry and SMI-32, therefore further visual validation is required in the future. Validation is essential as it is not uncommon for the 3.6 kb mouse *Hb9* promoter to show some ectopic expression as noted in the original published paper (Nakano *et al.*, 2005).

Although this expression is low, it would still be beneficial to check whether ectopic expression is observed in iPSC-derived motor neuron cultures. In addition to ectopic expression validation is important due to the use of the Cre/Lox system. Any inappropriate Cre expression will cause rearrangement of the floxed ChR2 cassette and false positive labelling of motor neurons.

Finally, further validation needs to assess whether the mouse *Hb9* promoter is effective enough to drive Cre. This is unlikely to be an issue as small levels of Cre expression will cause the inversion and excision of the cassette. However, in dual promoter constructs, the first promoter often suffers from reduced expression, this can be easily corrected by insertion of a second WPRE to stabilize mRNA production after the first promoter in addition to the final WPRE (Gascón *et al.*, 2008). This initial F2 construct does not include the WPRE in the center as this would increase the size of the already large plasmid and could lead to recombination within bacteria. However, this is something that could be tested if expression is not as high as desired from *Hb9* after further trials

Overall the constructs dual Cre/lox design and packaging into lentivirus was successful. Minor alterations to the fluorescent reporter as discussed above would be needed for its full functionality as a live cell reporter and further validation of its specific expression in mature motor neurons required.

Chapter 6:

Differential Allele expression at the

***C9ORF72* locus in Heterozygous**

Patients

6.1 Introduction

This chapter will generate data to contribute to the argument of whether *C9ORF72* gene expansion ALS, in part, is caused by a loss of function haploinsufficiency mechanism of the expanded *C9ORF72* allele using both techniques of 2D FISH and RT-qPCR.

6.1.1 Lymphoblastoid cell lines

Lymphoblastoid cell lines (LCLs) are often utilised as a model system for disease study, including *C9ORF72* ALS research (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). There are many advantages to the use of LCLs in disease modelling including: their ease of cell culture, ‘unlimited’ supply of biomolecules e.g. DNA, and their low somatic mutation rate in culture (Mohyuddin *et al.*, 2004). LCLs are created using the Epstein Barr Virus (EBV) by infecting peripheral blood lymphocytes resulting in an immortalized proliferative B cell population (Neitzel, 1986; Frisan, Levitsky and Masucci, 2001). The EBV LCLs carry the viral episome and on infection express viral proteins including six nuclear antigens EBNA1, EBNA2, EBNA3A, EBNA3B, EBND3C and EBNDLP, three membrane proteins LMP1, LMP2A and LMP2B as well as BamHIA region transcripts (BARTS) and non-coding RNAs EBER1 and EBER2 (Heller, van Santen and Kieff, 1982; Hennessy *et al.*, 1983, 1984, 1986; Fennewald, van Santen and Kieff, 1984; Hennessy and Kieff, 1985; Hennessy, Fennewald and Kieff, 1985; Longnecker and Kieff, 1990; Smith *et al.*, 1993; McFarland, Izumi and Mosialos, 1999). Although all proteins are expressed, not all are essential for the immortalisation of LCLs, EBNA2 and LMP1 are essential for the

process, EBNA1, EBNA1P, EBNA3A and EBNA3C also play a crucial role, while the remaining proteins are unnecessary (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989; Mannick *et al.*, 1991; Tomkinson and Kieff, 1992; Longnecker *et al.*, 1993; Tomkinson, Robertson and Kieff, 1993; Lee, Diamond and Yates, 1999; McFarland, Izumi and Mosialos, 1999).

6.1.2 Fluorescent in situ hybridisation

The use of 2D FISH to study *C9ORF72* gene expansion ALS allows for a novel approach to the question of haploinsufficiency. FISH is a powerful technique enabling researchers to visualize DNA or RNA sequences within cells. The advances in the in situ hybridisation (ISH) technique over the last ~50 years predominately revolve around the use and labelling of the probes. First described in 1969 in situ hybridisation was originally detected using radiolabelled sequences/probes (Gall and Pardue, 1969; John, Birnstiel and Jones, 1969). This technique although still used due to its high sensitivity suffers, from several issues including: safety of radioactive labels, short-half life and lengthy exposure time to detect signal. Non-radiolabelled probes were first explored in 1980s by multiple groups using several techniques. In 1980, RNA fluorescently labelled at the 3-prime end was used to probe kinetoplast DNA sequences in insects (Bauman *et al.*, 1980). One year later, Langer et al, developed amino-allyl modified probes that could later be conjugated to haptens or fluorophores (Langer, Waldrop and Ward, 1981). By 1982, several groups started using nick translation biotinylated probes in combination with secondary streptavidin conjugated fluorophores (Manuelidis, Langer-Safer and

Ward, 1982; Singer and Ward, 1982). The use of secondary labelling allows for amplification of the signal and visualisation of previously unobtainable sequences. There are many advantages to the use of FISH including: the high specificity of the technique for recognising specific DNAs/RNAs, its use in both interphase nuclei and metaphase chromosomes as well as enabling visualisation of target sequences at the single cell level (Cui, Shu and Li, 2016). Using FISH here allows both the expanded and WT alleles to be visualised at the single cell level individually and their location within the nucleus analysed separately. Studies have shown that the location of a gene within the nucleus is correlated to its transcriptional output, with lower output seen from genes residing at the nuclear lamina e.g. silenced immunoglobulins in mammals are mostly found at the periphery (Kosak *et al.*, 2002; Dillon, 2008). Several studies in mice support the idea of a relationship between gene activity and nuclear position, *GATA-3* and *c-maf* move from the periphery of the nucleus to a more central position during T-cell activation, *Mash1* follows the same path during neuronal differentiation (Hewitt *et al.*, 2004; Williams *et al.*, 2006; Takizawa, Meaburn and Misteli, 2008). The location of the WT and expanded alleles in *C9ORF72* ALS patient nuclei is currently unknown.

6.1.3 Aims of Chapter

This final chapter focuses on the haploinsufficiency hypothesis and will address the following:

- Optimisation of the technique of 2D FISH using LCLs.
- Application of 2D FISH to patient LCL lines.
- RT-qPCR looking at total *C9ORF72* expression and transcript variant expression on LCLs to assess validity of FISH.
- Extension of RT-qPCR analysis to both patient and control iPSC-derived motor neurons.

6.2 LCL Repeat Expansion Verification

Two patient and one control LCL line were selected for *C9ORF72* expression analysis. The two patients are unrelated, patient 1 referred to as ND06769 was a 46-year-old female at the time of sampling and patient 2 referred to as ND06751 was a 52-year old male at sampling (Figure 6.1A). The control referred to as ND07612 is the 61-year-old asymptomatic sister of patient ND06751 (Figure 6.1A).

To confirm the presence of the repeat expansion in the patients and a lack of expansion in the control, repeat primed PCR was carried out using the DeJesus-Hernandez *et al.*, 2011 protocol (as previously described for motor neurons Chapter 4). As expected, both ND06769 and ND06751 show repeat expansions with peaks continuing past the limit of detection of the technique decreasing in size from left to right (Figure 6.1B-C). As previously discussed, it would be expected for short amplicons of the expansion to be in highest abundance as amplification efficiency decreases as repeat length increases. This pattern is seen for ND06751 however, ND06769 showed a spike at ~390 base pairs rather than a smooth decrease indicating possibly a heterogeneous repeat length in the population of cells. Control ND07612 did not show the presence of a repeat expansion, evidenced by the lack of peaks expanding past the technique noise peaks ~160 base pairs (Figure 6.1D).

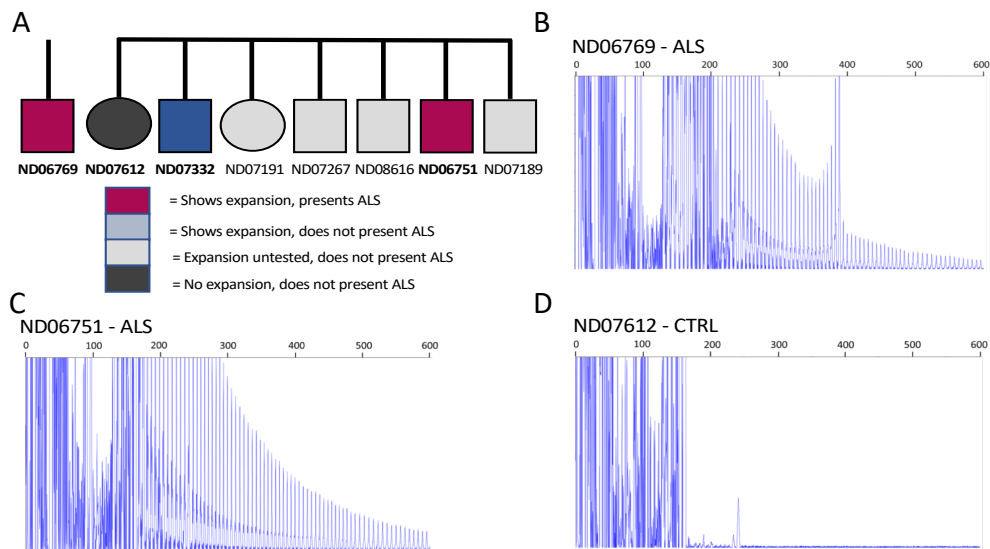


Figure 6.1: Repeat expansion present in LCL samples

A) Family tree of patients and controls used in RT-qPCR and FISH analysis on LCLs. Repeat primed PCR of two patient LCL lines B) ND06769 and C) ND06751 and one control line D) ND07612. X axis = base pairs. Y axis = zoomed to 2000 relative fluorescence units.

6.3 Optimisation of Expanded and WT Probes

As previously discussed the probes used in FISH are extremely important for the specificity and sensitivity of the technique. Correct sizing and labelling allow for reduced signal to noise ratio and easier identification of true probe spots.

To identify *C9ORF72* alleles the fosmid clone (WI2-2071M15 (G248P86104G8)) from BACPAC Resources was selected as it spans the entire length of the *C9ORF72* gene downstream of the repeat expansion allowing for identification of both the WT and expanded alleles. Following maxi preparation, expected restriction sizes and absence of other contaminating fosmids or plasmids were verified by digestion with *AfeI* (30,747 bp and 14,481 bp expected fragments) and *PmeI*+*XhoI* (26,829 bp and 18,401 bp expected fragments) followed by pulse field gel electrophoresis (PFGE) (Figure 6.2A). For FISH, the fosmid is digested into fragments of 200-500 nucleotides and tagged with Digoxigenin to enable fluorescent secondary antibody detection. Initial digest and labelling were performed by nick translation using the DIG-NICK kit (ROCHE). This procedure (Figure 6.2B-C) did not produce the desired fosmid fragments using either the recommended 1 µg of fosmid DNA or a reduced amount (0.5 µg DNA). Extended incubation with the kit from 90 to 180 minutes at 15°C also failed to produce the required fragment sizes. Supplementation with additional DNase I (Figure 6.2D-E) was required to obtain the correct fragment sizes required. DNase I addition ranging from 10 U-110 U and incubation for 90mins, 120mins and 180 minutes was assessed, 80 U for 90 minutes produced fragments of acceptable (200-500 nucleotides) use for 2D FISH.

To detect the expanded *C9ORF72* allele, a synthetic oligonucleotide (oligo) probe with 5 hexanucleotide repeats (GGCCCCGGCCCCGGCCCCGGCCCCGGCCCC) was prelabelled with Biotin at the 5-prime end. It must be noted that an oligo with 100% GC content has a high affinity to form hair pins and self-aggregate (Calculated using OligoCalc Software (Kibbe, 2007)).

With our approach, a successful FISH labelled nuclei should contain two FITC labelled fosmid spots with one spot – corresponding to the expanded allele - also colocalised with the CY3 oligo.

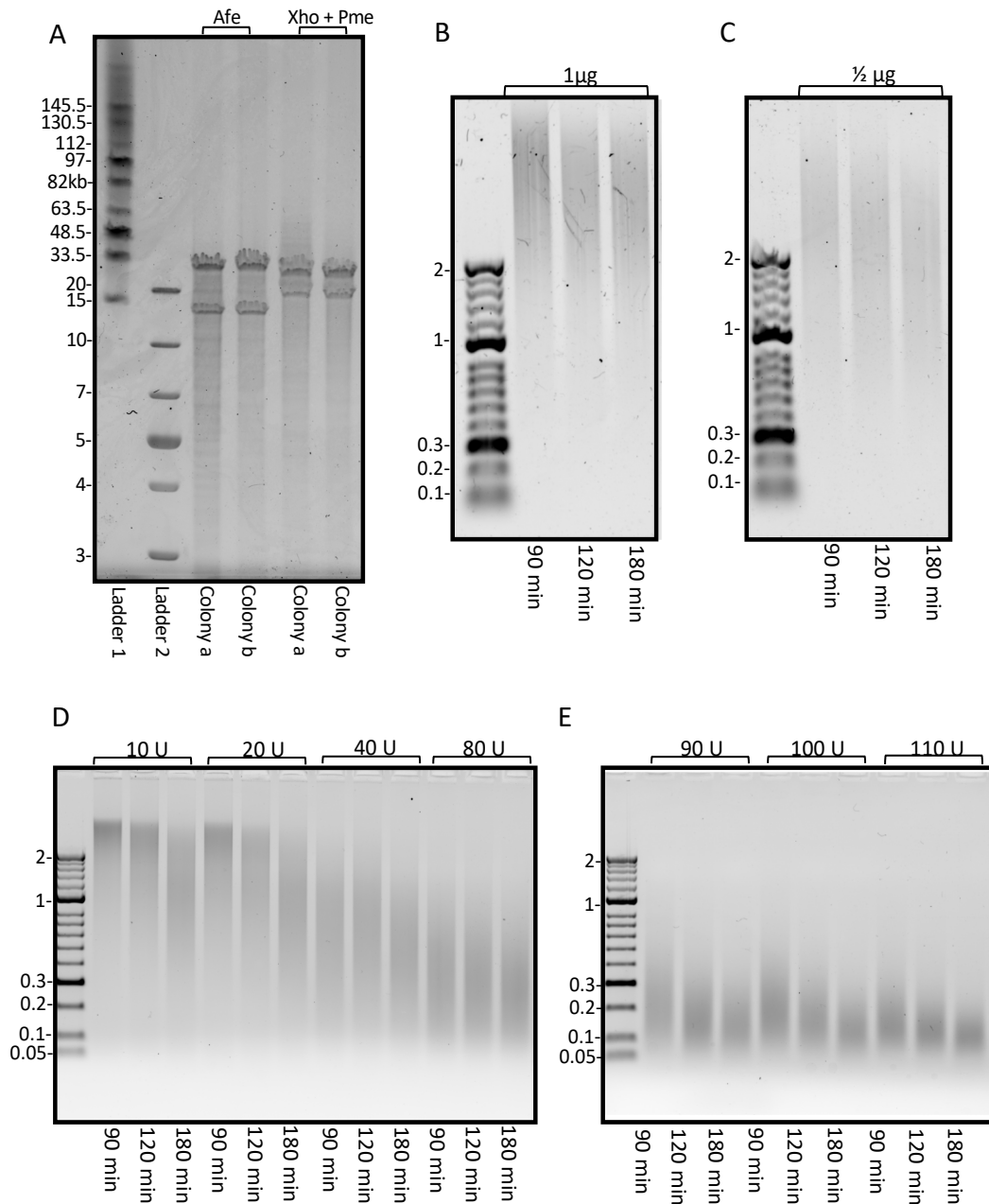


Figure 6.2 Labelling C9ORF72 fosmid for 2D FISH

A) Pulse field gel of C9ORF72 fosmid digested with restriction enzymes AfeI (lane 3 & 4) and XhoI+PmeI (lane 5&6). Lane 1: Ladder 1 – NEB MidRange PFG Marker. Lane 2: Ladder 2-GeneRuler 1kb plus ladder (ThermoFisher). B+C) DIG-NICK Translation kit (ROCHE) alone does not produce the correct size fragments whether using the recommended 1 µg or 0.5 µg DNA. D) Optimisation of DNase 1 supplementation to DIG-NICK translation kit incubating for 90 minutes, 120 minutes and 180 minutes.

Following probe preparation, 2D FISH trials were completed using patient ND06769 to establish the correct concentration of probes needed to correctly label the expanded and WT *C9ORF72* alleles. To visualise both probes, secondary antibodies were utilised, using FITC for fosmid detection and CY3 for oligo detection. Fosmid probes standardly are used at 100 ng/slide however, the concentration of oligo required must be optimised (Silva et al., 2015). Initial trials indicated the fosmid was working as expected, however, seemed to show a higher than anticipated background (Figure 6.3) while the oligo was undetectable at all amounts tested (10 ng-200 ng/slide Figure 6.3).

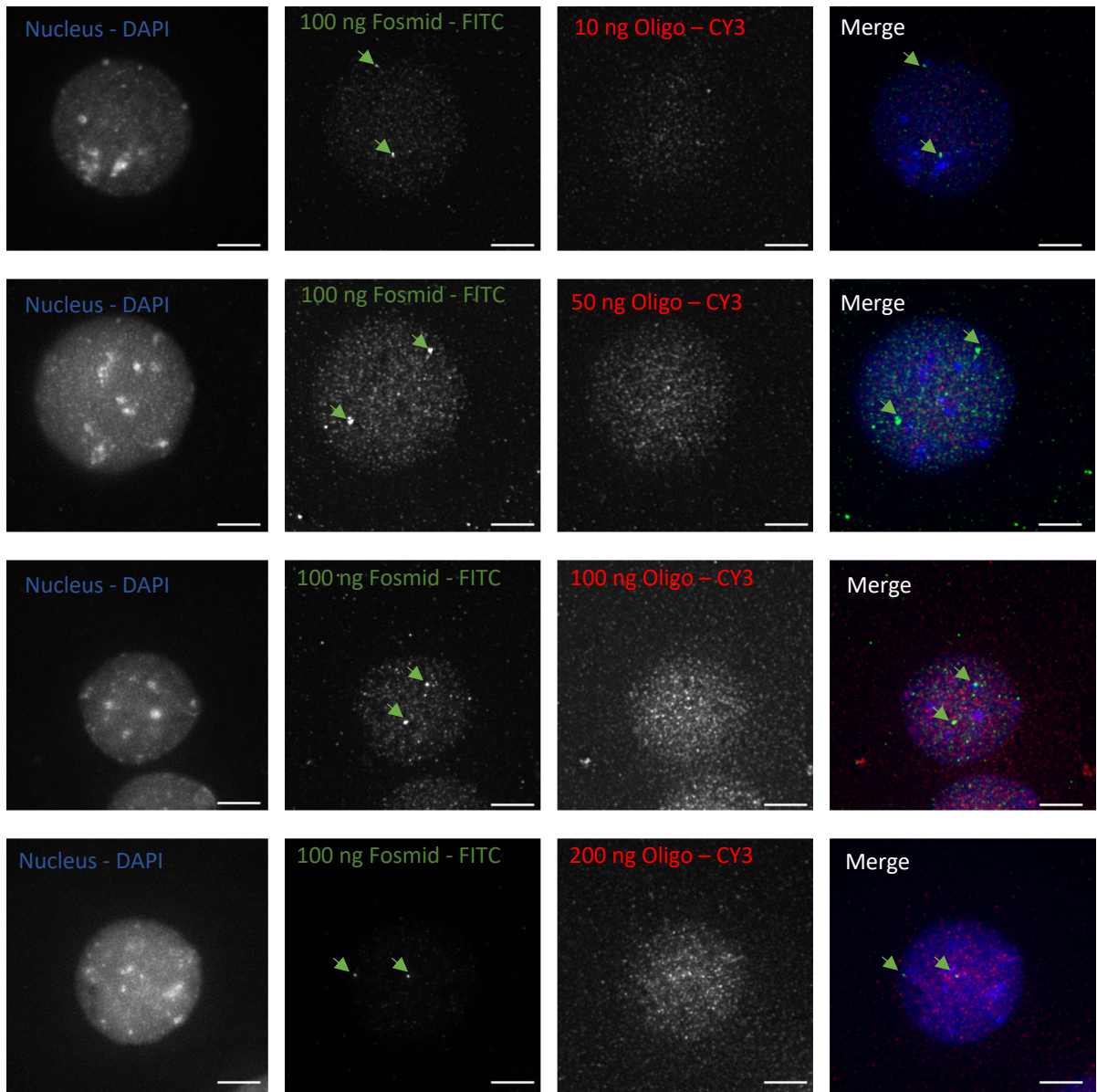


Figure 6.3: Optimisation of FISH oligos and probes in LCLs

100x Fluorescent images testing Fosmid detection at 100 ng application with double antibody detection and Oligo detection at 10 ng-200 ng application single layer antibody detection. Green arrows label fosmid spots. Scale bars – 5 μ m

6.4 2D DNA FISH enables detection of both expanded and WT *C9ORF72* alleles in patient LCLs

Building from the initial test round in which the oligo probe was undetectable and the fosmid probe produced higher than expected background several optimisation steps were performed.

The fosmid is visualised using a dual antibody detection method as we have previously described (Silva *et al.*, 2015). The biotin labelled fosmid fragments are detected with a sheep-anti-DIG-FITC antibody which is then labelled again with Rabbit-anti-Sheep-FITC. This dual antibody approach increases the signal but can also result in higher background staining due to unspecific binding. Due to the high background in the first trial, it was tested whether using one layer of antibody would be sufficient to detect the fosmid spots. Figure 6.4A-B shows that this approach was very successful as the fosmid spots could be detected by fluorescence microscopy.

Initial trials using the 5 repeat (5'-biotin-GGCCCCGGCCCCGGCCCCGGCCCCGGCCCC) probe were unsuccessful. Bioinformatic analysis using OligoCalc (Kibbe, 2007) indicates that this probe has the potential to form up to 14 internal hairpins. This secondary structure could limit the binding ability of the probe and explain the lack of signal detection. To address this issue, the length of the probe was reduced to 4.5 repeats (GGCCCCGGCCCCGGCCCCGGC) and 5-prime Alexa 555 labelled. Reducing the length of the probe allowed the formation of fewer hairpins (2 predicted). Directly labelling the probe also has the advantage of reducing the background as no additional antibody is

required. Finally, although using directly labelled probes can reduce the signal due to the lack of antibody amplification using the brighter Alexa 555 in place of CY3 was hoped to circumvent this issue (Berlier *et al.*, 2003).

In addition to improving the probes used for FISH analysis, the microscope used to capture the images was reconsidered. Originally captured at 100x magnification on a standard fluorescent widefield microscope (API DeltaVision), following images were captured using a 63 x objective on spinning disk confocal (Opera Phenix) as this provides a better resolution and reduces background fluorescence by removing 'out of focus' light from the imaging plane.

These changes resulted in successful FISH labelled nuclei for analysis (Figure 6.4A-B). As required, two fosmids were observed in nuclei with one co-localised to an oligo spot to label the expanded *C9ORF72* allele.

These nuclei were analysed automatically using the software CellProfiler to allow unbiased fosmid or oligo selection and measurement. The pipeline designed here is able to segment LCL nuclei then further identify fosmid and oligo spots located within each nucleus. Nuclei without the required two fosmid spots with one colocalised oligo spot are excluded from further analysis by the program. Finally, CellProfiler was used to measure the distance from the centre of the WT fosmid (centroid) to the nuclear centroid and to the nuclear periphery. The same measurements were performed for the oligo spot.

Once these distances are output from CellProfiler they can be further processed and the method of normalisation selected. The minimum distance can be expressed as a raw value converted into μm without normalisation (Figure 6.4C) or can be normalised in one of two methods (Figure 6.4D-F). Both methods express the distance from the probe

centroid to the periphery as a proportion of the total distance from nuclear centroid through probe to nuclear periphery (Figure 6.4C-F). The key difference is how the total distance is measured, this can be achieved by drawing a line straight line from the nuclear centroid through the probe to the periphery. This however, may not produce the minimum distance from probe to periphery if the nucleus is not a perfect sphere (Figure 6.4D-F). Therefore, this study opts to calculate the total by addition of nuclear centroid to probe centroid (1) and probe centroid minimum distance to periphery (2) (Figure 6.4F)

.

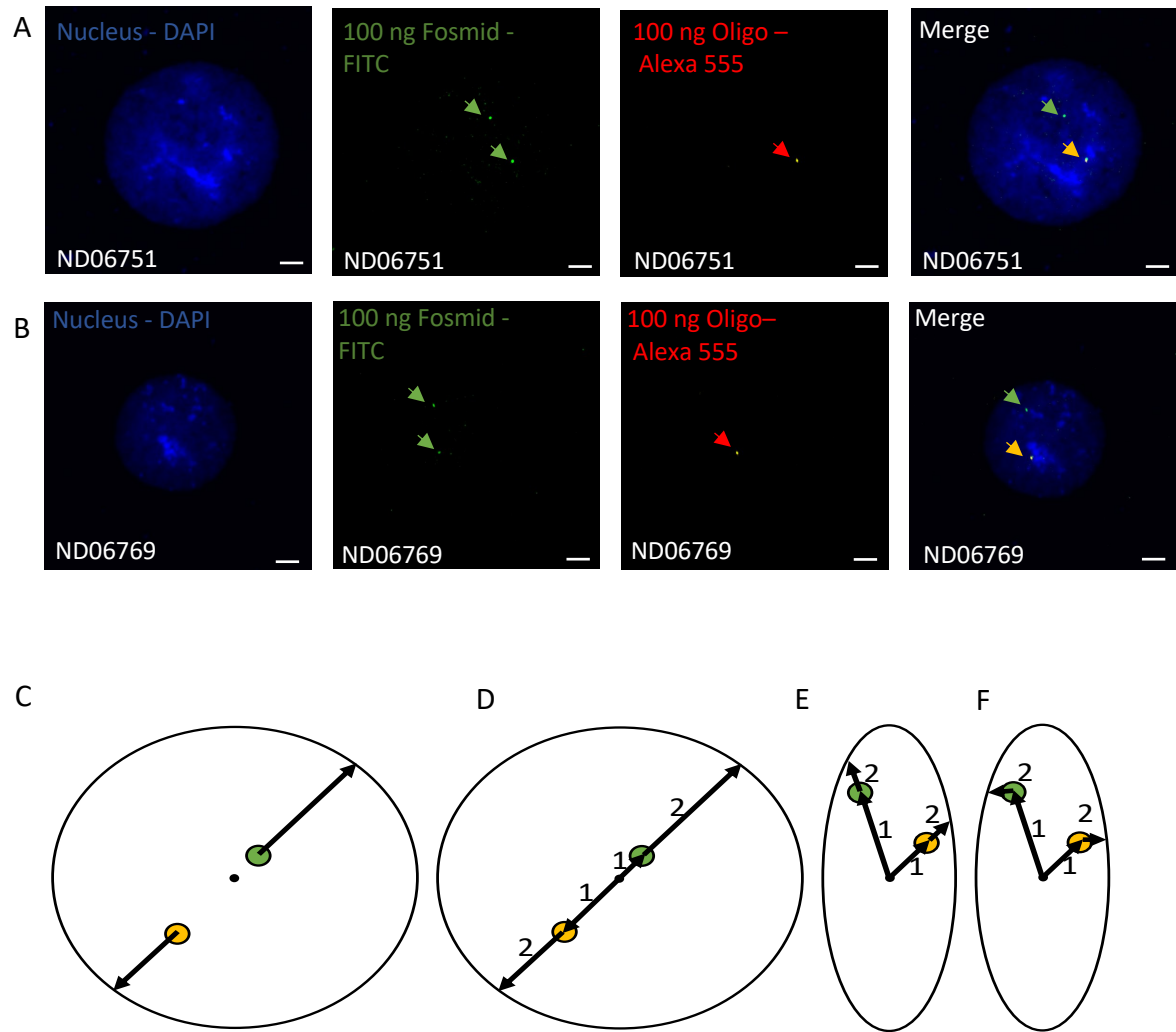


Figure 6.4: *C9ORF72* expression in LCLs using FISH and qPCR

A+B) 63 x FISH images captured using a spinning disk confocal. Fosmids were detected using single layer FITC antibody detection with 100 ng fosmid application. Oligos used here contained 4.5 repeats and were directly labelled with Alexa 555 and applied at 100 ng. Green arrows indicate fosmid spots, red arrows indicate oligo spots and yellow arrows indicate colocalised fosmid and oligo spots. Scale bars- 5 μ m

C-F) Diagram of analysis of FISH spots. Yellow and green spots represent Oligos and Fosmids respectively. Black dot represents the centre of nucleus. C) Shows un-normalised measurement of spot centroid to nuclear periphery. D) Indicates normalised measurement of least distance on round nucleus. Measurements 1 and 2 are added then measurement 2 is expressed as a proportion of (1+2). E) Shows normalisation using the straight-line method on a noncircular nucleus. F) Shows the normalisation method used here as discussed for 'D' on a non-circular nucleus.

6.5 Expanded *C9ORF72* shows a Heterogenous Pattern of Expression in LCLs

Following the optimisation of the CellProfiler pipeline on several test images, the pipeline was applied to image sets from both patient LCL lines (ND06769 and ND06751). Both raw distances from fosmid/oligo spot centroid to periphery (converted to μm) and normalised distances were analysed for both cell lines. A control cell line was not required due to the heterozygous nature of *C9ORF72* ALS, LCLs contain their own internal control as one allele is expanded and one WT.

LCL line ND06751 did not show any difference in both raw distance (Figure 6.5A) and normalised distance (Figure 6.5B) to nuclear periphery between expanded and WT *C9ORF72* alleles. However, ND06769 did show a significant difference in both the raw (Wilcoxon matched pairs: Expanded 2.782 ± 0.154 vs WT 3.127 ± 0.1549 $p= 0.0472$, N= 196) (Figure 6.5C) and normalised distances to the nuclear periphery (Wilcoxon matched pairs: Expanded 0.3167 ± 0.016 vs WT 0.3702 ± 0.017 $p= 0.0163$, N= 196) (Figure 6.5D). Indicating the expanded allele in ND06769 is significantly closer to the nuclear periphery than the WT allele.

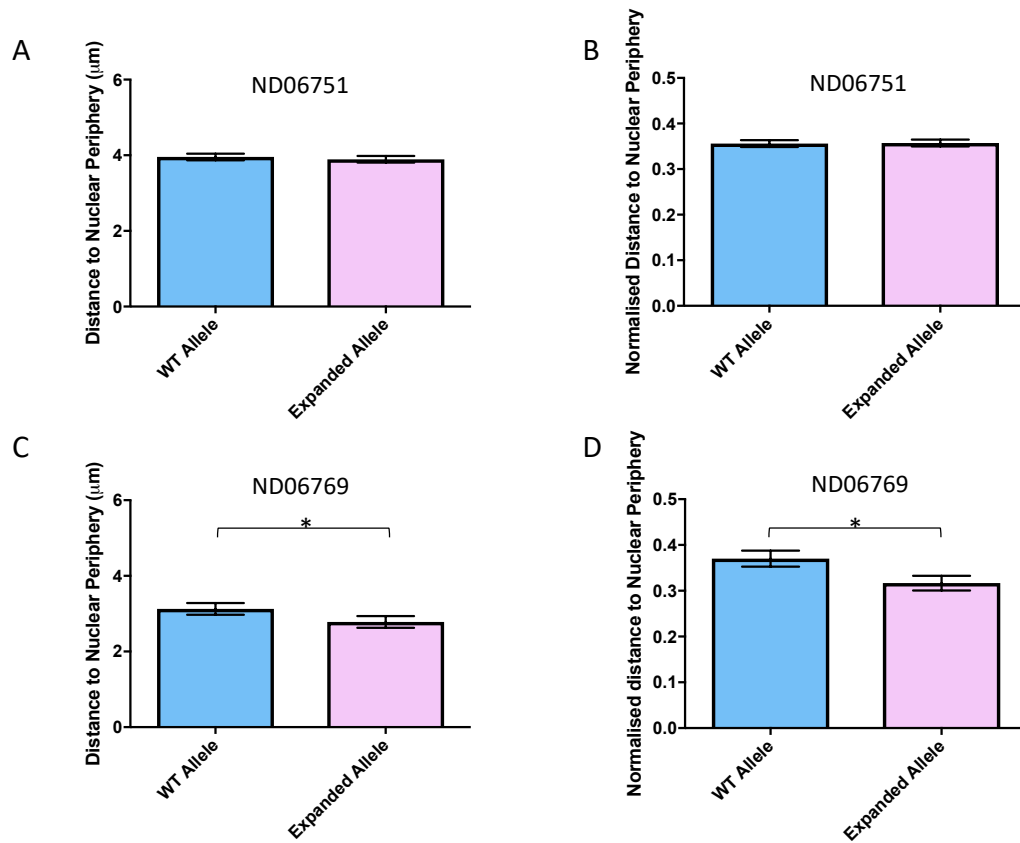


Figure 6.5: *C9ORF72* expression in LCLs using 2D FISH and qPCR

A-D) Data obtained from CellProfiler analysis using optimised pipeline for LCL line ND06769 (N=196 cells) and ND06751 (N=967 cells) (mean±SEM). Stats used Wilcoxon Matched-Pairs Signed Rank Test.

A+C) Show raw μm measurements from fosmid (WT allele) and Oligo (expanded allele) to nuclear periphery. B+D) Show normalised data using the least distance method.

The RT-qPCR analysis required the use of a control line (ND07612) to set a baseline for 'normal' expression, as this technique (used here with SYBR green) does not distinguish expression from the expanded and WT alleles, meaning the *C9ORF72* total (C-total) and variant expression is measured from both alleles together. Using the Livak ($\Delta\Delta CT$) method (Livak and Schmittgen, 2001) for analysis normalising to both a housekeeper gene (Beta-2-microglobulin (B2M)) and the control line (ND07612). No significant difference in mRNA expression was observed for total *C9ORF72* mRNA expression as well as variants 1 and 2 for patient ND06751 compared to control ND07612 (Figure 6.6A-C). Variant 3 (C3) however, showed a significant decrease in expression level compared to control line ND07612 (ND06752 0.8585 ± 0.02353 vs Control 1, One-way ANOVA with Tukey's test $p = 0.0215$ N=3 repeat samples)(Figure 6.6D).

ND06769 showed no significant difference in total *C9ORF72* mRNA expression compared to control line ND07612, however, significantly increased expression compared to the control line was seen for variant 1 (C1) (ND06769 5.206 ± 0.8937 vs Control 1, One-way ANOVA with Tukey's test $p = 0.003$, N=3 repeat samples) and variant 2 (C2) (ND06769 5.6769 ± 1.356 vs Control 1, One-way ANOVA with Tukey's test $p = 0.0135$, N=3 repeat samples) with a decrease seen for variant 3 (C3) (ND06769 0.5137 ± 0.03933 vs Control 1, One-way ANOVA with Tukey's test $p = <0.0001$, N=3 repeat samples)(Figure 6.6 A-D).

Interestingly, C1, C2 and C3 expression was also significantly different between both patient lines (C1: ND06769 5.206 ± 0.8937 vs ND06751 1.528 ± 0.09737 , One-way ANOVA with Tukey's test $p = 0.0058$, N=3, C2: ND06769 5.679 ± 1.356 vs ND06751 1.836 ± 0.1644 , One-way ANOVA with Tukey's test $p = 0.0317$, N=3 repeat samples, C3: ND06769 0.5137 ± 0.03933 vs ND06751 0.8585 ± 0.02353 , One-way ANOVA with Tukey's test $p = 0.0002$ N=3 repeat samples).

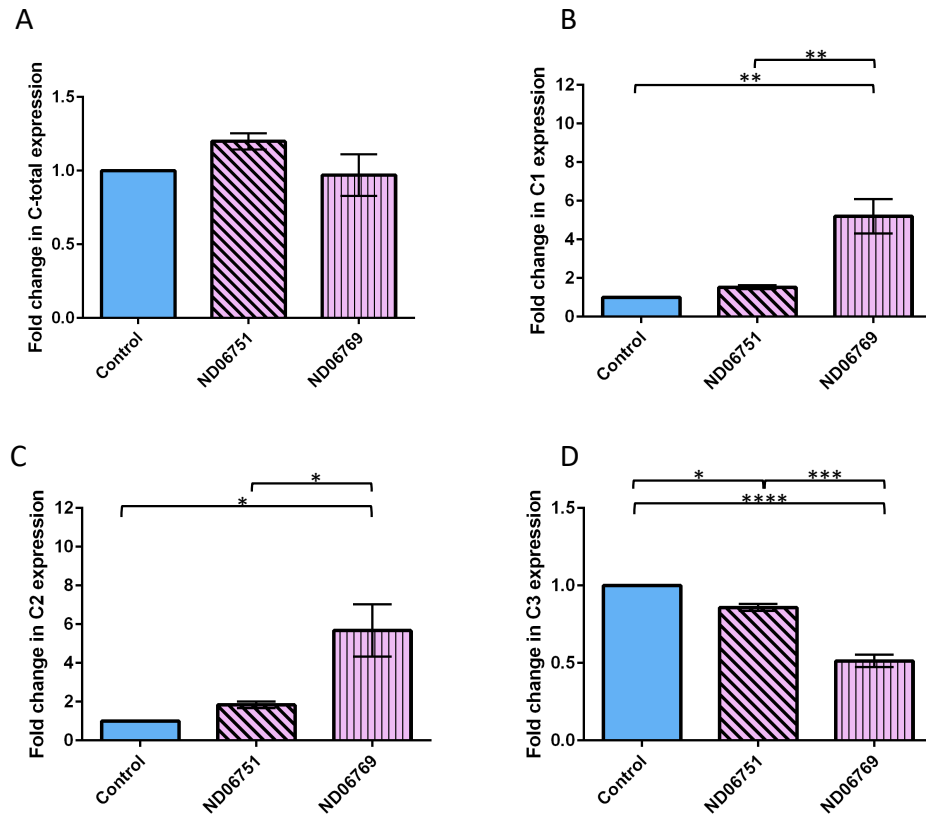


Figure 6.6: *C9ORF72* expression in LCLs using 2D FISH and qPCR

A-D) SYBR green reagent was used to run the RT-qPCRS here and were analysed using the Livak method of analysis (mean $\pm$ SEM). Samples obtained from 3 batches of cells for each patient and control.

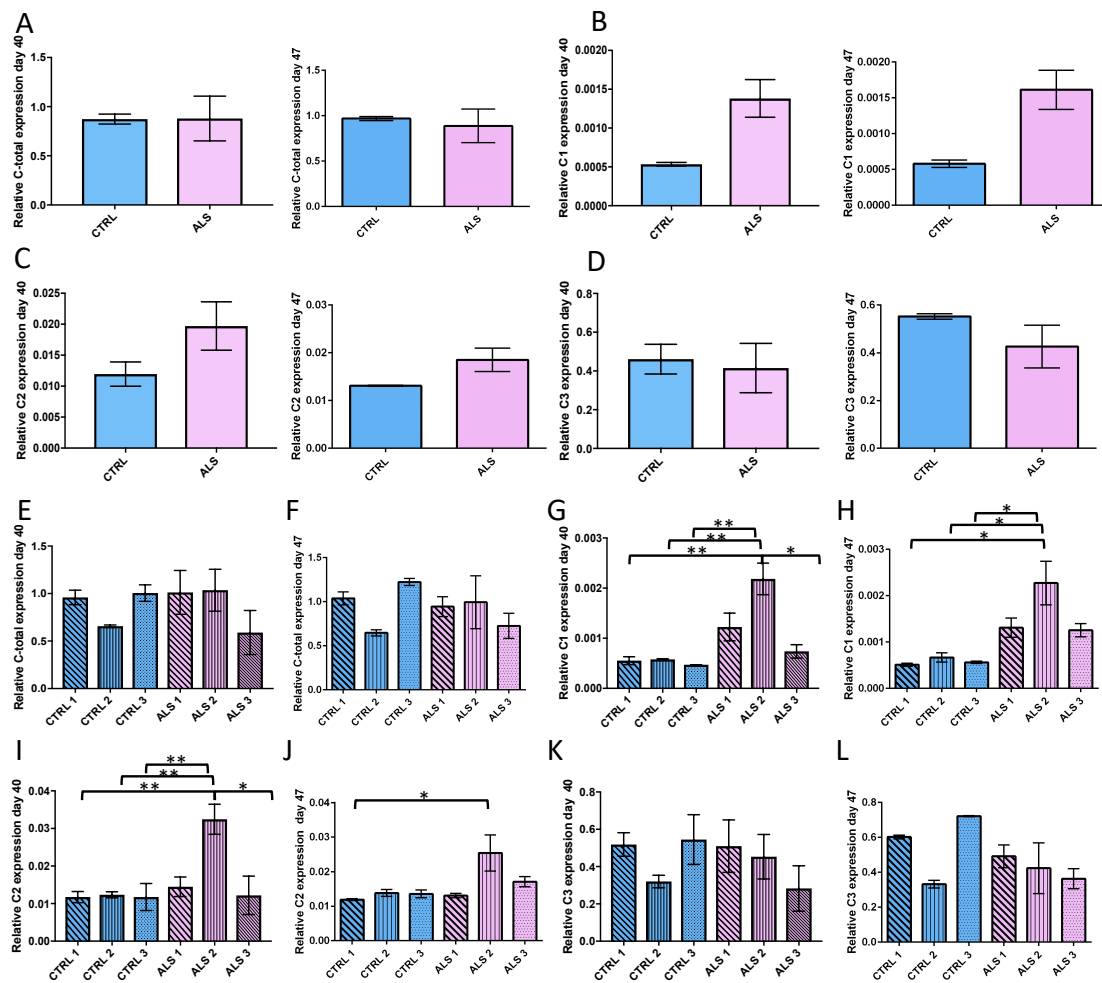
Stats used One-Way ANOVA with Tukey's multiple comparisons test.

6.6 Motor Neurons Show Heterogeneous *C9ORF72*

Variant Expression change

Extending this work to patient and control iPSC derived motor neurons allows for the study of *C9ORF72* expression in a more disease relevant context. 2D FISH analysis was not possible on these cells due to the high cellular density of these cultures. For FISH the nuclear periphery needs to be easily identifiable and distinguishable from neighbouring cells to allow accurate measurement of the distance of the fosmid/oligos to periphery accurately. Additionally, as the 2D FISH technique solely preserves the nuclei and no surrounding cytoplasm, identification of motor neurons amongst the mixed cell population would be difficult without an additional motor neuron specific DNA probe. However, RT-qPCR analysis of the expression levels of C9 variants within these cells is possible. Three patients and three controls were used in this study, analysed using the Δ CT method (a variation of the Livak method) to allow for differences in control variation to be viewed (normalised to Hypoxanthine Phosphoribosyltransferase housekeeper gene (HPRT)). Pooled data for controls and patients shows there is no difference in C-total expression, C1, C2 or C3 expression for either day 40 or day 47 cells, although a trend to increased C1 and C2 expression can be observed for patients (Figure 6.7A-D). When analysed separately against the controls, patients show no difference in C-total or C3 for either day 41 or 47. However, ALS 2 showed a significant increase in expression compared to all three controls for C1 expression at both time points (Day 40 One-way ANOVA, $p=0.0036$, with posthoc Tukey's, CTRL 1 0.0005 ± 0.0000795 vs ALS 2

0.002184 ± 0.000316 $p=0.0057$, CTRL 2 0.00058 ± 0.00001 vs ALS 2 0.002184 ± 0.000316
 $p= 0.0062$, CTRL 3 0.00047 ± 0.0000015 vs ALS 2 0.002184 ± 0.000316 $p= 0.0044$, N=2,
 One-way ANOVA Day 47 $p=0.0089$ with posthoc Tukey's CTRL 1 0.00051 ± 0.000033 vs
 ALS 2 0.0023 ± 0.0005 $p=0.0103$, CTRL 2 0.00067 ± 0.0001 vs ALS 2 0.0023 ± 0.0005 $p=$
 0.0163 , CTRL 3 0.00056 ± 0.000025 vs ALS 2 0.0023 ± 0.0005 $p= 0.0120$ N=2
 differentiations) (Figure 6.6K-L). At day 40 ALS 2 also showed a significant increase in C1
 expression compared to ALS 3 (ALS 2 0.00218 ± 0.000316 vs ALS 3 0.00074 ± 0.00013 ,
 $p=0.0104$, N=2). ALS 2 C2 expression at day 40 was also significantly increased compared
 to all three controls (Day 40 One-way ANOVA, $p=0.00226$, with posthoc Tukey's, CTRL 1
 0.01174 ± 0.00148 vs ALS 2 0.03244 ± 0.004 $p=0.0303$, CTRL 2 0.0123 ± 0.000789 vs ALS
 2 0.03244 ± 0.004 $p= 0.0347$, CTRL 3 0.0117 ± 0.0036 vs ALS 2 0.03244 ± 0.004 $p=$
 0.0303 , N=2). Additionally, AL2 was significantly increase compared to ALS 3 (ALS 2
 0.03244 ± 0.004 vs ALS 3 0.0122 ± 0.0051 $p=0.336$ N=2 differentiations) (Figure 6.7I).
 However, at day 47 only CTRL 1 was significantly decreased compared to ALS 2 (One-way
 ANOVA $p= 0.0435$ with posthoc Tukey's CTRL 1 0.0119 ± 0.0029 vs ALS 2 $0.02543 \pm$
 0.005232 , $p= 0.0430$, N=2 differentiations) (Figure 6.7J). Reasons for this are explored in
 the end of chapter discussion.



6.7 Discussion

The overall aim of this chapter was to explore the haploinsufficiency hypothesis using the novel approach of fluorescent in situ hybridisation, combined with RT-qPCR to probe the expression levels of the different *C9ORF72* mRNA variants.

There are three mechanisms hypothesised as to why the *C9ORF72* gene expansion causes disease including, haploinsufficiency (Shi *et al.*, 2018), toxic RNA (Donnelly *et al.*, 2013; Sareen *et al.*, 2013) and toxic protein (K. Mori *et al.*, 2013; Kwon *et al.*, 2014; Wen *et al.*, 2014). Therapeutic efforts have predominantly focused on RNA foci, RNA/DNA G-quadruplexes and dipeptide repeat proteins, however, as discussed in the general introduction chapter 1.3.1 it appears these toxic species alone do not cause *C9ORF72* gene expansion ALS. Therefore, targeting these species may not fully halt motor neuron degeneration.

Fluorescent in situ hybridisation is a novel approach to visually explore the haploinsufficiency hypothesis. Association of an allele to the nuclear periphery or nuclear lamina is thought to correlate with a reduced transcriptional output of that gene (Dillon, 2008). Many in vivo examples support the correlation between gene position and transcriptional state, one example includes the two alleles of the monoallelically expressed GFAP. A more peripheral positioning of the inactive allele is reported compared to the active allele (Takizawa *et al.*, 2008). However, whether transcription level is due to nuclear positioning or positioning is due to transcription level is still an area of debate.

Our results using FISH to visualise the position of both the expanded and WT *C9ORF72* alleles in two patient LCL lines suggested a more peripheral position of the expanded *C9ORF72* gene allele towards the periphery of the nucleus in patient ND06769 when compared to the WT allele. However, the second patient ND06751 showed no difference in nuclear positioning of the expanded or WT alleles.

Since nuclear position does not categorically implicate reduced gene expression (Takizawa, Meaburn and Misteli, 2008), the mRNA expression of *C9ORF72* was analysed by RT-qPCR to assess levels of transcription of total *C9ORF72* and the three variants. No difference in total *C9ORF72* expression was observed for either patient, however, patient ND06769 showed a significant increase in expression of both transcript variants 1 and 2 and reduced expression in transcript variant 3. Considering variant 1 is lowly expressed, this increase may have limited biological effects, however, variant 2 and 3 have relatively high expression. Variant 2 encodes the short form of the *C9ORF72* protein and variant 3 the long form. Interestingly patient ND06769 also showed the nuclear periphery positioning of the expanded *C9ORF72* allele. This data validates the differences observed in the patient FISH images and suggests abnormal transcriptional regulation at the expanded *C9ORF72* locus.

As motor neurons are the cell type at the centre of the pathophysiological mechanisms of ALS, the levels of transcription of total *C9ORF72* and the three variants were assessed by RT-qPCR in iPSC-derived motor neurons. Overall the same expression pattern was observed as for patient ND06769 LCLs – although in this case the differences did not reach statistical significance when all patients were analysed together. However, when considering iPSC-derived lines individually, it appears that there is considerable variation

between the lines. For example, although ALS 2 did show a significant increase in variant 1 and 2 compared to controls, the levels in the other patients were unchanged.

As with the LCL data it appears the expression level of *C9ORF72* gene expansion is highly variable among patients. This is also reflected in the literature where several studies report conflicting expression levels (DeJesus-Hernandez *et al.*, 2011; Almeida *et al.*, 2013; Rizzu *et al.*, 2016; McGoldrick *et al.*, 2018). It would be interesting to assess the repeat length of these patients to assess whether it correlates with *C9ORF72* mRNA expression.

One of the limitations when interpreting the RT-qPCR results is the inability to distinguish between the expression arising from the expanded and non-expanded alleles. It is not known whether the increase or decrease in variants is due to the hexanucleotide expansion itself or to a compensatory mechanism from the WT allele. However, as the function of the *C9ORF72* gene is still unknown, what functional impact these differences in transcript variant expression may have is unknown.

Overall the successful application of 2D DNA FISH on LCLs shows that the probes are effective in discriminating both the WT and expanded alleles. Building from previous studies from the Wade-Martins Laboratory using 3D FISH to study Friedreich's ataxia (Silva *et al.*, 2015), it would be of interest to use this technique here. 3D FISH maintains the structure of the nuclei and allows for combined immunofluorescence to visualise the nuclear lamina. Direct visualisation of the nuclear lamina will allow for co-localisation studies to assess whether the expanded allele is in contact with the lamina. Additionally, this technique could be adapted for use on iPSC-derived motor neurons. 2D FISH at present is not appropriate for mixed cultures as it does discriminate motor neurons from

other cell types as only the nucleus is maintained during processing. Whereas 3D FISH maintains the entire cell, so ICC could be used to label cytoplasmic motor neuron markers.

In light of the recent studies suggesting a larger role for haploinsufficiency (Shi *et al.*, 2018) as a cause for *C9ORF72* gene expansion, the need for drug screening assays to reverse this phenotype becomes more prominent. Optimising the FISH protocol to study iPSC-derived motor neurons in a 96-well plate format could allow for medium throughput drug screening. Re-localisation of the allele back to the inner nucleus would be the positive indicator of an effective drug

Chapter 7:

General Discussion

7.1 Overall

Much is still unknown in regards to the C9ORF72 protein's biological function and C9ORF72 gene expansion disease mechanism. This lack of understanding compared to other forms of ALS such as SOD1, is partly justified by the relatively recent discovery only in 2011 (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011) but emphasises the need for further research.

This project aimed to explore the C9ORF72 gene expansion from two approaches. Firstly, the downstream dysfunction caused by the expansion in neuronal excitability and calcium deficits. Secondly, looking at the gene itself using fluorescent in situ hybridisation (FISH) to analyse whether there is evidence to suggest haploinsufficiency as a disease mechanism.

7.2 The reduced PF protocol produced cells suitable for electrophysiology

A crucial element to the project was the selection of the iPSC-derived motor neuron maturation protocol. After comparison of the (Qu *et al.*, 2014) and the (Du *et al.*, 2015) protocols, it was found that the Du protocol produced neurons more conducive to electrophysiological assessment. The cultures did not form a dense monolayer, produced >30% motor neurons and expressed clear motor neuron markers including HB9, SMI-32 and ISL1. Four adaptations to the original protocol, 'Standard', 'Reduced PF', 'DAPT' and

'Synaptojuice' were trialled to assess whether these conditions could increase the maturation of the neurons for physiological assessments. Overall the Reduced PF protocol produced motor neurons with an acceptable level of maturity confirmed by both protein and mRNA expression of mature motor neuron markers (HB9, ChAT, ISL1 and SMI-32) and production of evoked action potentials using whole-cell patch clamp electrophysiology.

Although the Synaptojuice protocol was not selected for further use, this protocol could be of interest for future optimisation and electrophysiology studies. This protocol produced a monolayer of cells with large numbers of processes. Using cultures with few aggregations is of great benefit for electrophysiology as it allows better visualisation of the neuronal morphology to be patched. This protocol was published showing high action potential firing with Synaptojuice treatment (Telezhkin *et al.*, 2016). It would be interesting to see if the high level of activity recapitulated in motor neurons, as this protocol is optimised for medium spiny neurons (Telezhkin *et al.*, 2016).

7.3 Patient cells show hyperexcitability and calcium phenotypes

Three patient and control lines were differentiated using the reduced PF protocol and maintained for 40 to 47 days in vitro. Characterisation of the lines indicated that all six differentiated similarly in terms of mature motor neuron marker expression determined by ICC and RT-qPCR. However, a significant increase in SMI-32 protein percentage was observed at day 40 compared to day 47 in patients vs controls. Intrinsic neuronal properties such as cell capacitance, membrane resistance and resting membrane potential all showed no difference between patients and controls or over time, indicating a similar maturity across the groups.

Analysing both spontaneous action potential firing and evoked firing showed a trend (with significance for evoked action potentials at day 40) towards hyperexcitability. This supports previous findings which show hyperexcitability in iPSC-derived motor neurons from *C9ORF72* gene expansion patients (Wainger *et al.*, 2014; Devlin *et al.*, 2015).

However, no difference was observed in either voltage-gated sodium channel or voltage-gated-potassium channel current at day 40 implying that a different mechanism is responsible for this increased excitability. As described in the discussion chapter 4, this increase in excitability could be due to a combination of excitotoxic glutamate overstimulation causing increased patient spontaneous action potential activity and other intrinsic mechanisms such as increased voltage-gated calcium channel opening increasing patient evoked action potentials.

Additionally, calcium phenotypes were observed at day 40 and 47. Increased calcium release from all internal stores via ionomycin in patients was reported at day 40 which diminished at day 47. Whereas, at day 47 patient cells responded differently over time to FCCP compared to controls. This chapter overall lends support to the hypothesis made by previous studies that changes in excitability lead to a toxic shift of calcium from the ER to the mitochondria (Berridge, 2002; Grosskreutz *et al.*, 2007; Grosskreutz, Van Den Bosch and Keller, 2010).

Overall there seems to be a theme between excitotoxicity and calcium dysfunction linked to hyperexcitability.

This study and previous studies also highlight the importance of thorough characterisation of the final culture. The percentage of motor neurons present can have a great impact on the phenotype observed as evidenced by (Selvaraj *et al.*, 2018) in which pure neuronal cultures did not display the hyperexcitability previously reported in mixed neural cultures (Devlin *et al.*, 2015). For future studies it may be of interest to form more defined co-culture studies with controlled percentages of neurons and other iPSC patient-derived cell types such as astrocytes, interneurons or muscle cells to allow for more reproducible studies and phenotype observations.

7.4 Dual promoter and Cre/Lox lentiviral constructs are successful in motor neuron differentiations

In parallel to the optimisation of an iPSC-derived motor neuron protocol and preliminary patient vs control studies, a dual optogenetic-reporter lentiviral system was designed and cloned. This virus is particularly useful of electrophysiological studies both for the inclusion of optogenetics to allow manipulation of the activation of neurons but also the fluorescent reporter.

Typically for electrophysiology, cells are patched and assumed to be the cell type of interest from analysis of intrinsic properties (e.g. cell size and activity), morphology or by filling the patched cell with biocytin and posthoc staining. Without the use of post-hoc staining, which can be time consuming, this can lead to the inclusion of non-cell types of interest in the analysis, which could affect the overall phenotypes observed. Post-hoc staining circumvents this issue but adds an extra time element to the experiment, as the cells have to be stained after patching. A fluorescent reporter negates these issues and allows direct patching of the cell type of interest.

The successful F2 version of this construct was designed to improve on the unsuccessful F1 version in which the mouse *Hb9* promoter was directly driving the optogenetic reporter. Both CTRL 1 in the maturation chapter 3 and patient vs control study Chapter 4 emphasised the need for the Cre/Lox system of *HB9* control as RT-qPCR results from both showed a decrease in human *HB9* expression towards the end phases of the

protocol as the cells mature. Therefore, the F2 version was designed so that even transient *HB9* expression would result in long lasting ChR2-mCherry expression. The construct design and generation has proved successful but requires minor alterations to the fluorescent reporter for full functionality in live cells, as it currently requires antibody detection. Further validation of its specific expression in mature motor neurons is also required.

7.5 Fluorescent in situ hybridisation highlights the heterogeneous expression levels of *C9ORF72* in patients

Finally, the disease mechanism of haploinsufficiency was explored. Overall the successful application of the 2D DNA FISH technique on LCLs demonstrates that the probes are effective in targeting the both the WT and expanded alleles. Results from chapter 6 show a correlation between DNA FISH and differential *C9ORF72* mRNA variant expression. LCL patient line ND06769 showed both significantly peripheral localisation towards to nuclear lamina and also dysregulation of mRNA expression levels compared to the control line. Patient ND06751 showed no difference in allele localisation or mRNA expression levels for total *C9ORF72*, variant C1 and C2. However, it did show a decrease in C3 expression but not as reduced as ND06769 (patients showed significantly different expression). This suggests that transcriptional regulation at the *C9ORF72* locus is likely modulated by nuclear localisation and that this can be affected by the hexanucleotide

expansion. However, this would need to be confirmed in samples from additional expansion carriers.

In light of the recent studies suggesting a larger role for haploinsufficiency (Shi *et al.*, 2018) as the pathophysiological mechanism triggered by *C9ORF72* gene expansion, the need for drug testing assays to reverse this phenotype become more prominent.

7.6 Concluding Remarks

The disease mechanism caused by the *C9ORF72* gene expansion in ALS patients is complicated. This thesis aimed to explore both the expression level of the gene itself and the downstream implications of mutant *C9ORF72* at the synapse, as well as generate a novel optogenetic/reporter lentivirus for future electrophysiological studies.

Overall, this study using the optimised version of the Du et al iPSC-derived motor neuron protocol, reduced PF, showed a trend towards increased spontaneous action potential firing in patients as well as a significant increase in patient evoked action potentials, in parallel with internal calcium storage dysfunction.

Interestingly a recent study has linked calcium permeable AMPAR mediated excitotoxicity as previously discussed to haploinsufficiency. They found reduced *C9ORF72* protein led to an increase in calcium permeable GluR1 (Shi *et al.*, 2018).

This study determined *C9ORF72* gene expression using both FISH and RT-qPCR and found a correlation between the two techniques as well as heterogenous expression levels of *C9ORF72* mRNA variants among patients. With increasing evidence of haploinsufficiency

linked with excitotoxicity, its importance as a mechanism to drug target is increased. The FISH technique itself could form an interesting future drug screening technique.

References

- Abe, K. *et al.* (1997) 'Cognitive function in amyotrophic lateral sclerosis', *Journal of the Neurological Sciences*. Elsevier, 148(1), pp. 95–100. doi: 10.1016/S0022-510X(96)05338-5.
- Abrahams, S. *et al.* (1997) 'Relation between cognitive dysfunction and pseudobulbar palsy in amyotrophic lateral sclerosis', *Neurosurgery, and Psychiatry*, 62, pp. 464–472. doi: 10.1136/jnnp.62.5.464.
- Abrahams, S. *et al.* (2000) 'Verbal fluency and executive dysfunction in amyotrophic lateral sclerosis (ALS)', *Neuropsychologia*. Pergamon, 38(6), pp. 734–747. doi: 10.1016/S0028-3932(99)00146-3.
- Abrahams, S. *et al.* (2005) 'Frontotemporal white matter changes in amyotrophic lateral sclerosis', *Journal of Neurology*. Steinkopff-Verlag, 252(3), pp. 321–331. doi: 10.1007/s00415-005-0646-x.
- Abremski, K. and Hoess, R. (1984) 'Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein.', *The Journal of biological chemistry*, 259(3), pp. 1509–14. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6319400> (Accessed: 6 September 2018).
- Del Aguila, M. A. *et al.* (2003) *Prognosis in amyotrophic lateral sclerosis A population-based study*. Available at: www.neurology.org (Accessed: 27 July 2018).
- Al-Chalabi, A. *et al.* (2010) 'An estimate of amyotrophic lateral sclerosis heritability using twin data', *Journal of Neurology, Neurosurgery & Psychiatry*, 81(12), pp. 1324–1326. doi: 10.1136/jnnp.2010.207464.
- Al-Mahdawi, S. *et al.* (2007) 'The Friedreich ataxia GAA repeat expansion mutation induces comparable epigenetic changes in human and transgenic mouse brain and heart tissues', *Human Molecular Genetics*, 17(5), pp. 735–746. doi: 10.1093/hmg/ddm346.
- Almeida, S. *et al.* (2013) 'Modeling key pathological features of frontotemporal dementia with C9ORF72 repeat expansion in iPSC-derived human neurons', *Acta Neuropathologica*. Springer Berlin Heidelberg, 126(3), pp. 385–399. doi: 10.1007/s00401-013-1149-y.
- Altshuler, D., Daly, M. J. and Lander, E. S. (2008) 'Genetic mapping in human disease.', *Science (New York, N.Y.)*. NIH Public Access, 322(5903), pp. 881–8. doi: 10.1126/science.1156409.
- Amin, N. D. *et al.* (2015) 'Loss of motoneuron-specific microRNA-218 causes systemic

neuromuscular failure', *Science*, 350(6267), pp. 1525–1529. doi: 10.1126/science.aad2509.

Amoroso, M. W. *et al.* (2013) 'Accelerated High-Yield Generation of Limb-Innervating Motor Neurons from Human Stem Cells', *Journal of Neuroscience*, 33(2), pp. 574–586. doi: 10.1523/JNEUROSCI.0906-12.2013.

Arai, T. *et al.* (2006) 'TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis', *Biochemical and Biophysical Research Communications*, 351(3), pp. 602–611. doi: 10.1016/j.bbrc.2006.10.093.

Arber, S. *et al.* (1999) 'Requirement for the Homeobox Gene Hb9 in the Consolidation of Motor Neuron Identity', *Neuron*, 23(4), pp. 659–674. doi: 10.1016/S0896-6273(01)80026-X.

Ash, P. E. A. *et al.* (2013) 'Unconventional Translation of C9ORF72 GGGGCC Expansion Generates Insoluble Polypeptides Specific to c9FTD/ALS', *Neuron*, 77(4), pp. 639–646. doi: 10.1016/j.neuron.2013.02.004.

Atanasio, A. *et al.* (2016) 'C9orf72 ablation causes immune dysregulation characterized by leukocyte expansion, autoantibody production and glomerulonephropathy in mice', *Scientific Reports*. Nature Publishing Group, 6(1), p. 23204. doi: 10.1038/srep23204.

Ausubel, L. J. *et al.* (2012) 'Production of CGMP-Grade Lentiviral Vectors.', *BioProcess international*. NIH Public Access, 10(2), pp. 32–43. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22707919> (Accessed: 3 September 2018).

Bai, Q. *et al.* (2015) 'Temporal analysis of genome alterations induced by single-cell passaging in human embryonic stem cells.', *Stem cells and development*. Mary Ann Liebert, Inc., 24(5), pp. 653–62. doi: 10.1089/scd.2014.0292.

Bannwarth, S. *et al.* (2014) 'A mitochondrial origin for frontotemporal dementia and amyotrophic lateral sclerosis through CHCHD10 involvement', *Brain*. Oxford University Press, 137(8), pp. 2329–2345. doi: 10.1093/brain/awu138.

Bardy, C. *et al.* (2015) 'Neuronal medium that supports basic synaptic functions and activity of human neurons in vitro.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 112(20), pp. E2725–34. doi: 10.1073/pnas.1504393112.

Barson, F. . *et al.* (2000) 'A neuropsychological investigation of dementia in motor neurone disease (MND)', *Journal of the Neurological Sciences*. Elsevier, 180(1–2), pp. 107–113. doi: 10.1016/S0022-510X(00)00413-5.

Bauman, J. *et al.* (1980) 'A new method for fluorescence microscopical localization of specific DNA sequences by in situ hybridization of fluorochrome-labelled RNA', *Experimental Cell Research*. Academic Press, 128(2), pp. 485–490. doi: 10.1016/0014-4827(80)90087-7.

- Belzil, V. V. *et al.* (2013) 'Reduced C9orf72 gene expression in c9FTD/ALS is caused by histone trimethylation, an epigenetic event detectable in blood', *Acta Neuropathologica*, 126(6), pp. 895–905. doi: 10.1007/s00401-013-1199-1.
- Bensimon, G., Lacomblez, L. and Meininger, V. (1994) 'A Controlled Trial of Riluzole in Amyotrophic Lateral Sclerosis', *New England Journal of Medicine*, 330(9), pp. 585–591. doi: 10.1056/NEJM199403033300901.
- Berlier, J. E. *et al.* (2003) 'Quantitative Comparison of Long-wavelength Alexa Fluor Dyes to Cy Dyes: Fluorescence of the Dyes and Their Bioconjugates', *Journal of Histochemistry & Cytochemistry*, 51(12), pp. 1699–1712. doi: 10.1177/002215540305101214.
- Berridge, M. J. (2002) 'The endoplasmic reticulum: a multifunctional signaling organelle', *Cell Calcium*. Churchill Livingstone, 32(5–6), pp. 235–249. doi: 10.1016/S0143416002001823.
- Bindels, D. S. *et al.* (2017) 'mScarlet: a bright monomeric red fluorescent protein for cellular imaging', *Nature Methods*, 14(1), pp. 53–56. doi: 10.1038/nmeth.4074.
- Borghese, L. *et al.* (2010) 'Inhibition of Notch Signaling in Human Embryonic Stem Cell-Derived Neural Stem Cells Delays G1/S Phase Transition and Accelerates Neuronal Differentiation In Vitro and In Vivo', *STEM CELLS*, 28(5), pp. 955–964. doi: 10.1002/stem.408.
- Bourke, S. C. *et al.* (2006) 'Effects of non-invasive ventilation on survival and quality of life in patients with amyotrophic lateral sclerosis: a randomised controlled trial', *The Lancet Neurology*. Elsevier, 5(2), pp. 140–147. doi: 10.1016/S1474-4422(05)70326-4.
- Boxer, A. L. *et al.* (2011) 'Clinical, neuroimaging and neuropathological features of a new chromosome 9p-linked FTD-ALS family', *Journal of Neurology, Neurosurgery & Psychiatry*, 82(2), pp. 196–203. doi: 10.1136/jnnp.2009.204081.
- Boyden, E. S. *et al.* (2005) 'Millisecond-timescale, genetically targeted optical control of neural activity.', *Nature neuroscience*. Nature Publishing Group, 8(9), pp. 1263–8. doi: 10.1038/nn1525.
- Bradley Dm, W. G., Bradley, W. G. and Mash, D. C. (2009) 'Beyond Guam: The cyanobacteria/BMAA hypothesis of the cause of ALS and other neurodegenerative diseases', *Amyotrophic Lateral Sclerosis*, (2), pp. 7–20. doi: 10.3109/17482960903286009.
- Branda, C. S. and Dymecki, S. M. (2004) 'Talking about a Revolution: The Impact of Site-Specific Recombinases on Genetic Analyses in Mice', *Developmental Cell*. Cell Press, 6(1), pp. 7–28. doi: 10.1016/S1534-5807(03)00399-X.
- Broach, J. R., Guarascio, V. R. and Jayaram, M. (1982) 'Recombination within the yeast plasmid 2mu circle is site-specific.', *Cell*, 29(1), pp. 227–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6286142> (Accessed: 6 September 2018).

- Brooks, A. R. *et al.* (2004) 'Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle', *The Journal of Gene Medicine*, 6(4), pp. 395–404. doi: 10.1002/jgm.516.
- Brustovetsky, N. *et al.* (2002) 'Calcium-induced Cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane', *Journal of Neurochemistry*. Wiley/Blackwell (10.1111), 80(2), pp. 207–218. doi: 10.1046/j.0022-3042.2001.00671.x.
- Bukrinsky, M. I. *et al.* (1993) 'A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells', *Nature*, 365(6447), pp. 666–669. doi: 10.1038/365666a0.
- Buratti, E. and Baralle, F. E. (2001) 'Characterization and Functional Implications of the RNA Binding Properties of Nuclear Factor TDP-43, a Novel Splicing Regulator of *CFTR* Exon 9', *Journal of Biological Chemistry*, 276(39), pp. 36337–36343. doi: 10.1074/jbc.M104236200.
- Burberry, A. *et al.* (2016) 'Loss-of-function mutations in the C9ORF72 mouse ortholog cause fatal autoimmune disease', *Science Translational Medicine*, 8(347), p. 347ra93-347ra93. doi: 10.1126/scitranslmed.aaf6038.
- Bush, W. S. and Moore, J. H. (2012) 'Chapter 11: Genome-Wide Association Studies', *PLoS Computational Biology*. Edited by F. Lewitter and M. Kann, 8(12), p. e1002822. doi: 10.1371/journal.pcbi.1002822.
- Byrne, S. *et al.* (2012) 'Cognitive and clinical characteristics of patients with amyotrophic lateral sclerosis carrying a C9orf72 repeat expansion: a population-based cohort study', *The Lancet Neurology*, 11(3), pp. 232–240. doi: 10.1016/S1474-4422(12)70014-5.
- Cai, J., Yang, J. and Jones, D. (1998) 'Mitochondrial control of apoptosis: the role of cytochrome c', *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. Elsevier, 1366(1–2), pp. 139–149. doi: 10.1016/S0005-2728(98)00109-1.
- Carriedo, S. G. *et al.* (1995) 'In vitro kainate injury to large, SMI-32(+) spinal neurons is Ca²⁺ dependent.', *Neuroreport*, 6(6), pp. 945–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7612889> (Accessed: 9 September 2018).
- Carriedo, S. G., Yin, H. Z. and Weiss, J. H. (1996) 'Motor neurons are selectively vulnerable to AMPA/kainate receptor-mediated injury in vitro.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 16(13), pp. 4069–79. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8753869> (Accessed: 9 September 2018).
- de Carvalho, M. *et al.* (1996) 'Motor neuron disease presenting with respiratory failure', *Journal of the Neurological Sciences*. Elsevier, 139, pp. 117–122. doi: 10.1016/0022-510X(96)00089-5.
- Chambers, S. M. *et al.* (2009) 'Highly efficient neural conversion of human ES and iPS

cells by dual inhibition of SMAD signaling', *Nature Biotechnology*. Nature Publishing Group, 27(3), pp. 275–280. doi: 10.1038/nbt.1529.

Chang, Q. and Martin, L. J. (2016) 'Voltage-gated calcium channels are abnormal in cultured spinal motoneurons in the G93A-SOD1 transgenic mouse model of ALS', *Neurobiology of Disease*, 93, pp. 78–95. doi: 10.1016/j.nbd.2016.04.009.

Chao-min, L. and Theron, H. E. (1978) 'Characterization of ionomycin as a calcium ionophore', *J. Biol. Chem.*, 253(17), pp. 5892–5894. doi: VL-253.

Chew, J. *et al.* (2015) 'Neurodegeneration. C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits.', *Science (New York, N.Y.)*. NIH Public Access, 348(6239), pp. 1151–4. doi: 10.1126/science.aaa9344.

Chia, R., Chiò, A. and Traynor, B. J. (2018) 'Novel genes associated with amyotrophic lateral sclerosis: diagnostic and clinical implications.', *The Lancet. Neurology*. Elsevier, 17(1), pp. 94–102. doi: 10.1016/S1474-4422(17)30401-5.

Chio, A. *et al.* (2012) 'Clinical characteristics of patients with familial amyotrophic lateral sclerosis carrying the pathogenic GGGGCC hexanucleotide repeat expansion of C9ORF72', *Brain*. Oxford University Press, 135(3), pp. 784–793. doi: 10.1093/brain/awr366.

Chiò, A. *et al.* (2009) 'Prognostic factors in ALS: A critical review', *Amyotrophic Lateral Sclerosis*. Taylor & Francis, 10(5–6), pp. 310–323. doi: 10.3109/17482960802566824.

Ciura, S. *et al.* (2013) 'Loss of function of C9orf72 causes motor deficits in a zebrafish model of Amyotrophic Lateral Sclerosis', *Annals of Neurology*, 74(2), p. n/a-n/a. doi: 10.1002/ana.23946.

Cohen-Hadad, Y. *et al.* (2016) 'Marked Differences in C9orf72 Methylation Status and Isoform Expression between C9/ALS Human Embryonic and Induced Pluripotent Stem Cells.', *Stem cell reports*. Elsevier, 7(5), pp. 927–940. doi: 10.1016/j.stemcr.2016.09.011.

Cohen, J. I. *et al.* (1989) 'Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation.', *Proceedings of the National Academy of Sciences of the United States of America*, 86(23), pp. 9558–62. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2556717> (Accessed: 29 August 2018).

Cooper-Knock, J. *et al.* (2014) 'Sequestration of multiple RNA recognition motif-containing proteins by C9orf72 repeat expansions', *Brain*. Oxford University Press, 137(7), pp. 2040–2051. doi: 10.1093/brain/awu120.

Crick, F. (1999) 'The impact of molecular biology on neuroscience.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. The Royal Society, 354(1392), pp. 2021–5. doi: 10.1098/rstb.1999.0541.

Cui, C., Shu, W. and Li, P. (2016) 'Fluorescence In situ Hybridization: Cell-Based Genetic Diagnostic and Research Applications.', *Frontiers in cell and developmental biology*.

Frontiers Media SA, 4, p. 89. doi: 10.3389/fcell.2016.00089.

Dafinca, R. *et al.* (2016) 'C9orf72 Hexanucleotide Expansions Are Associated with Altered Endoplasmic Reticulum Calcium Homeostasis and Stress Granule Formation in Induced Pluripotent Stem Cell-Derived Neurons from Patients with Amyotrophic Lateral Sclerosis and Frontotemporal Dementia', *Stem Cells*. Wiley-Blackwell, 34(8), pp. 2063–2078. doi: 10.1002/stem.2388.

Damiano, M. *et al.* (2006) 'Neural mitochondrial Ca²⁺ capacity impairment precedes the onset of motor symptoms in G93A Cu/Zn-superoxide dismutase mutant mice', *Journal of Neurochemistry*, 96(5), pp. 1349–1361. doi: 10.1111/j.1471-4159.2006.03619.x.

Davenport, R. J. *et al.* (1996) 'Avoiding false positive diagnoses of motor neuron disease: lessons from the Scottish Motor Neuron Disease Register', *Neurol Neurosurg Psychiatry*, 60(7), pp. 147–151. doi: 10.1136/jnnp.60.2.147.

Davidson, Y. S. *et al.* (2014) 'Brain distribution of dipeptide repeat proteins in frontotemporal lobar degeneration and motor neurone disease associated with expansions in C9ORF72', *Acta Neuropathologica Communications*, 2(1), p. 70. doi: 10.1186/2051-5960-2-70.

Davis, B. M. *et al.* (1997) 'Expansion of a CUG trinucleotide repeat in the 3' untranslated region of myotonic dystrophy protein kinase transcripts results in nuclear retention of transcripts', *Proceedings of the National Academy of Sciences*, 94(14), pp. 7388–7393. doi: 10.1073/pnas.94.14.7388.

Van Deerlin, V. M. *et al.* (2010) 'Common variants at 7p21 are associated with frontotemporal lobar degeneration with TDP-43 inclusions.', *Nature genetics*, 42(3), pp. 234–9. doi: 10.1038/ng.536.

Deisseroth, K. (2010) 'Controlling the brain with light', *Scientific American*. Nature Publishing Group, 303(5), pp. 48–55. doi: 10.1038/scientificamerican1110-48.

Deisseroth, K. and Hegemann, P. (2017) 'The form and function of channelrhodopsin', *Science*, 357(6356), p. eaan5544. doi: 10.1126/science.aan5544.

DeJesus-Hernandez, M. *et al.* (2011) 'Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS', *Neuron*. Cell Press, 72(2), pp. 245–256. doi: 10.1016/J.NEURON.2011.09.011.

DeJesus-Hernandez, M. *et al.* (2017) 'In-depth clinico-pathological examination of RNA foci in a large cohort of C9ORF72 expansion carriers.', *Acta neuropathologica*. Springer, 134(2), pp. 255–269. doi: 10.1007/s00401-017-1725-7.

Deng, H.-X. *et al.* (2011) 'Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia.', *Nature*. Nature Publishing Group, 477(7363), pp. 211–5. doi: 10.1038/nature10353.

Deutschbauer, A. M. *et al.* (2005) 'Mechanisms of haploinsufficiency revealed by

genome-wide profiling in yeast.’, *Genetics*. Genetics Society of America, 169(4), pp. 1915–25. doi: 10.1534/genetics.104.036871.

Devlin, A.-C. *et al.* (2015) ‘Human iPSC-derived motoneurons harbouring TARDBP or C9ORF72 ALS mutations are dysfunctional despite maintaining viability’, *Nature Communications*. Nature Publishing Group, 6(1), p. 5999. doi: 10.1038/ncomms6999.

Dillon, N. (2008) ‘The Impact of Gene Location in the Nucleus on Transcriptional Regulation’, *Developmental Cell*. Cell Press, 15(2), pp. 182–186. doi: 10.1016/J.DEVCEL.2008.07.013.

Dimos, J. T. *et al.* (2008) ‘Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons’, *Science*. American Association for the Advancement of Science (New Series), 321(5893), pp. 1218–1221. doi: 10.2307/20144706.

Do-Ha, D., Buskila, Y. and Ooi, L. (2018) ‘Impairments in Motor Neurons, Interneurons and Astrocytes Contribute to Hyperexcitability in ALS: Underlying Mechanisms and Paths to Therapy’, *Molecular Neurobiology*. Springer US, 55(2), pp. 1410–1418. doi: 10.1007/s12035-017-0392-y.

Donnelly, C. J. *et al.* (2013) ‘RNA Toxicity from the ALS/FTD C9ORF72 Expansion Is Mitigated by Antisense Intervention’, *Neuron*, 80(2), pp. 415–428. doi: 10.1016/j.neuron.2013.10.015.

Du, Z.-W. *et al.* (2015) ‘Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells.’, *Nature communications*. Nature Publishing Group, 6, p. 6626. doi: 10.1038/ncomms7626.

Dull, T. *et al.* (1998) ‘A third-generation lentivirus vector with a conditional packaging system.’, *Journal of virology*. American Society for Microbiology (ASM), 72(11), pp. 8463–71. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9765382> (Accessed: 6 September 2018).

Erickson, J. D. *et al.* (1994) ‘Functional identification of a vesicular acetylcholine transporter and its expression from a ‘cholinergic’ gene locus.’, *The Journal of biological chemistry*, 269(35), pp. 21929–32. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8071310> (Accessed: 9 September 2018).

Ericson, J. *et al.* (1992) ‘Early stages of motor neuron differentiation revealed by expression of homeobox gene *Islet-1*’, *Science*, 256(5063), pp. 1555–1560. doi: 10.1126/science.1350865.

Ericson, J. *et al.* (1996) ‘Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity.’, *Cell*, 87(4), pp. 661–73. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8929535> (Accessed: 15 August 2018).

Ericson, J. *et al.* (1997) ‘Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling’, *Cell*. Elsevier, 90(1), pp. 169–180. doi: 10.1016/S0092-

8674(00)80323-2.

van Es, M. A. *et al.* (2009) 'Genome-wide association study identifies 19p13.3 (UNC13A) and 9p21.2 as susceptibility loci for sporadic amyotrophic lateral sclerosis.', *Nature genetics*, 41(10), pp. 1083–7. doi: 10.1038/ng.442.

Evans, M. J. and Kaufman, M. H. (1981) 'Establishment in culture of pluripotential cells from mouse embryos.', *Nature*, 292(5819), pp. 154–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7242681> (Accessed: 14 August 2018).

Fang, T. *et al.* (2018) 'Stage at which riluzole treatment prolongs survival in patients with amyotrophic lateral sclerosis: a retrospective analysis of data from a dose-ranging study.', *The Lancet. Neurology*. Elsevier, 17(5), pp. 416–422. doi: 10.1016/S1474-4422(18)30054-1.

Fecto, F. *et al.* (2011) 'SQSTM1 Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis', *Archives of Neurology*. American Medical Association, 68(11), p. 1440. doi: 10.1001/archneurol.2011.250.

Fennewald, S., van Santen, V. and Kieff, E. (1984) 'Nucleotide sequence of an mRNA transcribed in latent growth-transforming virus infection indicates that it may encode a membrane protein.', *Journal of virology*. American Society for Microbiology (ASM), 51(2), pp. 411–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6086953> (Accessed: 29 August 2018).

Frankel, A. D. and Young, J. A. T. (1998) 'HIV-1: Fifteen Proteins and an RNA', *Annual Review of Biochemistry*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA , 67(1), pp. 1–25. doi: 10.1146/annurev.biochem.67.1.1.

Fratta, P. *et al.* (2012) 'C9orf72 hexanucleotide repeat associated with amyotrophic lateral sclerosis and frontotemporal dementia forms RNA G-quadruplexes.', *Scientific reports*. Nature Publishing Group, 2, p. 1016. doi: 10.1038/srep01016.

Freischmidt, A. *et al.* (2015) 'Haploinsufficiency of TBK1 causes familial ALS and frontotemporal dementia', *Nature Neuroscience*. Nature Publishing Group, 18(5), pp. 631–636. doi: 10.1038/nn.4000.

Frisan, T., Levitsky, V. and Masucci, M. (2001) 'Generation of Lymphoblastoid Cell Lines (LCLs)', in *Epstein-Barr Virus Protocols*. New Jersey: Humana Press, pp. 125–127. doi: 10.1385/1-59259-227-9:125.

Fritz, E. *et al.* (2013) 'Mutant SOD1-expressing astrocytes release toxic factors that trigger motoneuron death by inducing hyperexcitability', *Journal of Neurophysiology*. American Physiological Society Bethesda, MD, 109(11), pp. 2803–2814. doi: 10.1152/jn.00500.2012.

Gall, J. G. and Pardue, M. L. (1969) 'Formation and detection of RNA-DNA hybrid molecules in cytological preparations.', *Proceedings of the National Academy of Sciences of the United States of America*, 63(2), pp. 378–83. Available at:

<http://www.ncbi.nlm.nih.gov/pubmed/4895535> (Accessed: 29 August 2018).

Gallassi, R. *et al.* (1989) *Neuropsychological, Electroencephalogram and Brain Computed Tomography Findings in Motor Neuron Disease*, *Eur Neurol*. Available at: <https://www.karger.com/Article/PDF/116391> (Accessed: 28 July 2018).

Gama-Norton, L. *et al.* (2011) 'Lentivirus Production Is Influenced by SV40 Large T-Antigen and Chromosomal Integration of the Vector in HEK293 Cells', *Human Gene Therapy*. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA, 22(10), pp. 1269–1279. doi: 10.1089/hum.2010.143.

Gao, B. X. and Ziskind-Conhaim, L. (1995) 'Development of glycine- and GABA-gated currents in rat spinal motoneurons.', *Journal of neurophysiology*. American Physiological Society Bethesda, MD, 74(1), pp. 113–21. doi: 10.1152/jn.1995.74.1.113.

Gascón, S. *et al.* (2008) 'Dual-promoter lentiviral vectors for constitutive and regulated gene expression in neurons.', *Journal of neuroscience methods*, 168(1), pp. 104–12. doi: 10.1016/j.jneumeth.2007.09.023.

Gendron, T. F. *et al.* (2013) 'Antisense transcripts of the expanded C9ORF72 hexanucleotide repeat form nuclear RNA foci and undergo repeat-associated non-ATG translation in c9FTD/ALS', *Acta Neuropathologica*, 126(6), pp. 829–844. doi: 10.1007/s00401-013-1192-8.

Gibson, D. G. *et al.* (2009) 'Enzymatic assembly of DNA molecules up to several hundred kilobases', *Nature Methods*. Nature Publishing Group, 6(5), pp. 343–345. doi: 10.1038/nmeth.1318.

Gijselink, I. *et al.* (2012) 'A C9orf72 promoter repeat expansion in a Flanders-Belgian cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum: a gene identification study', *The Lancet Neurology*, 11(1), pp. 54–65. doi: 10.1016/S1474-4422(11)70261-7.

Di Giorgio, F. P. *et al.* (2007) 'Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model.', *Nature neuroscience*. NIH Public Access, 10(5), pp. 608–14. doi: 10.1038/nn1885.

Di Giorgio, F. P. *et al.* (2008) 'Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation.', *Cell stem cell*. Elsevier, 3(6), pp. 637–48. doi: 10.1016/j.stem.2008.09.017.

Goetz, C. G. (2000) 'Amyotrophic lateral sclerosis: early contributions of Jean-Martin Charcot.', *Muscle & nerve*, 23(3), pp. 336–43. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10679709> (Accessed: 25 June 2018).

Gradinaru, V., Thompson, K. R. and Deisseroth, K. (2008) 'eNpHR: a Natronomonas halorhodopsin enhanced for optogenetic applications', *Brain Cell Biology*, 36(1–4), pp. 129–139. doi: 10.1007/s11068-008-9027-6.

Greenway, M. J. *et al.* (2006) 'ANG mutations segregate with familial and "sporadic" amyotrophic lateral sclerosis', *Nature Genetics*. Nature Publishing Group, 38(4), pp. 411–413. doi: 10.1038/ng1742.

Grosskreutz, J. *et al.* (2007) 'Role of mitochondria in kainate-induced fast Ca²⁺ transients in cultured spinal motor neurons', *Cell Calcium*. Churchill Livingstone, 42(1), pp. 59–69. doi: 10.1016/J.CECA.2006.11.010.

Grosskreutz, J., Van Den Bosch, L. and Keller, B. U. (2010) 'Calcium dysregulation in amyotrophic lateral sclerosis', *Cell Calcium*. Churchill Livingstone, 47(2), pp. 165–174. doi: 10.1016/J.CECA.2009.12.002.

Gurdon, J. B. (1962) 'The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles', *Journal of embryology and experimental morphology*, 10, pp. 622–640. Available at: <http://www.scopus.com/inward/record.url?eid=2-s2.0-0000155376&partnerID=tZOtx3y1>.

Haeusler, A. R. *et al.* (2014) 'C9orf72 nucleotide repeat structures initiate molecular cascades of disease', *Nature*. Nature Publishing Group, 507(7491), pp. 195–200. doi: 10.1038/nature13124.

Hamburger, V. (1989) 'The Heritage of Experimental Embryology: Hans Spemann and the Organizer', *Journal of the History of Biology*, 22(1), pp. 179–180. Available at: <https://philpapers.org/rec/HAMTHO-2> (Accessed: 14 August 2018).

Hamill, O. P. *et al.* (1981) 'Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches', *Pflügers Archiv - European Journal of Physiology*. Springer-Verlag, 391(2), pp. 85–100. doi: 10.1007/BF00656997.

Hammerschmidt, W. and Sugden, B. (1989) 'Genetic analysis of immortalizing functions of Epstein–Barr virus in human B lymphocytes', *Nature*, 340(6232), pp. 393–397. doi: 10.1038/340393a0.

Hantash, F. M. *et al.* (2010) 'Qualitative assessment of FMR1 (CGG)_n triplet repeat status in normal, intermediate, premutation, full mutation, and mosaic carriers in both sexes: Implications for fragile X syndrome carrier and newborn screening', *Genetics in Medicine*, 12(3), pp. 162–173. doi: 10.1097/GIM.0b013e3181d0d40e.

Harvey, A. M. and MacIntosh, F. C. (1940) 'Calcium and synaptic transmission in a sympathetic ganglion', *The Journal of Physiology*. Wiley/Blackwell (10.1111), 97(3), pp. 408–416. doi: 10.1113/jphysiol.1940.sp003818.

Heller, M., van Santen, V. and Kieff, E. (1982) 'Simple repeat sequence in Epstein-Barr virus DNA is transcribed in latent and productive infections.', *Journal of virology*, 44(1), pp. 311–20. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6292493> (Accessed: 29 August 2018).

Hemmati-Brivanlou, A. and Melton, D. (1997) 'Vertebrate Embryonic Cells Will Become

Nerve Cells Unless Told Otherwise', *Cell*. Cell Press, 88(1), pp. 13–17. doi: 10.1016/S0092-8674(00)81853-X.

Hennessy, K. *et al.* (1983) 'Simple repeat array in Epstein-Barr virus DNA encodes part of the Epstein-Barr nuclear antigen.', *Science (New York, N.Y.)*, 220(4604), pp. 1396–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6304878> (Accessed: 29 August 2018).

Hennessy, K. *et al.* (1984) 'A membrane protein encoded by Epstein-Barr virus in latent growth-transforming infection.', *Proceedings of the National Academy of Sciences of the United States of America*, 81(22), pp. 7207–11. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6095274> (Accessed: 29 August 2018).

Hennessy, K. *et al.* (1986) 'Definitive identification of a member of the Epstein-Barr virus nuclear protein 3 family.', *Proceedings of the National Academy of Sciences of the United States of America*, 83(15), pp. 5693–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/3016714> (Accessed: 29 August 2018).

Hennessy, K., Fennewald, S. and Kieff, E. (1985) 'A third viral nuclear protein in lymphoblasts immortalized by Epstein-Barr virus.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 82(17), pp. 5944–8. doi: 10.1073/PNAS.82.17.5944.

Hennessy, K. and Kieff, E. (1985) 'A second nuclear protein is encoded by Epstein-Barr virus in latent infection.', *Science (New York, N.Y.)*, 227(4691), pp. 1238–40. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2983420> (Accessed: 29 August 2018).

Hewitt, S. *et al.* (2004) 'Nuclear repositioning marks the selective exclusion of lineage-inappropriate transcription factor loci during T helper cell differentiation', *European Journal of Immunology*. Wiley-Blackwell, 34(12), pp. 3604–3613. doi: 10.1002/eji.200425469.

Higgins, C. M., Jung, C. and Xu, Z. (2003) 'ALS-associated mutant SOD1G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes.', *BMC Neuroscience*, 4(1), p. 16. doi: 10.1186/1471-2202-4-16.

Hinckley, C. A. *et al.* (2005) 'Locomotor-Like Rhythms in a Genetically Distinct Cluster of Interneurons in the Mammalian Spinal Cord', *Journal of Neurophysiology*, 93(3), pp. 1439–1449. doi: 10.1152/jn.00647.2004.

Hodgkin, A. L. and Huxley, A. F. (1990) 'A quantitative description of membrane current and its application to conduction and excitation in nerve', *Bulletin of Mathematical Biology*. Wiley/Blackwell (10.1111), 52(1–2), pp. 25–71. doi: 10.1007/BF02459568.

Hoess, R. H., Ziese, M. and Sternberg, N. (1982) 'P1 site-specific recombination: nucleotide sequence of the recombining sites.', *Proceedings of the National Academy of Sciences*, 79(11), pp. 3398–3402. doi: 10.1073/pnas.79.11.3398.

- Howland, D. S. *et al.* (2002) 'Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS)', *Proceedings of the National Academy of Sciences*, 99(3), pp. 1604–1609. doi: 10.1073/pnas.032539299.
- Hu, B.-Y. and Zhang, S.-C. (2009) 'Differentiation of spinal motor neurons from pluripotent human stem cells.', *Nature protocols*. NIH Public Access, 4(9), pp. 1295–304. doi: 10.1038/nprot.2009.127.
- Iacoangeli, A. and Tiedge, H. (2013) 'Translational control at the synapse: role of RNA regulators', *Trends in Biochemical Sciences*, 38(1), pp. 47–55. doi: 10.1016/j.tibs.2012.11.001.
- Ikenaka, K. *et al.* (2012) 'Disruption of axonal transport in motor neuron diseases.', *International journal of molecular sciences*. Multidisciplinary Digital Publishing Institute (MDPI), 13(1), pp. 1225–38. doi: 10.3390/ijms13011225.
- Irwin, D. J. *et al.* (2013) 'Cognitive decline and reduced survival in *C9orf72* expansion frontotemporal degeneration and amyotrophic lateral sclerosis', *Journal of Neurology, Neurosurgery & Psychiatry*, 84(2), pp. 163–169. doi: 10.1136/jnnp-2012-303507.
- Ishiura, H. *et al.* (2012) 'C9ORF72 Repeat Expansion in Amyotrophic Lateral Sclerosis in the Kii Peninsula of Japan', *Archives of Neurology*. American Medical Association, 69(9), pp. 1154–1158. doi: 10.1001/archneurol.2012.1219.
- Ishizuka, T. *et al.* (2006) 'Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels', *Neuroscience Research*. Elsevier, 54(2), pp. 85–94. doi: 10.1016/J.NEURES.2005.10.009.
- Ji, A.-L. *et al.* (2017) 'Genetics insight into the amyotrophic lateral sclerosis/frontotemporal dementia spectrum', *Journal of Medical Genetics*, 54(3), pp. 145–154. doi: 10.1136/jmedgenet-2016-104271.
- Jiang, H. *et al.* (2004) 'Myotonic dystrophy type 1 is associated with nuclear foci of mutant RNA, sequestration of muscleblind proteins and deregulated alternative splicing in neurons', *Human Molecular Genetics*. Oxford University Press, 13(24), pp. 3079–3088. doi: 10.1093/hmg/ddh327.
- Jiang, J. *et al.* (2016) 'Gain of Toxicity from ALS/FTD-Linked Repeat Expansions in C9ORF72 Is Alleviated by Antisense Oligonucleotides Targeting GGGGCC-Containing RNAs', *Neuron*, 90(3), pp. 535–550. doi: 10.1016/j.neuron.2016.04.006.
- John, H. A., Birnstiel, M. L. and Jones, K. W. (1969) 'RNA-DNA Hybrids at the Cytological Level', *Nature*. Nature Publishing Group, 223(5206), pp. 582–587. doi: 10.1038/223582a0.
- Johnson, J. O. *et al.* (2010) 'Exome Sequencing Reveals VCP Mutations as a Cause of Familial ALS', *Neuron*. Cell Press, 68(5), pp. 857–864. doi: 10.1016/J.NEURON.2010.11.036.

Johnson, J. O. *et al.* (2014) 'Mutations in the Matrin 3 gene cause familial amyotrophic lateral sclerosis', *Nature Neuroscience*. Nature Publishing Group, 17(5), pp. 664–666. doi: 10.1038/nn.3688.

Kabashi, E. *et al.* (2008) 'TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis', *Nature Genetics*. Nature Publishing Group, 40(5), pp. 572–574. doi: 10.1038/ng.132.

Kaczmarczyk, S. J. and Green, J. E. (2001) 'A single vector containing modified cre recombinase and LOX recombination sequences for inducible tissue-specific amplification of gene expression.', *Nucleic acids research*. Oxford University Press, 29(12), pp. E56–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11410679> (Accessed: 6 September 2018).

Karumbayaram, S. *et al.* (2009) 'Human embryonic stem cell-derived motor neurons expressing SOD1 mutants exhibit typical signs of motor neuron degeneration linked to ALS.', *Disease models & mechanisms*. Company of Biologists, 2(3–4), pp. 189–95. doi: 10.1242/dmm.002113.

Kemp, P. J. *et al.* (2016) 'Improving and accelerating the differentiation and functional maturation of human stem cell-derived neurons: role of extracellular calcium and GABA.', *The Journal of physiology*. Wiley-Blackwell, 594(22), pp. 6583–6594. doi: 10.1113/JP270655.

Kew, J. J. M. *et al.* (1993) 'The relationship between abnormalities of cognitive function and cerebral activation in amyotrophic lateral sclerosis: A neuropsychological and positron emission tomography study', *Brain*, 116(6), pp. 1399–1423. doi: 10.1093/brain/116.6.1399.

Kibbe, W. A. (2007) 'OligoCalc: an online oligonucleotide properties calculator', *Nucleic Acids Research*, 35(Web Server), pp. W43–W46. doi: 10.1093/nar/gkm234.

Kiernan, M. C. (2007) *The Motor Neurone Disease Handbook*. Australasian Medical Pub. Co. Limited.

Kim, H. J. *et al.* (2013) 'Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS', *Nature*. Nature Publishing Group, 495(7442), pp. 467–473. doi: 10.1038/nature11922.

Kim, V. N. *et al.* (1998) 'Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1.', *Journal of virology*, 72(1), pp. 811–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9420292> (Accessed: 6 September 2018).

Knibb, J. A. *et al.* (2016) 'A clinical tool for predicting survival in ALS', *Journal of Neurology, Neurosurgery & Psychiatry*, 87(12), pp. 1361–1367. doi: 10.1136/jnnp-2015-312908.

Koppers, M. *et al.* (2015) 'C9orf72 ablation in mice does not cause motor neuron degeneration or motor deficits', *Annals of Neurology*. Wiley-Blackwell, 78(3), pp. 426–

438. doi: 10.1002/ana.24453.

Kornfeld, S. and Mellman, I. (1989) 'The Biogenesis of Lysosomes', *Annual Review of Cell Biology*, 5(1), pp. 483–525. doi: 10.1146/annurev.cb.05.110189.002411.

Kornreich, B. G. (2007) 'The patch clamp technique: Principles and technical considerations', *Journal of Veterinary Cardiology*. Elsevier, 9(1), pp. 25–37. doi: 10.1016/j.jvc.2007.02.001.

Kosak, S. T. *et al.* (2002) 'Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 296(5565), pp. 158–62. doi: 10.1126/science.1068768.

Kriks, S. *et al.* (2011) 'Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease', *Nature*. Nature Publishing Group, 480(7378), pp. 547–551. doi: 10.1038/nature10648.

Krosnowski, K. *et al.* (2012) 'Diverse populations of intrinsic cholinergic interneurons in the mouse olfactory bulb', *Neuroscience*, 213, pp. 161–178. doi: 10.1016/j.neuroscience.2012.04.024.

Kuhne, J. *et al.* (2015) 'Early Formation of the Ion-Conducting Pore in Channelrhodopsin-2', *Angewandte Chemie International Edition*. Wiley-Blackwell, 54(16), pp. 4953–4957. doi: 10.1002/anie.201410180.

Kumar, M. *et al.* (2001) 'Systematic determination of the packaging limit of lentiviral vectors.', *Human gene therapy*. Mary Ann Liebert, Inc., 12(15), pp. 1893–905. doi: 10.1089/104303401753153947.

Kwiatkowski, T. J. *et al.* (2009) 'Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 323(5918), pp. 1205–8. doi: 10.1126/science.1166066.

Kwon, I. *et al.* (2014) 'Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 345(6201), pp. 1139–45. doi: 10.1126/science.1254917.

Laaksovirta, H. *et al.* (2010) 'Chromosome 9p21 in amyotrophic lateral sclerosis in Finland: a genome-wide association study.', *The Lancet. Neurology*, 9(10), pp. 978–85. doi: 10.1016/S1474-4422(10)70184-8.

Lacomblez, L. *et al.* (1996) 'Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis/Riluzole Study Group II.', *Lancet (London, England)*, 347(9013), pp. 1425–31. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8676624> (Accessed: 26 June 2018).

Lagier-Tourenne, C. *et al.* (2013) 'Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of

Sciences, 110(47), pp. E4530-9. doi: 10.1073/pnas.1318835110.

Lam, R. S. *et al.* (2017) 'Functional Maturation of Human Stem Cell-Derived Neurons in Long-Term Cultures', *PLOS ONE*. Edited by H.-C. I. Chen. Public Library of Science, 12(1), p. e0169506. doi: 10.1371/journal.pone.0169506.

Langer, P. R., Waldrop, A. A. and Ward, D. C. (1981) 'Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 78(11), pp. 6633–7. doi: 10.1073/PNAS.78.11.6633.

Lee, H. *et al.* (2007) 'Directed Differentiation and Transplantation of Human Embryonic Stem Cell-Derived Motoneurons', *Stem Cells*, 25(8), pp. 1931–1939. doi: 10.1634/stemcells.2007-0097.

Lee, M. A., Diamond, M. E. and Yates, J. L. (1999) 'Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus.', *Journal of virology*, 73(4), pp. 2974–82. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10074147> (Accessed: 29 August 2018).

Lee, Y. B. *et al.* (2013) 'Hexanucleotide repeats in ALS/FTD form length-dependent RNA Foci, sequester RNA binding proteins, and are neurotoxic', *Cell Reports*, 5(5), pp. 1178–1186. doi: 10.1016/j.celrep.2013.10.049.

Levivier, E. *et al.* (2001) 'uDENN, DENN, and dDENN: Indissociable Domains in Rab and MAP Kinases Signaling Pathways', *Biochemical and Biophysical Research Communications*. Academic Press, 287(3), pp. 688–695. doi: 10.1006/BBRC.2001.5652.

Lewinski, F. von and Keller, B. U. (2005) 'Ca²⁺, mitochondria and selective motoneuron vulnerability: implications for ALS', *Trends in Neurosciences*. Elsevier Current Trends, 28(9), pp. 494–500. doi: 10.1016/J.TINS.2005.07.001.

Li, W. *et al.* (2011) 'Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors', *Proceedings of the National Academy of Sciences*, 108(20), pp. 8299–8304. doi: 10.1073/pnas.1014041108.

Li, X.-J. *et al.* (2005) 'Specification of motoneurons from human embryonic stem cells', *Nature Biotechnology*. Nature Publishing Group, 23(2), pp. 215–221. doi: 10.1038/nbt1063.

Liem, K. F., Tremml, G. and Jessell, T. M. (1997) 'A role for the roof plate and its resident TGF β -related proteins in neuronal patterning in the dorsal spinal cord', *Cell*. Elsevier, 91(1), pp. 127–138. doi: 10.1016/S0092-8674(01)80015-5.

Lin, C. L. *et al.* (1998) 'Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis.', *Neuron*, 20(3), pp. 589–602. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9539131> (Accessed: 17 August 2018).

- Lin, J. Y. (2011) 'A user's guide to channelrhodopsin variants: features, limitations and future developments.', *Experimental physiology*, 96(1), pp. 19–25. doi: 10.1113/expphysiol.2009.051961.
- Liu-Yesucevitz, L. *et al.* (2011) 'Local RNA translation at the synapse and in disease.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 31(45), pp. 16086–93. doi: 10.1523/JNEUROSCI.4105-11.2011.
- Liu, J.-P., Laufer, E. and Jessell, T. M. (2001) 'Assigning the Positional Identity of Spinal Motor Neurons: Rostrocaudal Patterning of Hox-c Expression by FGFs, Gdf11, and Retinoids', *Neuron*. Cell Press, 32(6), pp. 997–1012. doi: 10.1016/S0896-6273(01)00544-X.
- Liu, Q. *et al.* (2016) 'Whole-exome sequencing identifies a missense mutation in hnRNPA1 in a family with flail arm ALS.', *Neurology*. Wolters Kluwer Health, Inc. on behalf of the American Academy of Neurology, 87(17), pp. 1763–1769. doi: 10.1212/WNL.0000000000003256.
- Liu, Y. *et al.* (2016) 'C9orf72 BAC Mouse Model with Motor Deficits and Neurodegenerative Features of ALS/FTD', *Neuron*. Elsevier, 90(3), pp. 521–534. doi: 10.1016/j.neuron.2016.04.005.
- Livak, K. J. and Schmittgen, T. D. (2001) 'Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method', *Methods*, 25(4), pp. 402–408. doi: 10.1006/meth.2001.1262.
- Lomen-Hoerth, C. *et al.* (2003) 'Are amyotrophic lateral sclerosis patients cognitively normal?', *NEUROLOGY*, 60, pp. 1094–1097. Available at: <http://n.neurology.org/content/neurology/60/7/1094.full.pdf> (Accessed: 22 July 2018).
- Longnecker, R. *et al.* (1993) 'The last seven transmembrane and carboxy-terminal cytoplasmic domains of Epstein-Barr virus latent membrane protein 2 (LMP2) are dispensable for lymphocyte infection and growth transformation in vitro.', *Journal of virology*, 67(4), pp. 2006–13. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8383224> (Accessed: 29 August 2018).
- Longnecker, R. and Kieff, E. (1990) 'A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and colocalizes with LMP1.', *Journal of virology*, 64(5), pp. 2319–26. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2157888> (Accessed: 29 August 2018).
- Louwerse, E. S. *et al.* (1997) 'Amyotrophic lateral sclerosis: mortality risk during the course of the disease and prognostic factors', *Journal of the Neurological Sciences*. Elsevier, 152, pp. s10–s17. doi: 10.1016/S0022-510X(97)00238-4.
- Mackenzie, I. R. A. *et al.* (2007) 'Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations', *Annals of Neurology*, 61(5), pp. 427–434. doi: 10.1002/ana.21147.

- Mackenzie, I. R. A. *et al.* (2015) 'Quantitative analysis and clinico-pathological correlations of different dipeptide repeat protein pathologies in C9ORF72 mutation carriers', *Acta Neuropathologica*, 130(6), pp. 845–861. doi: 10.1007/s00401-015-1476-2.
- Mahoney, C. J. *et al.* (2012) 'Frontotemporal dementia with the C9ORF72 hexanucleotide repeat expansion: clinical, neuroanatomical and neuropathological features.', *Brain : a journal of neurology*. Oxford University Press, 135(Pt 3), pp. 736–50. doi: 10.1093/brain/awr361.
- Majounie, E. *et al.* (2012) 'Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study', *The Lancet Neurology*. Elsevier, 11(4), pp. 323–330. doi: 10.1016/S1474-4422(12)70043-1.
- Mali, S. (2013) 'Delivery systems for gene therapy.', *Indian journal of human genetics*. Wolters Kluwer -- Medknow Publications, 19(1), pp. 3–8. doi: 10.4103/0971-6866.112870.
- Mankodi, A. *et al.* (2001) 'Muscleblind localizes to nuclear foci of aberrant RNA in myotonic dystrophy types 1 and 2', *Human Molecular Genetics*. Oxford University Press, 10(19), pp. 2165–2170. doi: 10.1093/hmg/10.19.2165.
- Mannick, J. B. *et al.* (1991) 'The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation.', *Journal of virology*, 65(12), pp. 6826–37. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/1658376> (Accessed: 29 August 2018).
- Manuelidis, L., Langer-Safer, P. R. and Ward, D. C. (1982) 'High-resolution mapping of satellite DNA using biotin-labeled DNA probes.', *The Journal of cell biology*. Rockefeller University Press, 95(2 Pt 1), pp. 619–25. doi: 10.1083/JCB.95.2.619.
- Marin, B. *et al.* (2017) 'Variation in worldwide incidence of amyotrophic lateral sclerosis: a meta-analysis.', *International journal of epidemiology*. Oxford University Press, 46(1), pp. 57–74. doi: 10.1093/ije/dyw061.
- Martin-Caraballo, M. and Greer, J. J. (1999) 'Electrophysiological Properties of Rat Phrenic Motoneurons During Perinatal Development', *Journal of Neurophysiology*, 81(3), pp. 1365–1378. doi: 10.1152/jn.1999.81.3.1365.
- Martin, G. R. (1981) 'Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.', *Proceedings of the National Academy of Sciences of the United States of America*, 78(12), pp. 7634–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6950406> (Accessed: 14 August 2018).
- Maruyama, H. *et al.* (2010) 'Mutations of optineurin in amyotrophic lateral sclerosis.', *Nature*. Nature Publishing Group, 465(7295), pp. 223–6. doi: 10.1038/nature08971.
- Massman, P. J. *et al.* (1996) 'Prevalence and correlates of neuropsychological deficits in

amyotrophic lateral sclerosis', *J Neurol Neurosurg Psychiatry*, 61, pp. 450–455. doi: 10.1136/jnnp.61.5.450.

Matsuno-Yagi, A. and Mukohata, Y. (1977) 'Two possible roles of bacteriorhodopsin; a comparative study of strains of *Halobacterium halobium* differing in pigmentation.', *Biochemical and biophysical research communications*, 78(1), pp. 237–43. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20882> (Accessed: 13 August 2018).

Mattiazzi, M. *et al.* (2002) 'Mutated Human SOD1 Causes Dysfunction of Oxidative Phosphorylation in Mitochondria of Transgenic Mice', *Journal of Biological Chemistry*, 277(33), pp. 29626–29633. doi: 10.1074/jbc.M203065200.

Maury, Y. *et al.* (2015) 'Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes.', *Nature biotechnology*. Nature Publishing Group, 33(1), pp. 89–96. doi: 10.1038/nbt.3049.

McFarland, E. D. C., Izumi, K. M. and Mosialos, G. (1999) 'Epstein-Barr virus transformation: involvement of latent membrane protein 1-mediated activation of NF- κ B', *Oncogene*. Nature Publishing Group, 18(49), pp. 6959–6964. doi: 10.1038/sj.onc.1203217.

McGoldrick, P. *et al.* (2018) 'Unaffected mosaic *C9orf72* case', *Neurology*, 90(4), pp. e323–e331. doi: 10.1212/WNL.0000000000004865.

McLarnon, J. G. (1995) 'Potassium currents in motoneurons.', *Progress in neurobiology*, 47(6), pp. 513–31. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8787033> (Accessed: 26 November 2018).

McLaughlin, R. L., Vajda, A. and Hardiman, O. (2015) 'Heritability of Amyotrophic Lateral Sclerosis', *JAMA Neurology*. American Medical Association, 72(8), p. 857. doi: 10.1001/jamaneurol.2014.4049.

Mertens, J. *et al.* (2015) 'Directly Reprogrammed Human Neurons Retain Aging-Associated Transcriptomic Signatures and Reveal Age-Related Nucleocytoplasmic Defects', *Cell Stem Cell*, 17(6), pp. 705–718. doi: 10.1016/j.stem.2015.09.001.

Miles, G. B. *et al.* (2004) 'Functional properties of motoneurons derived from mouse embryonic stem cells.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 24(36), pp. 7848–58. doi: 10.1523/JNEUROSCI.1972-04.2004.

Miller, J. D. *et al.* (2013) 'Human iPSC-Based Modeling of Late-Onset Disease via Progerin-Induced Aging', *Cell Stem Cell*, 13(6), pp. 691–705. doi: 10.1016/j.stem.2013.11.006.

Miller, R. G., Mitchell, J. D. and Moore, D. H. (2012) 'Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND)', *Cochrane Database of Systematic Reviews*. John Wiley & Sons, Ltd. doi: 10.1002/14651858.CD001447.pub3.

- Mizielinska, S. *et al.* (2014) 'C9orf72 repeat expansions cause neurodegeneration in *Drosophila* through arginine-rich proteins.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 345(6201), pp. 1192–1194. doi: 10.1126/science.1256800.
- Mohyuddin, A. *et al.* (2004) 'Genetic instability in EBV-transformed lymphoblastoid cell lines.', *Biochimica et biophysica acta*, 1670(1), pp. 81–3. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14729144> (Accessed: 27 August 2018).
- Mok, K. *et al.* (2012) 'The chromosome 9 ALS and FTD locus is probably derived from a single founder', *Neurobiology of Aging*. Elsevier, 33(1), p. 209.e3-209.e8. doi: 10.1016/J.NEUROBIOLAGING.2011.08.005.
- Molecular Devices (2018) *What is an Action Potential | Molecular devices*. Available at: <https://www.moleculardevices.com/applications/patch-clamp-electrophysiology/what-action-potential#gref> (Accessed: 20 September 2018).
- Mor-Shaked, H. and Eiges, R. (2018) 'Reevaluation of FMR1 Hypermethylation Timing in Fragile X Syndrome.', *Frontiers in molecular neuroscience*. Frontiers Media SA, 11, p. 31. doi: 10.3389/fnmol.2018.00031.
- Mori, K., Arzberger, T., *et al.* (2013) 'Bidirectional transcripts of the expanded C9orf72 hexanucleotide repeat are translated into aggregating dipeptide repeat proteins', *Acta Neuropathologica*, 126(6), pp. 881–893. doi: 10.1007/s00401-013-1189-3.
- Mori, K., Lammich, S., *et al.* (2013) 'hnRNP A3 binds to GGGGCC repeats and is a constituent of p62-positive/TDP43-negative inclusions in the hippocampus of patients with C9orf72 mutations', *Acta Neuropathologica*, 125(3), pp. 413–423. doi: 10.1007/s00401-013-1088-7.
- Mori, K. *et al.* (2013) 'The C9orf72 GGGGCC Repeat Is Translated into Aggregating Dipeptide-Repeat Proteins in FTLD/ALS', *Science*, 339(6125), pp. 1335–1338. doi: 10.1126/science.1232927.
- Morita, M. *et al.* (2006) 'A locus on chromosome 9p confers susceptibility to ALS and frontotemporal dementia.', *Neurology*, 66(6), pp. 839–44. doi: 10.1212/01.wnl.0000200048.53766.b4.
- Van Mossevelde, S. *et al.* (2017) 'Relationship between C9orf72 repeat size and clinical phenotype', *Current Opinion in Genetics & Development*. Elsevier Current Trends, 44, pp. 117–124. doi: 10.1016/J.GDE.2017.02.008.
- Mulherkar, R. (2001) 'Gene therapy for cancer', *Current Science*, 81(5). Available at: <https://pdfs.semanticscholar.org/c0b9/e7c57e70cc6f0892b95f81c0ad646f851907.pdf> (Accessed: 2 September 2018).
- Müller, F., Blader, P. and Strähle, U. (2002) 'Search for enhancers: teleost models in comparative genomic and transgenic analysis of cis regulatory elements', *BioEssays*. Wiley-Blackwell, 24(6), pp. 564–572. doi: 10.1002/bies.10096.

Müller, M. *et al.* (2011) 'Projection Structure of Channelrhodopsin-2 at 6 Å Resolution by Electron Crystallography', *Journal of Molecular Biology*. Academic Press, 414(1), pp. 86–95. doi: 10.1016/J.JMB.2011.09.049.

Nagai, M. *et al.* (2007) 'Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons.', *Nature neuroscience*. Nature Publishing Group, 10(5), pp. 615–22. doi: 10.1038/nn1876.

Nagel, G. *et al.* (2002) 'Channelrhodopsin-1: A Light-Gated Proton Channel in Green Algae', *Science*, 296(5577), pp. 2395–2398. doi: 10.1126/science.1072068.

Nagel, G. *et al.* (2003) 'Channelrhodopsin-2, a directly light-gated cation-selective membrane channel.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(24), pp. 13940–5. doi: 10.1073/pnas.1936192100.

Nagel, G., Szellas, T., *et al.* (2005) 'Channelrhodopsins: directly light-gated cation channels', *Biochemical Society Transactions*, 33(4), pp. 863–866. doi: 10.1042/BST0330863.

Nagel, G., Brauner, M., *et al.* (2005) 'Light Activation of Channelrhodopsin-2 in Excitable Cells of *Caenorhabditis elegans* Triggers Rapid Behavioral Responses', *Current Biology*. Cell Press, 15(24), pp. 2279–2284. doi: 10.1016/J.CUB.2005.11.032.

Nakano, T. *et al.* (2005) 'Identification of a conserved 125 base-pair Hb9 enhancer that specifies gene expression to spinal motor neurons', *Developmental Biology*. Academic Press, 283(2), pp. 474–485. doi: 10.1016/J.YDBIO.2005.04.017.

NEB (2013) *DNA Modifying Enzymes Gibson Assembly Master Mix*. Available at: <https://www.neb.com/-/media/catalog/datacards-or-manuals/manuale2611.pdf> (Accessed: 3 September 2018).

Neely, M. D. *et al.* (2012) 'DMH1, a Highly Selective Small Molecule BMP Inhibitor Promotes Neurogenesis of hiPSCs: Comparison of PAX6 and SOX1 Expression during Neural Induction', *ACS Chemical Neuroscience*, 3(6), pp. 482–491. doi: 10.1021/cn300029t.

Neher, E. and Sakmann, B. (1976) 'Single-channel currents recorded from membrane of denervated frog muscle fibres', *Nature*. Nature Publishing Group, 260(5554), pp. 799–802. doi: 10.1038/260799a0.

Neitzel, H. (1986) 'A routine method for the establishment of permanent growing lymphoblastoid cell lines', *Human Genetics*. Springer-Verlag, 73(4), pp. 320–326. doi: 10.1007/BF00279094.

Neumann, M. *et al.* (2006) 'Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 314(5796), pp. 130–3. doi: 10.1126/science.1134108.

- Nishimura, A. L. *et al.* (2004) 'A Mutation in the Vesicle-Trafficking Protein VAPB Causes Late-Onset Spinal Muscular Atrophy and Amyotrophic Lateral Sclerosis', *The American Journal of Human Genetics*. Cell Press, 75(5), pp. 822–831. doi: 10.1086/425287.
- Nordström, U., Jessell, T. M. and Edlund, T. (2002) 'Progressive induction of caudal neural character by graded Wnt signaling', *Nature Neuroscience*, 5(6), pp. 525–532. doi: 10.1038/nn854.
- O'Rourke, J. G. *et al.* (2015) 'C9orf72 BAC Transgenic Mice Display Typical Pathologic Features of ALS/FTD.', *Neuron*. Elsevier, 88(5), pp. 892–901. doi: 10.1016/j.neuron.2015.10.027.
- Oesterhelt, D. and Stoeckenius, W. (1971) 'Rhodopsin-like protein from the purple membrane of Halobacterium halobium.', *Nature: New biology*, 233(39), pp. 149–52. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/4940442> (Accessed: 13 August 2018).
- O'Rourke, J. G. *et al.* (2016) 'C9orf72 is required for proper macrophage and microglial function in mice', *Science*, 351(6279), pp. 1324–1329. doi: 10.1126/science.aaf1064.
- Park, A. *et al.* (2016) 'Sendai virus, an RNA virus with no risk of genomic integration, delivers CRISPR/Cas9 for efficient gene editing.', *Molecular therapy. Methods & clinical development*. American Society of Gene & Cell Therapy, 3, p. 16057. doi: 10.1038/mtm.2016.57.
- Pasinelli, P. *et al.* (1998) 'Caspase-1 is activated in neural cells and tissue with amyotrophic lateral sclerosis-associated mutations in copper-zinc superoxide dismutase.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 95(26), pp. 15763–8. doi: 10.1073/pnas.95.26.15763.
- Patani, R. *et al.* (2011) 'Retinoid-independent motor neurogenesis from human embryonic stem cells reveals a medial columnar ground state.', *Nature communications*. Nature Publishing Group, 2, p. 214. doi: 10.1038/ncomms1216.
- Pearson, J. P. *et al.* (2011) 'Familial frontotemporal dementia with amyotrophic lateral sclerosis and a shared haplotype on chromosome 9p', *Journal of Neurology*, 258(4), pp. 647–655. doi: 10.1007/s00415-010-5815-x.
- Perlson, E. *et al.* (2009) 'A Switch in Retrograde Signaling from Survival to Stress in Rapid-Onset Neurodegeneration', *Journal of Neuroscience*, 29(31), pp. 9903–9917. doi: 10.1523/JNEUROSCI.0813-09.2009.
- Peters, O. M. *et al.* (2015) 'Human C9ORF72 Hexanucleotide Expansion Reproduces RNA Foci and Dipeptide Repeat Proteins but Not Neurodegeneration in BAC Transgenic Mice', *Neuron*, 88(5), pp. 902–909. doi: 10.1016/j.neuron.2015.11.018.
- Peviani, M. *et al.* (2012) 'Lentiviral vectors carrying enhancer elements of Hb9 promoter drive selective transgene expression in mouse spinal cord motor neurons.', *Journal of*

neuroscience methods, 205(1), pp. 139–47. doi: 10.1016/j.jneumeth.2011.12.024.

Phukan, J. *et al.* (2012) 'The syndrome of cognitive impairment in amyotrophic lateral sclerosis: a population-based study', *Journal of Neurology, Neurosurgery & Psychiatry*, 83(1), pp. 102–108. doi: 10.1136/jnnp-2011-300188.

Preux, P.-M. *et al.* (1996) 'Survival Prediction in Sporadic Amyotrophic Lateral Sclerosis', *Neuroepidemiology*, 15(3), pp. 153–160. doi: 10.1159/000109902.

Puls, I. *et al.* (2003) 'Mutant dynactin in motor neuron disease', *Nature Genetics*. Nature Publishing Group, 33(4), pp. 455–456. doi: 10.1038/ng1123.

Qu, Q. *et al.* (2014) 'High-efficiency motor neuron differentiation from human pluripotent stem cells and the function of Islet-1.', *Nature communications*. Nature Publishing Group, 5, p. 3449. doi: 10.1038/ncomms4449.

Rabin, S. J. *et al.* (2010) 'Sporadic ALS has compartment-specific aberrant exon splicing and altered cell–matrix adhesion biology', *Human Molecular Genetics*. Oxford University Press, 19(2), pp. 313–328. doi: 10.1093/hmg/ddp498.

Ramezani, A. and Hawley, R. G. (2002) 'Overview of the HIV-1 Lentiviral Vector System', in *Current Protocols in Molecular Biology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., p. 16.21.1-16.21.15. doi: 10.1002/0471142727.mb1621s60.

Reddy, K. *et al.* (2013) 'The disease-associated r(GGGGCC)_n repeat from the C9orf72 gene forms tract length-dependent uni- and multimolecular RNA G-quadruplex structures.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 288(14), pp. 9860–6. doi: 10.1074/jbc.C113.452532.

Renoncourt, Y. *et al.* (1998) 'Neurons derived in vitro from ES cells express homeoproteins characteristic of motoneurons and interneurons', *Mechanisms of Development*, 79(1–2), pp. 185–197. doi: 10.1016/S0925-4773(98)00189-0.

Renton, A. E. *et al.* (2011) 'A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD', *Neuron*. Cell Press, 72(2), pp. 257–268. doi: 10.1016/j.neuron.2011.09.010.

Ringholz, G. M. *et al.* (2005) 'Prevalence and patterns of cognitive impairment in sporadic ALS.', *Neurology*. Wolters Kluwer Health, Inc. on behalf of the American Academy of Neurology, 65(4), pp. 586–90. doi: 10.1212/01.wnl.0000172911.39167.b6.

Rizzu, P. *et al.* (2016) 'C9orf72 is differentially expressed in the central nervous system and myeloid cells and consistently reduced in C9orf72, MAPT and GRN mutation carriers.', *Acta neuropathologica communications*. BioMed Central, 4(1), p. 37. doi: 10.1186/s40478-016-0306-7.

Roelink, H. *et al.* (1995) 'Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis.', *Cell*, 81(3), pp. 445–55. Available at:

<http://www.ncbi.nlm.nih.gov/pubmed/7736596> (Accessed: 15 August 2018).

Rosen, D. R. *et al.* (1993) 'Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis.', *Nature*. Nature Publishing Group, 362(6415), pp. 59–62. doi: 10.1038/362059a0.

Rothstein, J. D. *et al.* (1995) 'Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis', *Annals of Neurology*, 38(1), pp. 73–84. doi: 10.1002/ana.410380114.

Sachs, F. and Auerbach, A. (1983) 'Single-Channel Electrophysiology: Use of the Patch Clamp', in *Methods in Enzymology*, pp. 147–176. doi: 10.1016/S0076-6879(83)03011-6.

Sances, S. *et al.* (2016) 'Modeling ALS with motor neurons derived from human induced pluripotent stem cells.', *Nature neuroscience*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 16(4), pp. 542–53. doi: 10.1038/nn.4273.

Sances, S. *et al.* (2018) 'Human iPSC-Derived Endothelial Cells and Microengineered Organ-Chip Enhance Neuronal Development.', *Stem cell reports*. Elsevier, 10(4), pp. 1222–1236. doi: 10.1016/j.stemcr.2018.02.012.

Sandor, C. *et al.* (2017) 'Transcriptomic profiling of purified patient-derived dopamine neurons identifies convergent perturbations and therapeutics for Parkinson's disease.', *Human molecular genetics*. Oxford University Press, 26(3), pp. 552–566. doi: 10.1093/hmg/ddw412.

Sareen, D. *et al.* (2013) 'Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion', *Science Translational Medicine*. NIH Public Access, 5(208). doi: 10.1126/scitranslmed.3007529.

Sasaki, S. and Iwata, M. (2007) 'Mitochondrial Alterations in the Spinal Cord of Patients With Sporadic Amyotrophic Lateral Sclerosis', *Journal of Neuropathology and Experimental Neurology*. Oxford University Press, 66(1), pp. 10–16. doi: 10.1097/nen.0b013e31802c396b.

Schludi, M. H. *et al.* (2015) 'Distribution of dipeptide repeat proteins in cellular models and C9orf72 mutation cases suggests link to transcriptional silencing', *Acta Neuropathologica*, 130(4), pp. 537–555. doi: 10.1007/s00401-015-1450-z.

Sellier, C. *et al.* (2016) 'Loss of C9ORF72 impairs autophagy and synergizes with polyQ Ataxin-2 to induce motor neuron dysfunction and cell death.', *The EMBO journal*. EMBO Press, 35(12), pp. 1276–97. doi: 10.15252/embj.201593350.

Selvaraj, B. T. *et al.* (2018) 'C9ORF72 repeat expansion causes vulnerability of motor neurons to Ca²⁺-permeable AMPA receptor-mediated excitotoxicity', *Nature Communications*. Nature Publishing Group, 9(1), p. 347. doi: 10.1038/s41467-017-02729-0.

- Sharma, K. *et al.* (1998) 'LIM Homeodomain Factors Lhx3 and Lhx4 Assign Subtype Identities for Motor Neurons', *Cell*, 95(6), pp. 817–828. doi: 10.1016/S0092-8674(00)81704-3.
- Sharma, R. *et al.* (2004) 'Gene Therapy : Current Concepts', *JK Science*, 6(2), pp. 62–6. Available at: <https://pdfs.semanticscholar.org/2d67/569b5406fa9319e5cf0cc95d1f3d5723006c.pdf> (Accessed: 2 September 2018).
- Shatunov, A. *et al.* (2010) 'Chromosome 9p21 in sporadic amyotrophic lateral sclerosis in the UK and seven other countries: a genome-wide association study.', *The Lancet. Neurology*, 9(10), pp. 986–94. doi: 10.1016/S1474-4422(10)70197-6.
- Shen, J. (2014) 'Function and Dysfunction of Presenilin', *Neurodegenerative Diseases*, 13(2–3), pp. 61–63. doi: 10.1159/000354971.
- Shi, Y. *et al.* (2018) 'Haploinsufficiency leads to neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons', *Nature Medicine*, 24(3), pp. 313–325. doi: 10.1038/nm.4490.
- Shin, S., Dalton, S. and Stice, S. L. (2005) 'Human Motor Neuron Differentiation from Human Embryonic Stem Cells', *Stem Cells and Development*, 14(3), pp. 266–269. doi: 10.1089/scd.2005.14.266.
- Silva, A. M. *et al.* (2015) 'Expanded GAA repeats impair FXN gene expression and reposition the FXN locus to the nuclear lamina in single cells.', *Human molecular genetics*, 24(12), pp. 3457–71. doi: 10.1093/hmg/ddv096.
- Singer, R. H. and Ward, D. C. (1982) 'Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinated nucleotide analog.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 79(23), pp. 7331–5. doi: 10.1073/PNAS.79.23.7331.
- Smith, B. N. *et al.* (2014) 'Exome-wide Rare Variant Analysis Identifies TUBA4A Mutations Associated with Familial ALS', *Neuron*. Cell Press, 84(2), pp. 324–331. doi: 10.1016/J.NEURON.2014.09.027.
- Smith, P. R. *et al.* (1993) 'Complex nature of the major viral polyadenylated transcripts in Epstein-Barr virus-associated tumors.', *Journal of virology*, 67(6), pp. 3217–25. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8098777> (Accessed: 29 August 2018).
- Sofroniew, M. V and Vinters, H. V (2010) 'Astrocytes: biology and pathology.', *Acta neuropathologica*. Springer, 119(1), pp. 7–35. doi: 10.1007/s00401-009-0619-8.
- Sreedharan, J. *et al.* (2008) 'TDP-43 Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis', *Science*, 319(5870), pp. 1668–1672. doi: 10.1126/science.1154584.
- Stenmark, H. (2009) 'Rab GTPases as coordinators of vesicle traffic', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 10(8), pp. 513–525. doi:

10.1038/nrm2728.

Stewart, H. *et al.* (2012) 'Clinical and pathological features of amyotrophic lateral sclerosis caused by mutation in the C9ORF72 gene on chromosome 9p', *Acta Neuropathologica*, 123(3), pp. 409–417. doi: 10.1007/s00401-011-0937-5.

Stifani, N. (2014) 'Motor neurons and the generation of spinal motor neuron diversity', *Frontiers in Cellular Neuroscience*, 8, p. 293. doi: 10.3389/fncel.2014.00293.

Sturtevant, A. H. (1913) 'The Linear Arrangement Of Six Sex-Linked Factors In *Drosophila*, As Shown By Their Mode Of Association', *JOURNAL OF EXPERIMENTAL ZOOLOGY*, 14, pp. 43--59. Available at: <http://citeseerx.ist.psu.edu/viewdoc/summary?doi=10.1.1.37.9595> (Accessed: 2 August 2018).

Suzuki, T. *et al.* (2003) 'Archaeal-type rhodopsins in *Chlamydomonas*: model structure and intracellular localization', *Biochemical and Biophysical Research Communications*. Academic Press, 301(3), pp. 711–717. doi: 10.1016/S0006-291X(02)03079-6.

Takahashi, K. *et al.* (2007) 'Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors', *Cell*. Cell Press, 131(5), pp. 861–872. doi: 10.1016/J.CELL.2007.11.019.

Takahashi, K. and Yamanaka, S. (2006) 'Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors', *Cell*, 126(4), pp. 663–676. doi: 10.1016/j.cell.2006.07.024.

Takazawa, T. *et al.* (2012) 'Maturation of Spinal Motor Neurons Derived from Human Embryonic Stem Cells', *PLoS ONE*. Edited by S. Hochman. Public Library of Science, 7(7), p. e40154. doi: 10.1371/journal.pone.0040154.

Takizawa, T. *et al.* (2008) 'Allele-specific nuclear positioning of the monoallelically expressed astrocyte marker GFAP.', *Genes & development*. Cold Spring Harbor Laboratory Press, 22(4), pp. 489–98. doi: 10.1101/gad.1634608.

Takizawa, T., Meaburn, K. J. and Misteli, T. (2008) 'The Meaning of Gene Positioning', *Cell*. Cell Press, 135(1), pp. 9–13. doi: 10.1016/J.CELL.2008.09.026.

Taneja, K. L. *et al.* (1995) 'Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues.', *The Journal of cell biology*. Rockefeller University Press, 128(6), pp. 995–1002. doi: 10.1083/JCB.128.6.995.

Taylor, J. P., Brown, R. H. and Cleveland, D. W. (2016) 'Decoding ALS: from genes to mechanism', *Nature*. Nature Publishing Group, 539(7628), pp. 197–206. doi: 10.1038/nature20413.

Telezhkin, V. *et al.* (2016) 'Forced cell cycle exit and modulation of GABA_A, CREB, and GSK3 β signaling promote functional maturation of induced pluripotent stem cell-derived neurons', *American Journal of Physiology - Cell Physiology*. American Physiological Society Bethesda, MD, 310(7), pp. C520–C541. doi: 10.1152/ajpcell.00166.2015.

- Teschendorf, C. *et al.* (2002) 'Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus.', *Anticancer research*, 22(6A), pp. 3325–3330. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12530082> (Accessed: 1 October 2018).
- Testa, D. *et al.* (2004) 'Survival of 793 patients with amyotrophic lateral sclerosis diagnosed over a 28-year period', *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*. Taylor & Francis, 5(4), pp. 208–212. doi: 10.1080/14660820410021311.
- Teyssou, E. *et al.* (2013) 'Mutations in SQSTM1 encoding p62 in amyotrophic lateral sclerosis: genetics and neuropathology', *Acta Neuropathologica*. Springer-Verlag, 125(4), pp. 511–522. doi: 10.1007/s00401-013-1090-0.
- Thor, S. *et al.* (1991) 'The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat', *Neuron*, 7(6), pp. 881–889. doi: 10.1016/0896-6273(91)90334-V.
- Tieng, V. *et al.* (2014) 'Engineering of Midbrain Organoids Containing Long-Lived Dopaminergic Neurons', *Stem Cells and Development*, 23(13), pp. 1535–1547. doi: 10.1089/scd.2013.0442.
- Tomkinson, B. and Kieff, E. (1992) 'Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro.', *Journal of virology*, 66(5), pp. 2893–903. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/1313908> (Accessed: 29 August 2018).
- Tomkinson, B., Robertson, E. and Kieff, E. (1993) 'Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation.', *Journal of virology*, 67(4), pp. 2014–25. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8445720> (Accessed: 29 August 2018).
- Valdmanis, P. N. *et al.* (2007) 'Three Families With Amyotrophic Lateral Sclerosis and Frontotemporal Dementia With Evidence of Linkage to Chromosome 9p', *Archives of Neurology*, 64(2), p. 240. doi: 10.1001/archneur.64.2.240.
- Vance, C. *et al.* (2006) 'Familial amyotrophic lateral sclerosis with frontotemporal dementia is linked to a locus on chromosome 9p13.2–21.3', *Brain*. Oxford University Press, 129(4), pp. 868–876. doi: 10.1093/brain/awl030.
- Vance, C. *et al.* (2009) 'Mutations in FUS, an RNA Processing Protein, Cause Familial Amyotrophic Lateral Sclerosis Type 6', *Science*. Europe PMC Funders, 323(5918), pp. 1208–1211. doi: 10.1126/science.1165942.
- Vink, C. A. *et al.* (2017) 'Eliminating HIV-1 Packaging Sequences from Lentiviral Vector Proviruses Enhances Safety and Expedites Gene Transfer for Gene Therapy', *Molecular Therapy*. doi: 10.1016/j.ymthe.2017.04.028.
- Vucic, S., Rothstein, J. D. and Kiernan, M. C. (2014) 'Advances in treating amyotrophic

lateral sclerosis: insights from pathophysiological studies', *Trends in Neurosciences*, 37(8), pp. 433–442. doi: 10.1016/j.tins.2014.05.006.

Waddington, C. H. (1936) 'Organizers in Mammalian Development', *Nature*. Nature Publishing Group, 138(3481), pp. 125–125. doi: 10.1038/138125a0.

Wainger, B. J. *et al.* (2014) 'Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons.', *Cell reports*. Elsevier, 7(1), pp. 1–11. doi: 10.1016/j.celrep.2014.03.019.

Waite, A. J. *et al.* (2014) 'Reduced C9orf72 protein levels in frontal cortex of amyotrophic lateral sclerosis and frontotemporal degeneration brain with the C9ORF72 hexanucleotide repeat expansion', *Neurobiology of Aging*. Elsevier, 35(7), p. 1779.e5-1779.e13. doi: 10.1016/J.NEUROBIOLAGING.2014.01.016.

Wang, G., Gilbert, J. and Man, H.-Y. (2012) 'AMPA receptor trafficking in homeostatic synaptic plasticity: functional molecules and signaling cascades.', *Neural plasticity*. Hindawi Limited, 2012, p. 825364. doi: 10.1155/2012/825364.

Watanabe, K. *et al.* (2007) 'A ROCK inhibitor permits survival of dissociated human embryonic stem cells', *Nature Biotechnology*, 25(6), pp. 681–686. doi: 10.1038/nbt1310.

Wen, X. *et al.* (2014) 'Antisense Proline-Arginine RAN Dipeptides Linked to C9ORF72-ALS/FTD Form Toxic Nuclear Aggregates that Initiate In Vitro and In Vivo Neuronal Death', *Neuron*. Elsevier, 84(6), pp. 1213–1225. doi: 10.1016/j.neuron.2014.12.010.

Westbrook, G. L. and Mayer, M. L. (1984) 'Glutamate currents in mammalian spinal neurons: resolution of a paradox.', *Brain research*, 301(2), pp. 375–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6145505> (Accessed: 26 November 2018).

Wichterle, H. *et al.* (2002) 'Directed Differentiation of Embryonic Stem Cells into Motor Neurons', *Cell*. Cell Press, 110(3), pp. 385–397. doi: 10.1016/S0092-8674(02)00835-8.

Wiedemann, F. R. *et al.* (2002) 'Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients', *Journal of Neurochemistry*. John Wiley & Sons, Ltd, 80(4), pp. 616–625. doi: 10.1046/j.0022-3042.2001.00731.x.

Williams, R. R. E. *et al.* (2006) 'Neural induction promotes large-scale chromatin reorganisation of the Mash1 locus.', *Journal of cell science*. The Company of Biologists Ltd, 119(Pt 1), pp. 132–40. doi: 10.1242/jcs.02727.

Williamson, T. L. and Cleveland, D. W. (1999) 'Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons', *Nature Neuroscience*. Nature Publishing Group, 2(1), pp. 50–56. doi: 10.1038/4553.

Wilmut, I. *et al.* (1997) 'Viable offspring derived from fetal and adult mammalian cells', *Nature*. Nature Publishing Group, 385(6619), pp. 810–813. doi: 10.1038/385810a0.

Witgert, M. *et al.* (2010) 'Frontal-lobe mediated behavioral dysfunction in amyotrophic lateral sclerosis', *European Journal of Neurology*. Wiley/Blackwell (10.1111), 17(1), pp.

103–110. doi: 10.1111/j.1468-1331.2009.02801.x.

Wong, J., Abilez, O. J. and Kuhl, E. (2012) 'Computational Optogenetics: A Novel Continuum Framework for the Photoelectrochemistry of Living Systems.', *Journal of the mechanics and physics of solids*. NIH Public Access, 60(6), pp. 1158–1178. doi: 10.1016/j.jmps.2012.02.004.

Wu, C.-H. *et al.* (2012) 'Mutations in the profilin 1 gene cause familial amyotrophic lateral sclerosis', *Nature*, 488(7412), pp. 499–503. doi: 10.1038/nature11280.

Wu, W. L., Ziskind-Conhaim, L. and Sweet, M. A. (1992) 'Early development of glycine- and GABA-mediated synapses in rat spinal cord.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 12(10), pp. 3935–45. doi: 10.1523/JNEUROSCI.12-10-03935.1992.

Xi, Z. *et al.* (2013) 'Hypermethylation of the CpG Island Near the G 4 C 2 Repeat in ALS with a C9orf72 Expansion', *The American Journal of Human Genetics*, 92, pp. 981–989. doi: 10.1016/j.ajhg.2013.04.017.

Xi, Z. *et al.* (2014) 'Identical twins with the C9orf72 repeat expansion are discordant for ALS.', *Neurology*. American Academy of Neurology, 83(16), pp. 1476–8. doi: 10.1212/WNL.0000000000000886.

Yoshimura, S. *et al.* (2010) 'Family-wide characterization of the DENN domain Rab GDP-GTP exchange factors.', *The Journal of cell biology*. Rockefeller University Press, 191(2), pp. 367–81. doi: 10.1083/jcb.201008051.

Yu, J. *et al.* (2007) 'Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells', *Science*, 318(5858), pp. 1917–1920. doi: 10.1126/science.1151526.

Zhang, Y.-J. *et al.* (2014) 'Aggregation-prone c9FTD/ALS poly(GA) RAN-translated proteins cause neurotoxicity by inducing ER stress', *Acta Neuropathologica*, 128(4), pp. 505–524. doi: 10.1007/s00401-014-1336-5.

Zhang, Y.-J. *et al.* (2016) 'C9ORF72 poly(GA) aggregates sequester and impair HR23 and nucleocytoplasmic transport proteins', *Nature Neuroscience*. Nature Publishing Group, 19(5), pp. 668–677. doi: 10.1038/nn.4272.

Zhang, Z.-N. *et al.* (2016) 'Layered hydrogels accelerate iPSC-derived neuronal maturation and reveal migration defects caused by MeCP2 dysfunction', *Proceedings of the National Academy of Sciences*, 113(12), pp. 3185–3190. doi: 10.1073/pnas.1521255113.

Zhao, S. *et al.* (2008) 'Improved expression of halorhodopsin for light-induced silencing of neuronal activity', *Brain Cell Biology*, 36(1–4), pp. 141–154. doi: 10.1007/s11068-008-9034-7.

Zhou, J. *et al.* (2010) 'High-Efficiency Induction of Neural Conversion in Human ESCs and Human Induced Pluripotent Stem Cells with a Single Chemical Inhibitor of Transforming

Growth Factor Beta Superfamily Receptors', *STEM CELLS*. Wiley-Blackwell, 28(10), pp. 1741–1750. doi: 10.1002/stem.504.

Zu, T. *et al.* (2013) 'RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia', *Proceedings of the National Academy of Sciences*, 110(51), pp. E4968–E4977. doi: 10.1073/pnas.1315438110.