

# Investigation of oligodendroglial pathology in amyotrophic lateral sclerosis

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# Abstract

*Background:* Amyotrophic lateral sclerosis (ALS) is a fatal, neurodegenerative disease. TDP-43 is found in pathological protein aggregates in neurons and glia in ALS and it is part of some mRNA transport granules. *MBP* messenger RNA (mRNA) must be transported to the oligodendrocyte processes for correct myelination. If TDP-43 were part of *MBP* mRNA transport granules, its aggregation could lead to loss of MBP in the CNS. Additionally, *C90rf72* is a gene whose GGGGCC expansion mutation causes ALS. The expansion binds hnRNP-A2, a protein essential for the transport of *MBP* mRNA. This interaction may lead to the sequestration of hnRNP-A2, reducing its availability and causing a shortage of MBP in the CNS.

*Aims:* To characterise the oligodendroglial pathology and the loss of MBP in our cohort of ALS cases, and to create zebrafish lines to investigate the development of oligodendroglial degeneration in sporadic ALS and *C9orf72* ALS. The overarching hypothesis of the project is that the observed oligodendrocyte degeneration in ALS is primarily caused by a dysfunction of *MBP* mRNA transport, causing demyelination.

*Methods & Results:* Using immunohistochemistry in human *post mortem* tissue, this project reports a greater, distinct ubiquitin-related glial pathology in the primary motor cortex of *C9orf72*-ALS cases. This *C9orf72*-related glial pathology was independent of the dipeptide-repeat protein inclusions usually found in the motor neurones of these patients. Using Western blot and qPCR, the levels of MBP and PLP, another myelin protein that is translated in the oligodendrocyte cell body, were measured. A reduction in the levels of MBP, but not those of PLP, was found in the spinal cord lateral corticospinal tracts of ALS cases, which was more pronounced in *C9orf72* ALS. This reduction of the MBP levels was not accompanied by either a reduction in the number of axons panning that area or the levels of *MBP* mRNA.

*Conclusions:* These results suggest that an impairment of mRNA transport is the main cause of the reduced MBP levels in the spinal cord of ALS patients and that oligodendrocyte degeneration precedes axonal degeneration in ALS. Were this confirmed using *in vivo* models of ALS, it would imply that ALS begins as a myelinopathy which later causes the apparition of neuronal pathology and death. None of the *in vivo* models trialled in this project were successful, but others have created promising models that will surely help understand oligodendroglial degeneration in ALS.

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# List of abbreviations

<b>3'E:</b> 3' entry	CNS: Central nervous system	
<b>5'E:</b> 5' entry	<b>CRISPR</b> : Clustered Regularly	
A2RE: A2 response element	Interspaced Short Palindromic Repeats	
AAV: Adeno associated virus	<b>CTP</b> : Cytosine triphosphate	
<b>AP:</b> Alkaline phosphatase	DAB: 3,3'-Diaminobenzidine	
<b>ABC</b> : Avidin-biotin complex	<b>DENN:</b> Differentially expressed in	
ALS: Amyotrophic lateral sclerosis	normal and neoplastic cells <b>DNA</b> : Deoxyribonucleic acid	
<b>C9ALS</b> : <i>C90rf72</i> ALS	·	
<b>fALS</b> : Familial ALS	<b>Dpf</b> : Days post-fertilisation	
sALS: Sporadic ALS	<b>dNTP</b> : Deoxynucleoside triphosphate	
<b>AMO:</b> Antisense morpholino oligonucleotides	<b>DPR</b> : Dipeptide repeat <b>EDTA</b> : Ethylenediaminetetraacetic acid	
<b>ATP</b> : Adenosine triphosphate	EGFR: Epidermal growth factor	
BAC: Bacterial artificial chromosome	receptor	
<b>BSA</b> : Bovine serum albumin	<b>ELISA</b> : enzyme-linked immunosorbent assay	
<b>C9HNR</b> : <i>C9orf72</i> hexanucleotide repeat	<b>FMR1</b> : fragile X mental retardation gene	
<b>C9orf72</b> : Chromosome 9 open reading	FTD: Frontotemporal dementia	
frame 72	<b>C9FTD</b> : <i>C90rf72</i> FTD	
<b>Cas9</b> : CRISPR associated protein 9	<b>FTLD</b> : Frontotemporal lobar	
<b>CNP</b> : 2',3'-Cyclic-nucleotide 3'-	degeneration	
phosphodiesterase		

FUS/TLS:	Fused	in	MCT1: Monocarboxy	late transporter 1
sarcoma/translocate	d in liposarcom	a	<b>ME:</b> Middle entry	
FXS: Fragile X synd:	rome		<b>MMEJ</b> : microhomol	logy-mediated end
FXTAS: Fragile X	-associated tre	mor	joining	
ataxia syndrome			<b>MND</b> : Motor neuron	e disease
<b>GDP</b> : Guanosine dip	ohosphate		MOBP:	Myelin-associated
<b>GFP</b> : Green fluoresc	ent protein		oligodendrocytic basi	c protein
<b>GM:</b> Grey matter			<b>MSP:</b> Multisystem pr	roteinopathy
GTP: Guanosine trip	ohosphate		NF: Neurofilament	
GWAS: Genome-wid	de association st	udy	NHEJ: non-homolog	gous end joining
HCM: Haematoxylir	n, clear and mou	int	Nogo-A: Neurite out	growth inhibitor A
HNR: Hexanucleotic	de repeat		NSC-34: Neuroblast	oma spinal cord
HR: homologous rec	combination		<b>OPC</b> : Oligodendrocy	te precursor cell
HRP: Horseradish p	oeroxidase		<b>OPTN</b> : Optineurin	
IgG: Immunoglobuli	in G		<b>OSP</b> : Oligodendrocyt	e-specific protein
IHC: Immunohistoc	hemistry		PAM: Protospacer-ad	ljacent motif
IMS: Industrial met	hylated spirit		<b>PBS</b> : Phosphate buff	er saline
<b>iPSC</b> : Induced pluri	potent stem cell		<b>PCR</b> : Polymerase cha	ain reaction
LMN: Lower motor	neurone		<b>qPCR</b> : Quant	itative PCR
MAG: Myelin-associ	iated glycoprote	in	<b>PFA:</b> Paraformaldehy	yde
<b>MAP</b> : Microtubule-a	associate protein	1	PLP: Proteolipid pro	tein
<b>MBP</b> : Myelin basic p	protein		<b>PSP</b> : Progressive sup	ranuclear palsy
MCS: Multicloning s	site			

**RanGAP**: Ran GTPase-activating protein

**RanGTP**: GTP-bound RAs-related nuclear protein

**RanGDP**: GDP-bound RAs-related nuclear protein

**RAN-translation**: Repeat-associated non-ATG translation

RBM45: RNA Binding Motif Protein 45

**RGNEF**: Rho guanine nucleotide exchange factor

**RIPA**: Radioimmunoprecipitation assay

**RLS**: RNA localisation signal

RNA: Ribonucleic acid

mRNA: Messenger RNA

rRNA: Ribosomal RNA

gRNA: Guide RNA

**RNP**: Ribonucleprotein

hnRNP: heteronuclear RNP

**ROI**: Region of interest

RRM: RNA recognition motif

**rSAP**: Recombinant shrimp alkaline phosphatase

**RT**: Room temperature

SSC: Salt-sodium citrate

sncRNA: Small non-coding RNA

SOD1: Superoxide dismutase 1

**ssODN**: single-stranded oligodeoxynucleotide donor template

**TALENS**: transcription activator-like effector nucleases

**TARDBP**: transactive response-DNA binding protein (gene)

TBS: Tris buffer saline

**TBST**: Tris buffer saline with tween

**TDP-43**: transactive response-DNA binding protein 43 KDa

**pTDP-43**: Phosphorylated TDP-

**TEMED**: N,N,N,N-tetramethylethylene diamine

TSC: Trisodium citrate

43

UMN: Upper motor neurone

UTP: Uracil triphosphate

UTR: Untranslated region

UV: Ultraviolet

WISH: Whole-mount in situ

hybridisation

**WM**: White matter

INTRODUCTION

#### **CHAPTER 1**

# Introduction

This project aims to determine whether impaired transport of the myelin basic protein (*MBP*) mRNA in oligodendrocytes is involved in the pathogenesis of amyotrophic lateral sclerosis (ALS).

#### 1.1 Amyotrophic lateral sclerosis

ALS is a fatal, adult-onset, degenerative motor neuron disease. It is the most common of the motor neuron diseases: the incidence of ALS found by studies using the commonlyused El Escorial criteria for ALS diagnosis (Brooks, 1994, Brooks et al., 2000) is 1.5-2 per 100,000 people per year (Marin et al., 2016), with a prevalence of 5-7 per 100,000 people worldwide (Mitchell and Borasio, 2007, McDermott and Shaw, 2008). About 95% of ALS cases have an unknown cause (sporadic ALS or sALS) while 5% have a family background of the disease (familial ALS or FALS; Byrne et al., 2011). There is an increasing number of genes which have been linked to ALS (**table 1.1**), some of which have been used to model this disease in animals (e.g. in mice or zebrafish) and in cell lines. Some of the most commonly linked and studied genes are Cu/Zn superoxide dismutase 1 (*SOD1*), chromosome 9 open reading frame 72 (*C90rf72*), transactive response-DNA binding protein 43 (*TARDBP*) and fused in sarcoma/translocated in liposarcoma (*FUS/TLS*). Although ALS has historically been regarded as a disorder of the neurones of the central nervous system (CNS), nowadays there is mounting evidence which supports the notion that ALS is also caused by a dysfunction of glial cells.

# 1.1.1 Clinical features of ALS

Classic ALS is characterized by a loss of the combined function of the upper (brain) and lower (brain stem and spinal cord) motor neurons (UMN and LMN respectively, (McDermott and Shaw, 2008)). However, some cases present with failure only in UMN, which can develop into primary lateral sclerosis or pseudobulbar palsy (central), or only in LMN, which can become a progressive muscular atrophy or a bulbar palsy (peripheral).

ALS is localized in onset, usually affecting a particular group of muscles first (McDermott and Shaw, 2008). Depending on the region of onset, it can be defined as limb, bulbar or respiratory. While respiratory onset is found in a small population of ALS cases (2.7%) and bulbar onset only affects about 1 in 5 cases, limb onset, is the most prevalent and affects 78% of cases. Limb onset is mainly characterised by a decrease in the motor function of the arms or legs. This can significantly hinder the ability to perform ordinary tasks such as standing up, walking, holding small objects or manipulating things with one hand. Bulbar onset usually results in deficits in glossal muscles. Because of this, patients tend to find trouble articulating words to form speech, often presenting with fasciculation of the tongue and with dysphagia at a later stage. Patients with respiratory onset disease present with apnoea, night hypoventilation, and other respiratory complications which are the cause of some sleeping disorders and, eventually, death by respiratory dysfunction.

# 1.1.2 Genetics of ALS

A number of genes have been found to harbour heritable pathogenic mutations in familial ALS, with *SOD1*, *TARDBP*, *FUS* and *C90rf72* being the most commonly mutated and the most studied. Although the ALS-causing mutations found in some of these genes are very common in familial ALS, they have also been found in many cases of sporadic ALS. Other genes which have also been linked to fALS and sALS are shown in **table 1.1**. Some of these genes whose mutations cause ALS could be related to mRNA transport, in

particular, *TARDBP*, *C9orf72* and *FUS*, whose main functional, mutational and pathological aspects will be discussed below.

# 1.1.3 Pathology of ALS

The pathological picture of ALS includes degeneration of the upper and lower motor neurones, with other brain areas involved in many cases. Neurones and glia in affected regions accumulate proteins in pathological cytoplasmic inclusions. In addition, the axons of the affected neurones degenerate. These cytoplasmic inclusions contain hyperphosphorylated TDP-43 (pTDP-43) and p62. p62 is a ubiquitin-binding protein involved in protein degradation through the proteasome pathway which can be labelled to visualise misfolded protein aggregation. Inclusions tend to be round and compact or skein-like in shape or take the form of preinclusions which show depletion of nuclear TDP-43 with translocation to the cytoplasm as a fine, diffuse granularity throughout the cell soma (Mackenzie et al., 2013). Four stages have been defined in ALS regarding TDP-43 pathology (Brettschneider et al., 2013):

In stage 1, projection neurones from the neocortex, neurones from layers II, III, V, and VI of the motor cortex, large motor neurones from the spinal cord ventral horns, and the nuclei for cranial nerves V, VII, X, XI, and XII, are all immunoreactive for pTDP-43 cytoplasmic inclusions. Oligodendrocytes supporting these neurones also show this type of inclusions.

In stage 2, the above-defined neuronal pathology extends to the prefrontal cortex, some of the precerebellar nuclei, the reticular formation, dopaminergic neurones of the substantia nigra pars compacta. Oligodendrocytes that accompany neuronal projections from the precerebellar nuclei to the cerebellum also display pTDP43 cytoplasmic aggregation in stage 2.

Stage 3 displayed pTDP-43 involvement of the prefrontal granular cortex, where only neurones in layer IV are spared, and by the extension of this pathology to sensory areas of the parietal and temporal cortices, where only pyramidal cells and their myelinating oligodendrocytes present pTDP-43 pathology. The inferior colliculus, the different nuclei of the striatum, and the projection neurones of the claustrum also present with pTDP-43 pathology.

Stage 4 is characterised by an extensive pTDP-43 pathology in certain neuronal groups of the anteromedial temporal lobe and in the hippocampus. The hippocampal involvement is outlined by pTDP-43 inclusions in the dentate fascia granular cells and in the pyramidal neurones of the Ammon's horn. Some stage 4 cases also show cerebellar involvement, particularly in the dentate nucleus.

Macroscopically, ALS brains do not usually have striking features, although many have atrophy of the ventral nerve roots and medullary pyramids. A few have atrophy of the precentral gyrus, and ALS patients with overlapping frontotemporal dementia (FTD) may show a distinctive atrophy of the temporal cortex (bilateral superior temporal gyri and right temporal pole) and the frontal cortex (left middle and inferior frontal gyri, and medial premotor cortex).

Some pathological marks are explicitly linked to certain ALS-causing genetic mutations, and they will be discussed below in their correspondent sections.

GENE	GENE NAME	CHROMOSOME
SYMBOL		
SOD1	Cu/Zn superoxide dismutase 1	21q22.11
ALS2	Amyotrophic lateral sclerosis 2	21q22.11
ALS3	Unknown	18q21
SETX	Senataxin	9q34.13
SPG11	Spastic paraplegia 11	15q14
FUS	Fused in sarcoma	16p11.2
ALS7	Unknown	20p13
VAPB	Vesicle-associated membrane protein-associated protein B	20q13.33
ANG	Angiogenin	14q11.1
TARDBP	TAR DNA binding protein	1p36.22
FIG4	FIG4 homolog, SAC1 lipid phosphatase domain containing	6q21
OPTN	Optineurin	10p13
ATXN2	Ataxin 2	12q23-q24.1
VCP	Valosin-containing protein	9p13
UBQLN2	Ubiquilin 2	Xp11.21
SIGMAR1	Sigma non-opioid intracellular receptor 1	9p13
CHMP2B	Chromatin modifying protein 2B	3p12.1
PFN1	Profilin 1	17p13.3
ERBB4	V-erb-b2 avian erythroblastic leukemia viral oncogene	2q33.3-q34
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	12q13.1
MATR3	Matrin 3	5q31.2
C90rf72	Chromosome 9 open reading frame 72	9p21.2
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10	22q11.23
UNC13A	Unc-13 homolog A	19p13.12
DAO	D-amino-acid oxidase	12q24
DCTN1	Dynactin	2p13
NEFH	Neurofilament, heavy polypeptide 200kda, heavy chain	22q12.1-q13.1
PRPH	Peripherin	12q12
SQSTM1	Sequestosome 1	5935
TAF15	TAF15 RNA polymerase II	17q11.1-q11.2
SPAST	Spastin	2p24
ELP3	Elongation protein 3 homolog	8p21.1
LMNB1	Lamin B1	5q23.2
SARM1	Sterile Alpha and TIR Motif Containing 1	17q11.2
MOBP	Myelin-associated oligodendrocytic basic protein	3p22.1

**Table 1.1: Major known ALS-linked genes.** List obtained from ALSoD (Abel et al., 2013, Abel, 2013) and the latest ALS GWAS study (van Rheenen et al., 2016).

#### 1.2 TDP-43

#### 1.2.1 TDP-43 structure and functions

*TARDBP* is the gene which encodes transactive response-DNA binding protein 43 kDa (TDP-43). TDP-43 is the protein which has most commonly been found in pathologic ubiquitinated neural inclusions in ALS. The proportion of ALS patients carrying at least one mutant *TARDBP* allele is 4% of fALS cases and 2.5% of sALS cases (Mackenzie et al., 2010).

TDP-43 is an RNA/DNA-binding protein involved in the regulation of many steps of RNA processing, from transcription and alternative splicing to transport, translation and degradation (reviewed by Laggier-Tourenne, 2010). TDP-43 exists mostly as a thermostable homodimer. It comprises two RNA recognition motif domains (RRM-1 and RRM-2), a glycine-rich C-terminus (Kuo et al., 2009), and nuclear export and import signals (figure 1.1). These allow TDP-43 to shuttle in and out of the nucleus in a manner dependent on its association with mRNA and/or other proteins (Ayala et al., 2008). The RRMs confer TDP-43 with the ability to bind preferentially to TG/UG-rich segments of mRNA or single-stranded (uncoiled) DNA, and thereby form dimers (Kuo et al., 2009, Buratti and Baralle, 2001). Multiple studies have found that only the longer glycine-rich domain-containing isoforms of TDP-43 are involved in splicing regulation, as this domain confers the ability to establish direct interactions with other mRNA-processing proteins (Ayala et al., 2005, Wang et al., 2004). Shorter recombinant isoforms which lack the glycine-rich C-terminus do not seem to have their (ribo)nucleic acid-binding function impaired (Buratti and Baralle, 2001), and therefore they could be responsible for other TDP-43 functions such as transcriptional and translational control of target genes and mRNAs.

In addition, Bentmann et al. (2012) have shown that the C-terminus is necessary for recruiting TDP-43 to stress granules in the cytoplasm. Stress granules are mRNAarresting RNP structures which are formed after stress to avoid translation and regulate cell metabolism. TDP-43 self-regulates its own mRNA transcription levels through the use of a negative feedback loop: TDP-43 protein binds the *TARDBP* mRNA transcript via the 3' untranslated region (UTR; Ayala et al., 2011).

#### 1.2.2 ALS pathology related to TDP-43

Immunohistochemical studies have shown that TDP-43 can be found in both the nucleus and the cytoplasm, although the nucleus harbours a much higher concentration in normal non-mutant cells (Ince et al., 2011). When motor neurons are stained with an anti-TDP-43 antibody, *TARDBP* ALS mutants, sporadic ALS cases and many fALS cases show nuclear depletion of TDP-43 in many neurones which instead can be found in the cytoplasm as aggregates of a hyperphosphorylated form (Neumann et al., 2006). This is a feature shared with other diseases, known as the TDP-43 proteinopathies (Geser et al., 2010), such as FTLD.

#### 1.3 FUS

#### 1.3.1 FUS structure and functions

The *FUS* gene encodes a protein known as Fused in sarcoma/translocated in liposarcoma (FUS/TLS, from now on referred to as FUS). Fifty-eight pathogenic mutations have been identified with a frequency of about 5% in fALS cases, and less than 1% in fALS cases (Lattante et al. 2013). Most of the pathogenic mutations found in ALS occur in the well-conserved C-terminus, which has been identified as an indispensable part of FUS for DNA/RNA binding and alternative splicing modulation (Zinszner et al., 1997, Perrotti et al., 1998, Crozat et al., 1993).

FUS is a DNA/RNA-binding protein. Among other functions, FUS regulates the transcription and splicing of thousands of target genes (Lagier-Tourenne et al., 2012), while outside the nucleus it has been shown to form part of stress granules and RNA transport granules, regulating localised translation in cell processes (Yasuda et al., 2013,

Dormann et al., 2010). The structural properties which confer FUS these properties are (**figure 1.1**): an RNA recognition motif; a zinc-finger domain; a nuclear localisation motif recognised by transportin; a glycine-rich domain; a glutamine-glycine-serine-tyrosine-rich domain; and an arginine-glycine-glycine-rich domain (Mackenzie et al., 2010, Dormann et al., 2010).

# 1.3.2 ALS pathology related to FUS

FUS is localised to both the nucleus and the cytoplasm and constantly shuttles between them (Zinszner et al., 1997, Andersson et al., 2008). FUS immunohistochemistry of control cases shows strong staining in the nucleus of neurons and glia, and fainter staining in their cytoplasm, indicating that a much higher concentration of FUS is usually found in the nucleus. Some of the FUS mutations lead to FUS aggregation in the form of cytoplasmic inclusions accompanied by nuclear depletion of FUS, but cases of ALS without mutant FUS showed just the standard pattern of FUS staining (Hewitt et al., 2010).

40 A.	A							
TDP-4	NLS 3 82 98 106	RRM1 RRM2	2 NES 262 274	G-Rich	414			
40 A.	A							
	Q-G-S-Y-Rich	G-Ric	ch NES	RRM	R-G-Rich	ZnF R-G-F	Rich NLS	
<sup>1</sup> FUS/T	LS	165	267 285	3	71 422	453	501 98 526	
Domain key		Glutamine-Gly	cine-Serine-Tyrosine-ric	ch				
RNA recognition motif		Arginine-Glyci	Arginine-Glycine-rich					
Nuclear export signal		Zinc finger	Zinc finger					
Glycine-rich		Nuclear localis	Nuclear localisation sequence					

**Figure 1.1: TDP-43 and FUS/TLS protein domains.** Each of the two TDP-43 monomers that make the protein contains a nuclear localisation sequence, an RNA recognition motif, a nuclear export sequence, and a glycine-rich N-terminus. FUS/TLS is similar, containing all of the TDP-43 domains in addition to a Glu-Gly-Ser-Tyr-rich domain, an Arg-Gly-rich domain, and a zinc finger.

#### 1.4 C9orf72

Chromosome 9 open reading frame 72 (*C9orf72*) is the gene which encodes the homonym protein C9orf72. *C9orf72* is an extremely well-conserved gene across species and the most commonly mutated gene in fALS and sALS accounting for around 37.6% of fALS cases, and 6.3% of sALS cases worldwide (Majounie et al., 2012). Around 29.3% of patients with *C9orf72* mutations develop signs of both ALS and FTD, and this phenotype is diagnosed as ALS-FTD. This gene also seems to be the most commonly mutated in FTD. It is unclear what factors determine whether a patient harbouring the mutation develops ALS, FTD or both (van Blitterswijk et al., 2012). This is exemplified by a case of genetically identical twins harbouring the mutant *C9orf72* gene, of whom one of them developed ALS while the other developed FTD (Xi et al., 2014). This could indicate that there must be *C9orf72*-independent factors which intervene in the development of either disease in patients with the mutation.

# 1.4.1 C9orf72 functions

The functions C9orf72 performs are starting to be understood. Under normal conditions, there are three transcript variants of the *C9orf72* mRNA (**figure 1.2A**). Two of these transcripts (V2, V3) encode for the 481 a.a. isoform (*C9orf72*L) while the remaining transcript (V1) encodes for the 222 a.a. isoform (*C9orf72*S) (DeJesus-Hernandez et al., 2011). At the structural level, Levine et al. (2013) determined that the C-terminus of C9orf72 contains the majority of the secondary structures found in "differentially expressed in normal and neoplastic cells" (DENN) proteins (**figure 1.2B**), which are known to interact and regulate proteins of the Rab family. They, therefore, predicted that C9orf72 might be associated with the fine tuning of membrane trafficking by modulating Rab GTPase activity.

This Rab autophagocytic pathway has recently been explored by multiple authors. Yang et al., (2016) and Sullivan et al. (2016) found that the DENN domain of C9orf72 allows it to form a complex with SMCR8, WDR41, and ATG101, which regulates ULK1-dependent activation of autophagosomes. At the same time, Webster et al. (2016) found that this complex is responsible for recruiting the ULK1-dependent complex to active Rab1a, thus initiating the formation of the autophagosome. The C9orf72/SMCR8 complex also acts as a GTP-exchange factor for Rab8a and Rab39b (Yang et al., 2016, Corbier and Sellier, 2016), thus facilitating other downstream reactions involved in the formation of the autophagosome. Total or partial ablation of C9orf72 in different cell types has been shown to impair the formation of autophagosomes (Yang et al., 2016, Webster et al., 2016, Ugolino et al., 2016, Sullivan et al., 2016), consistent with these findings.

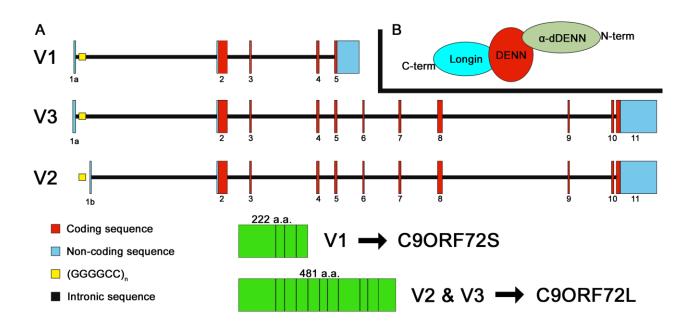
In addition, C9orf72 has also been recently shown to regulate actin dynamics in motor neurones by promoting the dephosphorylation of cofilin, a modulator of actin fibre synthesis (Sivadasan et al., 2016).

In summary, C9orf72 seems to play a role in the regulation of the immune system homeostasis, in the initiation of the autophagocytic pathway, and in the regulation of the synthesis of actin fibres in the neuronal growth cone.

# 1.4.2 C9orf72 mutation in ALS

The mutation harboured by the *C9orf72* gene in ALS and FTD is a large hexanucleotide (GGGGCC) expansion located in intron 1 (**figure 1.2A**). This was first reported by Renton et al. (2011) and DeJesus-Hernandez et al. (2011). However, linkage analysis done in multiple studies had reported that a locus in the short arm of chromosome 9 was linked to ALS and FTD (Vance et al., 2006, Morita et al., 2006, Boxer et al., 2011, Pearson et al., 2011). The mutant expansion is defined as anything more than 30 repeats according to Garcia-Redondo et al. (2013), but the range of the repeats usually is in the region of 700 to 1500 (Buchman et al. (2013).

The mechanisms by which expanded *C9orf72* exerts its pathogenic activity are still to be concluded. The three principal mechanisms which have been proposed so far are described below.



**Figure 1.2: Human C9orf72 mRNA and protein domains.** A shows the three different unspliced mRNA transcripts for *C9orf72*, including the (GGGGCC)<sub>n</sub> repeats in intron 1 of splice variants V1 and V3, but not in V2. The protein isoforms translated from each of the splice variants are below them in green. **B** depicts the three putative domains of C9orf72: A longin domain at the C terminus followed by a DENN domain and an alpha dDENN domain at the N terminus.

# 1.4.3 Toxic gain of function of the expanded mRNA

Nuclear foci formed of sense and antisense forms of the expanded *C9orf72* mRNA have been observed in neurones and glial cells from motor cortex, frontal cortex, hippocampus, spinal cord, and cerebellum of ALS patients harbouring the GGGGCC expansion (DeJesus-Hernandez et al., 2011, Donnelly et al., 2013, Gendron et al., 2013, Zu et al., 2013, Mizielinska et al., 2013). The foci have also been shown to occur in patient-derived fibroblasts, lymphoblasts, peripheral blood leukocytes, and pluripotent cell-derived neurones (Donnelly et al., 2013, Lagier-Tourenne et al., 2013, Zu et al., 2013). In addition, HEK293T cells transfected with (GGGGCC)<sub>66</sub> RNA (Gendron et al., 2013), and NSC34 (motor neurone-like) cells with tetracycline-induceable interrupted (GGGGCC)<sub>102</sub> repeats (Stopford et al., 2017) also show such foci. These foci are formed by the hexanucleotide repeat expansion mainly, without most of the flanking regions (Donnelly et al., 2013).

*In vitro*, sense (GGGGCC) repeats form tract length-dependent uni- and multimolecular RNA G-quadruplex structures and single and double R-loops during the transcription process between the nascent RNA and the template DNA strand (Fratta et al., 2012, Reddy et al., 2013, Sket et al., 2015, Reddy et al., 2014). Likewise, the antisense (CCCCGG) repeats form C-protonated hairpins and i-motifs (Kovanda et al., 2015). These structures are thermodynamically favoured by the formation of hydrogen bonds between four and two guanine bases respectively in the sense mRNA, and between two cytosine residues in the antisense mRNA. Nuclear foci formed by these stable structures have been hypothesised to bind and sequester RNA-binding proteins.

Multiple studies have shown that a vast number of RNA-binding proteins are capable of binding sense and antisense GGGGCC tracts in vitro (Haeusler et al., 2014, Donnelly et al., 2013, Lee et al., 2013, Sareen et al., 2013, Xu et al., 2013, Cooper-Knock et al., 2015, Cooper-Knock et al., 2014, Mori et al., 2013b, Rossi et al., 2015). So far, only some of these proteins have been shown to co-localise with (GGGGCC)<sub>n</sub> nuclear foci in tissue derived from patients. These include members of the heterogeneous nuclear

ribonucleoproteins family (A1, F, H, K, U) as well as ALYREF, FUS, TDP-43, Pur- $\alpha$ , ADARB2, SC35, nucleolin, SRSF2 and ILF3. The binding of different proteins to *C9orf72* foci such as hnRNPs-A2/B1, hnRNP-H, eIF2 $\alpha$ , eIF2 $\beta$ , and RAX has been confirmed by pull-down assays using cell lysates (Rossi et al., 2015, Conlon et al., 2016). Additionally, spectrophotometric assays determined that the heme factor binds to (GGGGCC)<sub>4</sub> quadruplexes, activating its catalytic activity as an oxidative molecule (Grigg et al., 2014).

#### 1.4.4 Haploinsufficiency

Expansions in the *C9orf72* gene cause an inefficient translation of both isoforms of the *C9orf72* mRNA. At the transcriptional level, transcript variant 2 of *C9orf72*, which encodes the long isoform and is the main *C9orf72* transcript, appears to undergo reduced transcription in C9ALS patients' tissue (DeJesus-Hernandez et al., 2011, Gijselinck et al., 2012, van Blitterswijk et al., 2015, Donnelly et al., 2013, Haeusler et al., 2014, Ciura et al., 2013, Tran et al., 2015, Belzil et al., 2013, Waite et al., 2014, Fratta et al., 2013). This was also observed for human iPSC-derived motor neurones (Donnelly et al., 2013).

Whether a reduction in C9orf72 protein levels is the primary cause of C9ALS has been tested using knock-out and knock-down models. In *c. elegans*, ablation of the *C9orf72* ortholog, *alfa-1* resulted in the specific degeneration of GABAergic motor neurones, which led to age-dependent motor defects (Therrien et al., 2013). However, drawing conclusions about possible haploinsufficiency in human C9ALS from this model seems unreliable since its ortholog shares limited homology with that of humans (23%). In zebrafish, the *zgc:100846* gene is the ortholog for *C9orf72*, sharing a 76% homology. Knockdown of *zgc:100846* which targeted the ATG initiation codons, resulted in a reduced motility and axonopathy (Ciura et al., 2013). Zebrafish knockdown, however, requires confirmation with genetic mutants due to possible off-target effects (Robu et al., 2007). Some other groups have studied the effects of *C9orf72* ablation both ubiquitously and in neuronal and glial cells specifically using mouse models, whose *C9orf72* ortholog (henceforth *C9orf72*) is 98% similar to humans. The studies which targeted only brain cells show that a depletion of *C9orf72* in the brain is not sufficient to cause ALS

phenotypes or pathology (Lagier-Tourenne et al., 2013, Koppers et al., 2015). Total ablation of *C90rf72* in mice resulted in immune dysregulation, but the mice did not develop motor neurone disease phenotypes or pathology (O'Rourke et al., 2016, Sullivan et al., 2016, Sudria-Lopez et al., 2016, Burberry et al., 2016). One study showed mild neuronal deficits and multiple anomalies in the immune system in knock-out mice, but not a motor neurone disease phenotype (Atanasio et al., 2016). Amongst the immune system anomalies, they detected a generalised increase in the amount of immune cells and a rise in the levels of some cytokines and chemokines such as interleukins, tumour necrosis factor alpha, and monocyte chemoattractant protein 1. Interestingly, they also found that knock-out mice produce a significantly higher amount of autoantibodies for the rheumatoid factor protein. Therefore, the current consensus appears to be that haploinsufficiency contributes to the development of the C9ALS, but it is not the primary cause of the disease.

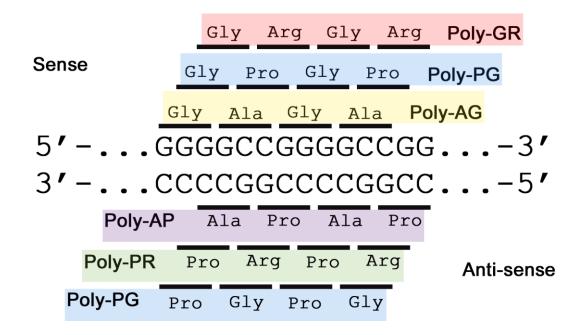
# 1.4.5 Toxic accumulation of RAN-translated polypeptides

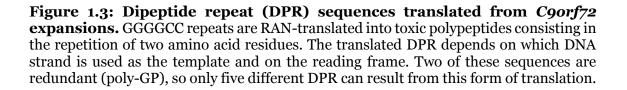
Repeat-associated non-ATG initiated (RAN) translation is a form of polypeptide synthesis which does not depend on an AUG codon. RAN-translation has been demonstrated in other repeat-associated diseases such as fragile X-associated tremor ataxia syndrome (FXTAS) and fragile X syndrome (FXS): A CGG repeat expansion in the 5' UTR of the fragile X mental retardation gene (*FMR1*) causes the translation of poly-Gly or poly-Ala, depending on the reading frame used by the ribosome. The size of this expansion determines whether the carrier will be more prone to develop FXTAS or FXS. FXS is associated with what is known as a full mutation, meaning that the expansion contains over 200 repeats (Yu et al., 1991, Oberle et al., 1991). However, carriers of the premutation, consisting of 55-200 repeats, are more prone to develop FXTAS, and the penetrance will be dependent on age and repeat size (Jacquemont et al., 2004). Initiation of RAN translation of the *FMR1* expansion seems to occur at a near-cognate ACG codon upstream the CGG repeats (Sellier et al., 2017). RAN-translation of the C9ALS-associated GGGGCC expansion also causes the synthesis of sense and antisense insoluble dipeptide

repeats (DPR) which aggregate in neurones and glia in the brain of both C9ALS and C9FTD patients (Mori et al., 2013a, Mori et al., 2013c, Ash et al., 2013, Zu et al., 2013). The protein sequence of the DPR depends on the reading frame from which it is translated, which gives rise to a total of 5 different DPR (figure 1.3). These are -in descending order of the frequency in which they are found aggregated in the brain-poly-GA, poly-GP, poly-GR, poly-PA and poly-PR. From those, arginine-containing DPR (poly-GR and poly-PR) have been shown to be the most neurotoxic when aggregated after overexpression in cultured neurones, yeast, and drosophila. Arg-rich DPR have been mainly found to localise within the nucleolus. They interact with nuclear and nucleolar proteins, inducing nucleolar stress, which causes impairment of normal gene expression, including the dysregulation and silencing of mRNAs (Tao et al., 2015). Nucleolar stress is usually denoted by the cytoplasmic translocation of B23 (Yao et al., 2010), which is responsible for ribosome biogenesis, promotes cell survival and regulates cell cycle progression (Lee et al., 2008, Lindstrom, 2011). Arg-rich DPR aggregation in the nucleus also causes the enlargement and dysfunction of the nucleolus in neurones (Mizielinska et al., 2017). The relevance of poly-GR aggregation seems more relevant than that of poly-PR because while poly-GR is found frequently in post mortem ALS/FTD brains, poly-PR is rarely found (Mackenzie et al., 2015, Schludi et al., 2015).

Surprisingly, poly-GA, the most commonly found form of DPR, appears to reduce the toxicity caused by arginine-rich DPR in human induced-pluripotent stem cell-derived neurones and *drosophila*. Poly-GA (15 and 50 repeats) was also found to form toxic fibrils in solution and to cause motor deficits in mice when expressed using an adeno associated virus (AAV) viral system injected in the cerebral ventricles. It remains to be ascertained whether the level of expression reached using viral vectors is similar to that of human C9ALS and whether it produces neurotoxicity at normal pathological levels (as opposed to overexpression levels) in the human brain.

It would be expected to find correlations between neuronal death or disease severity and the degree of DPR accumulation if DPR were indeed the main cause of C9ALS/FTD pathogenesis. However, even with DPR being prevalent in some key areas of these patients' brains (Mackenzie et al., 2013), the relative infrequency of the most toxic, Argrich DPR species, suggests that they could merely be pathological hallmarks in some regions of the brain, but be of no neurotoxic consequence. Opposing this view, there is also the possibility that most of the neurones and glial cells containing these aggregates die before the patient does, causing ALS/FTD phenotypes and rendering neuropathologists unable to detect them *post mortem*.





# 1.4.6 Nucleocytoplasmic transport and the C9orf72 expansion

Common ground for the mRNA and DPR toxic gain of function hypotheses seems to be the impairment of nucleocytoplasmic transport. As stated above, the most commonly aggregated protein in ALS cytoplasmic inclusions, TDP-43, is constantly shuttled between the nucleus and the cytoplasm, and so do other proteins and molecules. This transport requires GTP-bound RAs-related nuclear protein (RanGTP) to hydrolyse its bound GTP to GDP, forming RanGDP. Otherwise, importins would be inhibited in the cytoplasm, disabling cytoplasm-to-nucleus transport. Ran GTPase-activating protein (RanGAP) is essential to activate the catalytic function of RanGTP. The cytoplasmic accumulation and dysfunction of RanGAP and RanGTP, and their co-localisation with expanded C90rf72 mRNA foci have been demonstrated in C9ALS brain tissue as well as in patient iPSC-derived neurones and Drosophila models harbouring expanded C9orf72 (Zhang et al., 2015). Also, the severity of RanGTP mislocalisation correlates with the severity of TDP-43 nuclear depletion (Winton et al., 2008), meaning that TDP-43 keeps being transported out of the nucleus without making its way back in. Also, nuclear depletion of TDP-43 aggregation seems to accumulate in cells with expanded C9orf72 mRNA foci (Cooper-Knock et al., 2015). This all seems to indicate that expanded C9orf72 mRNA binds and sequesters some essential factors for the active transport of macromolecules between the nucleus and the cytoplasm. However, there is also evidence that RAN-translated poly-GA DPR can cause mislocalisation of RanGAP and nuclear envelope proteins just by itself (Zhang et al., 2016b). Cytoplasmic poly-GA also inhibits the nuclear import of transcription factor p65 and promotes the cytoplasmic aggregation of TDP-43 in neurones (Khosravi et al., 2017). This phenotype can be rescued by inducing the overexpression of importin-alpha and nuclear pore components. Furthermore, directing poly-PA to the nucleus avoids TDP-43 mislocalisation to the cytoplasm (Khosravi et al., 2017). Another study showed a lower import rate in the nucleus caused by poly-PR and poly-GR (arginine-rich) using NSC-34 cells (Shani et al., 2017). Therefore, cytoplasmic aggregation of poly-GA and expanded C90rf72 mRNA both contribute to ALS pathogenesis by disrupting nucleocytoplasmic transport.

# 1.4.7 Pathology in C9orf72 expansion-carrying patients

Surprisingly, ALS cases harbouring *C90rf72* mutations show a pathological pattern which seems qualitatively very similar (but quantitatively greater) from patients not harbouring the expansion. Additionally, as described above, RAN-translated DPR also aggregate in C9ALS to form cytoplasmic and intranuclear neuronal inclusions which are negative for TDP-43, but positive for proteins related to the proteasome such as p62 and ubiquitin (Mackenzie et al., 2013, Zu et al., 2013, Mori et al., 2013c, Gendron et al., 2013, Ash et al., 2013). This neuronal aggregation accompanied by dystrophic neurites is extended in the cortex from the prefrontal cortex all the way to the visual cortex in the occipital lobe. The hippocampus is an extramotor area where TDP-43 and OPTN are usually good markers for protein inclusion pathology at stage 4, showing little involvement in the rest of the stages. However, neither of them can pick up all the abundant intra- and extra-nuclear neuronal inclusions which are labelled for p62 and DPR in granular and Purkinje cells of the cerebellum and the pyramidal neurones of the hippocampus found in C9ALS (Cooper-Knock et al., 2012, Mackenzie et al., 2014, Ash et al., 2013).

## 1.5 Oligodendrocytes and their dysfunction in ALS

There are four main types of glial cell in the adult CNS: microglia, astrocytes, ependymal cells, and oligodendrocytes. The latter are responsible for forming the myelin sheaths which wrap the neuronal axons and make them deliver the nerve impulse considerably faster than unsheathed axons. It has been shown that many ALS cases and SOD1 (G93A) mice show demyelination of motor fibres (Verstraete et al., 2014, Ahdab et al., 2013, Kang et al., 2013, Borisow et al., 2013, Nishijima et al., 2012). Furthermore, myelin degeneration has been seen in higher mammal models of ALS. For example, canine degenerative myelopathy (CDM) is a condition that affects dogs with a naturallyoccurring mutation in their SOD1 homologue gene (Awano et al., 2009). CMD is considered a model of human ALS because affected dogs show the same type of spinal lesions and the adult-onset progressive loss of motor function (Nardone et al., 2016). One of the main pathological features of CDM is the evidence of demyelination in the spinal cord funiculi, especially in the dorsal area of the lateral funiculi, where the lateral motor tracts are located (Griffiths and Duncan, 1975). Additionally, a transgenic line of pigs expressing ALS-causing mutant human G93A SOD1 has been made (Yang et al., 2014). These pigs showed myelin sheath degeneration in the spinal cord along with all the other common features of ALS -e.g. muscle wasting, motor disfunction and SOD1 neuronal and glial inclusions. Demyelination in these models and in human ALS patients can be caused by a number of factors, including a lack of myelin production, a lack of the appropriate receptors which allow oligodendrocyte processes to attach to the axon or other layers, or an inefficacy of the oligodendrocytes to produce competent processes to wrap the axons.

### 1.5.1 The role of MBP in myelin formation

Oligodendrocytes develop multiple processes which project from the oligodendrocyte cell body to axons which they wrap in a process known as myelination: this is where multiple membrane layers containing myelin stack around the axon to isolate the axon from the external medium. This membrane assembly has recently been shown to be triggered by a phase transition of myelin basic protein (MBP), from a soluble form to a form which makes the cytoplasm extremely viscous (Aggarwal et al., 2013).

MBP is the main protein responsible for compaction and biogenesis of myelin. This was established in the *shiverer* mouse which lacks myelin due to *Mbp* mutations and accordingly, shows hypomyelination and incorrect compaction of myelin were present in the central, but not the peripheral nervous system. This results in the mouse showing the characteristic shivering gait. Axonal remyelination was restored following the expression of the MBP protein using an *MBP*-containing cosmid plasmid that was injected into fertilised eggs (Readhead et al., 1987, Allinquant et al., 1991). Consisting mostly of polar and charged amino acids which exert self-repulsive forces, MBP has almost no secondary structure in aqueous solution, appearing as a flexible coil, (a so-called 'intrinsically unstructured protein'; Tompa, 2002). Once it binds to the cell membrane, the charges are neutralised and MBP polymerises into a meshwork, with non-polar phenylalanine groups playing a crucial role in this polymerisation. When polymerised, MBP shows typical alpha-helix and beta-sheet folding patterns.

## 1.5.2 The role of MOBP in myelin formation

A protein very similar to MBP, myelin-associated oligodendrocytic basic protein (MOBP), regulates the radial growth of axons and the radial component of the myelin sheath, a network of interlamellar tight junctions that is present in the central nervous system only (Sadahiro et al., 2000). MOBP is not necessary for the compaction of the myelin sheath, unlike MBP, but mice lacking MOBP have more sheath damage than wild-type mice after treatment with hexachlorophene, a myelin sheath disruptor (Yoshikawa, 2001). As a regulator of the tight junction distance between the myelin layers, MOBP probably confers the membrane protection against external agents by keeping the lamellae attached to each other. Interestingly, three single nucleotide polymorphisms in *MOBP*, the gene encoding MOBP, have been recently denoted as ALS risk loci by a GWAS study (van Rheenen et al., 2016). The same polymorphisms had previously been linked to FTD and progressive supranuclear palsy (PSP). FTD patients harbouring the *MOBP* SNPs

presented with an increased white matter atrophy which shortened the duration of the disease compared to noncarriers (Irwin et al., 2014). However, in PSP patients, the *MOBP* SNPs impaired the expression of the neighbouring gene *SLC25A38* (Hoglinger et al., 2011), a mitochondrial carrier for glycine (Lunetti et al., 2016). This is not an issue for FTD because this *SLC25A38* knock-down effect affects mainly the cerebellum and not so much the frontal cortex.

# 1.5.3 Oligodendrocytes support axons metabolically, and this function is impaired in ALS

In addition to being electrical insulators of axons, oligodendrocytes have also been shown to support axon upkeep and metabolic support. This is done by releasing neurotrophic factors and maintaining the energetic balance inside the axon, thus performing some roles classically attributed only to astrocytes in the CNS (Funfschilling et al., 2012, Lee et al., 2012). Indeed, the lack of metabolic support of axons has been proposed to be more important than the lack of electric insulation in some diseases such as hereditary spastic paraplegia and multiple sclerosis (reviewed by Soderblom et al., 2006).

Monocarboxylate transporter 1 (MCT1) is a myelin sheath-localised protein which helps oligodendrocytes perform their axonal metabolic support functions via export/import of lactate. In a study using cell culture, human tissue, and ALS rat tissue, Lee et al. (2012) showed that ALS patients generally show a reduction of more than 50% of MCT1 in the motor cortex, compared to healthy controls. They also revealed that a loss of MCT1 leads to a deficiency in axonal function and neuronal degeneration, making it crucial to maintain the energetic requirements of axons. Furthermore, Kang et al. (2013) found oligodendroglial degeneration and protein aggregates in human ALS motor cortex and determined that specific demyelination of the motor cortex is a feature of ALS.

There are very few studies which have investigated the mechanism underlying dysfunction of oligodendrocytes and myelin in ALS pathogenesis compared to the vast

literature dedicated to ALS pathogenic mechanisms involving neurones, astrocytes and microglia. This makes it an unexplored field yet to be investigated, but the current evidence points towards oligodendrocytes playing a role in the pathogenesis of ALS.

## 1.6 Modelling ALS and demyelination using Zebrafish

Monitoring the development of the brain pathology along the life of a patient with a neurodegenerative disease such as ALS raises ethical concerns due to the risk of significantly altering the life of the patient upon the extraction of brain tissue samples. Nonetheless, in vitro models using cultured brain cells differentiated from patients' fibroblasts have been trying to circumvent this limitation in the last decade, allowing us to study the impact of these diseases in patients' neurones and glia while the patients are still alive. These patient-derived cells also provide a good balance between the level of throughput and the translatability to patients. However, these in vitro models lack the complexity that a full organism has, and that is one of the reasons why in vivo models are still used today. Regarding ALS, numerous models have been created to help understand the different aspects of the disease since 1993, when the first ALS-causing mutations were identified in the SOD1 gene (Rosen et al., 1993). In particular, distinct features of familial ALS have been modelled in yeast, small invertebrates, such as C. elegans or D. melanogaster; and vertebrates with a high degree of genetic homology, such as zebrafish and mice (Van Damme et al., 2017). In this project, zebrafish was used as a model species, and the relevant characteristics why this organism was chosen are described in this section.

#### 1.6.1 Zebrafish as a model species

Zebrafish (*Danio rerio*) is a small teleost fish which has proven advantageous as a model species for the human central and peripheral nervous system. Zebrafish go through rapid embryonic growth, generating a nervous system that is of experimental utility in a less than a week in contrast to the mouse, which takes a few weeks to reach such a stage. Moreover, the zebrafish embryo is transparent during embryonic stages, which allows for a clear visualisation of internal organs and cells at early stages of growth. Hundreds to thousands of embryos are easily harvested from a few mating couples in one day, thanks to the external fertilisation. This is really useful when a large number of individuals are needed in high-throughput experiments such as drug screening (McGown et al., 2016) or large-scale mutational studies (Pichler et al., 2003).

Regarding genetic manipulation, zebrafish can be subject to a variety of techniques including CRISPR/Cas9, TALENS and Zinc fingers for site-specific mutations; or tools such as the modular Gateway system (Kwan et al., 2007) or bacterial artificial chromosomes (BACs) (Ramesh et al., 2010) for transgenesis. In this aspect, they can serve as models of human mutations due to the high degree of homology many zebrafish genes share with mammals.

Overall, zebrafish represents an excellent option to investigate the effects of diseasecausing mutations *in vivo* in a way that allows a diversity of manipulating techniques to be used, especially when *in vitro* models are not an option due to the complexity of the whole organism. For example, this includes the instances when signalling cascades, molecular gradients, and interaction with different types of cells are involved in the fate of the investigated cells. This is particularly relevant in the context of myelination, where all of those are relevant –as explained in the next section.

#### 1.6.2 Zebrafish as a model of myelination

Zebrafish oligodendrocytes are developed within the first days of embryonic life from oligodendrocyte precursor cells (OPCs) while the embryo is still transparent. Due to the quick growth of the central nervous system, it is crucial that axonal myelination occurs promptly, and it can be observed from 4 dpf with the expression of *mbp*, the zebrafish ortholog of *MBP* (Jung et al., 2010). There are many aspects of zebrafish myelination which replicate those of human myelination. Firstly, zebrafish myelin contains homologous proteins to those of human myelin, namely mbp, Plp/DM20, and Mpz corresponding to human MBP, PLP, and myelin protein zero respectively (Nawaz et al., 2013, Schweitzer et al., 2006, Bai et al., 2011, Avila et al., 2007). In addition, the properties of myelin sheaths are very similar across both species, including lipid composition, sheath thickness, periodicity and membrane packing (Avila et al., 2007, Kirschner et al., 1989). The relationship between axonal diameter and the radial

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component of myelin sheaths are positively correlated across multiple vertebrate species, including mammals and fish (Hildebrand and Hahn, 1978).

Regarding myelination signalling pathways, zebrafish share with humans the Notch regulatory signal which allows for the maintenance of radial glia and the formation of oligodendrocytes and primary motor neurones (Kim et al., 2008). Additionally, the Wnt pathway has been shown to drive the expression of myelinating genes and to be essential for myelination (Tawk et al., 2011), and is conserved in zebrafish (Azim and Butt, 2011). The same can be said for the hedgehog pathway: Inhibition of sonic hedgehog signalling leads to a decrease in the levels of OLIG2, which is essential for the early differentiation of motor neurones and OPCs (Park et al., 2004, Chung et al., 2013). Inhibition of the hedgehog pathway also results in a reduction in the expression of proteins which are crucial for myelination in both the central and peripheral nervous system -such as MBP and MAG (Yoshimura and Takeda, 2012, Wang and Almazan, 2016). This pathway is conserved between humans and zebrafish (Schebesta and Serluca, 2009) and has been thoroughly studied in zebrafish. Another well-studied pathway that regulates myelination is the ErBb pathway. The different members of the ErBb family collaborate towards the differentiation of oligodendrocytes from OPCs. For example, EGFR plays a role in the asymmetrical polarisation of neural precursor cells into different classes of neural cells (Sun et al., 2005), and plays a major role in remyelination in mammals (Aguirre et al., 2007, Brinkmann et al., 2008). The ErBb signalling pathway is also shared by zebrafish (Lyons et al., 2005, Pruvot et al., 2014) and the effect of overexpression and inhibition of the different ErBb proteins in vivo is an area of ongoing research in this model animal.

## 1.6.3 Zebrafish as a model of ALS

The genetic homology to humans that zebrafish have and the relatively high throughput they offer compared to other vertebrates have allowed multiple mutations that cause ALS to be modelled in zebrafish to investigate their pathogenic mechanisms. Most of the genes found mutated in fALS and sALS have known zebrafish orthologs with usually high

degrees of genetic homology and protein residue conservation. Therefore, this allows for the genetic manipulation of the endogenous zebrafish orthologs to investigate the effect of ALS-causing mutations. For example, the use of antisense morpholino oligonucleotides (AMOs) to knock down the expression of the FUS, TARDBP, SOD1, and C90rf72 orthologs (fus, tardbp, sod1, and C13H90rf72) has been useful to confirm that their loss of function leads to motor deficits and axonopathy (Kabashi et al., 2011, Armstrong and Drapeau, 2013, Ciura et al., 2013). Random mutagenesis and gene mutation screening have also been used to identify a *tardbp* allele that contains an early stop codon (Hewamadduma et al., 2013). This strain was used to demonstrate that Tardbp depletion in zebrafish leads to the alternative splicing of the mRNA of another TARDBP ortholog, tardbpl, which replaces tardbp when the latter is not expressed. For that strain, a *tardbpl*-targeted AMO in a high concentration produced defects in axonal guidance and motor function, also reducing zebrafish survival. Similar results were found in a study where zinc finger nucleases were used to knock out tardbp and tardbpl (Schmid et al., 2013). Additionally, CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated system 9) technology have been used to knock-in human ALS-causing mutations in conserved amino acids of the endogenous Tardbp and Fus zebrafish proteins (Armstrong et al., 2016). However, these strains still remain to be fully characterised regarding any ALS-related pathology. Regarding SOD1, transgenesis of wild type human SOD1 and of SOD1 containing an ALS-causing mutation in zebrafish was detrimental to motor neurones, causing motor deficits, abnormal neuromuscular junctions and premature death (Ramesh et al., 2010). A similar approach also showed that mutant SOD1 can provoke a heat shock response in zebrafish without the need of a heat shock, affecting mainly the inhibitory input of the glycinergic interneurons in the spinal cord (McGown et al., 2013). Transient expression of human SOD1 harbouring ALS-causing mutations has also been used to show that it causes the deficiency of motor neurone axons in zebrafish embryos (Sakowski et al., 2012). Concerning C90rf72, the injection DNA constructs containing 38 and 72 GGGGCC repeats in zebrafish embryos increased the number of apoptotic cells in zebrafish compared to constructs containing 8 repeats and controls (Lee et al., 2013). Embryos injected with the  $72 \times$  GGGGCC construct also displayed GGGGCC-positive RNA foci in the majority of the apoptotic cells.

In summary, using zebrafish as a model to investigate human CNS myelination and ALS has proven to be a successful approach regarding the proteins and differentiating and specialising regulatory pathways involved.

### 1.7 Myelin and MBP production

This section is focused on how the CNS myelination is controlled. It covers diverse aspects of myelination which are relevant to eventually understand the mechanisms that can be susceptible to be dysregulated or impaired by ALS-causing mutations.

#### 1.7.1 The different models of CNS myelination

The insulating and metabolic functions performed by oligodendrocytes are dependent on the integrity of the myelin sheath and its physical contact with the axonal membrane. Since 1954, when glial cells were associated with the production of myelin (Geren and Schmitt, 1954), the mechanisms of myelination have been mostly in the dark due to the lack of the necessary technology to visualise the wrapping of the oligodendroglial processes around axons. A first model -the "carpet crawler" model- proposed that upon contact with the axon, oligodendrocytes and Schwann cells extend the myelin compartment all along the internode prior to wrapping the axon (Bunge et al., 1961, Bunge et al., 1989). This was observed using light microscopy to observe the remyelinating axons of cats upon which a myelin injury had been caused. However, this contradicted the nonuniformity in sheath thickness during early ensheathment (Knobler et al., 1976) and would imply that the leading membrane would have to use a large amount of force to displace the layers in contact with the axon. With the development of new visualisation techniques, the "liquid croissant" model was proposed (Sobottka et al., 2011) using confocal and electron microscopy. In this model, the axon guides the oligodendrocyte's process laterally along the internode as new layers "pour" on top of each other, resembling the layered structure of a croissant. However, the observations made by Snaidero et al. (2014) using a reporter of membrane trafficking to visualise membrane growth suggest that the myelin growth cone does follow the "carpet crawler" type of spiralling around the axon. Nonetheless, as new layers are formed, they begin growing laterally along the axonal internode from the oligodendrocyte-axon contact point. Thus, the shorter inner layers grow under the longer outer layers until they all cover the whole of the internode. This explains the observation that the sheath is not uniform during its development, but it is later on after it is fully compacted. They also observed 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNP) regulates the MBPmediated compaction of the layers. The mRNA encoding MBP is transported in protein granules to the myelin sheath (Colman et al., 1982), where MBP is synthesised to produce compact myelin. However, if this compaction occurred at all times during the growth of the sheath, the leading edge would not be able to continue growing due to the constraints caused by myelin compaction. While MBP is indeed synthesised along the sheath during its development, CNP concentration modulates the compaction rate so that the leading edge can grow while the outer layers are compacted as they deposit along the axon (Gravel et al., 1996, Snaidero et al., 2014). Therefore, compaction seems to happen from the outside in.

#### 1.7.2 Relevant characteristics of the MBP mRNA

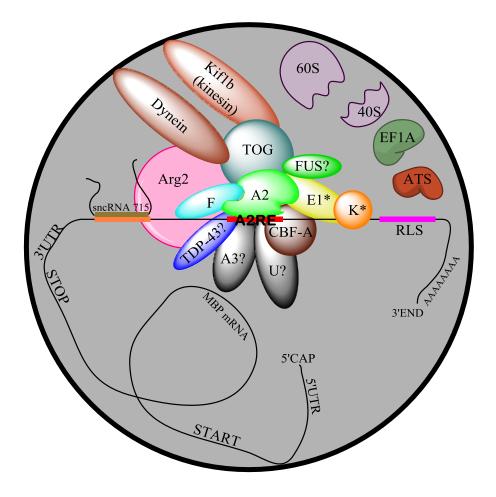
As noted above, MBP is not synthesised in the cell body, where it would cause a fatal compaction of the cellular organelle membranes. Instead, its mRNA is silenced by the action of some proteins and small RNA molecules (described below), and another number of proteins bind to its 3' untranslated region (UTR) in order to facilitate its transport to the myelin compartment, where it is translated (Muller et al., 2013). This protein complex is known as the transport granule, whose current model is depicted in **figure 1.4**. There are a number of features of *MBP* mRNA which have been shown to be essential for the assembly of the transport granule, and for its transport along microtubules towards the myelin compartment.

**Lack of exon II:** MBP has a number of isoforms. Of all these, only those lacking exon II seem to be localized in the oligodendrocyte distal cell membrane in adults, while all other isoforms, which include exon II, account for only about 5% of MBP in adults and are located in the cell body (de Vries et al., 1997). Transcripts that retain exon II are probably predominantly present in a subset of OPCs which are ready for remyelination in the event of a loss of myelin: Allinquant et al. (1991) transfected cultured oligodendrocytes from *shiverer* mice with pRSV viral vectors containing the sequence

for each of the MBP isoforms, and their transcription was stimulated using sodium butyrate. They showed that OPCs in *shiverer* mice expressed almost exclusively exon IIcontaining MBP isoforms which were located in the cell soma. However mature oligodendrocytes only show exon II-lacking MBP isoforms, and these were located in the distal processes.

**Cis-acting elements** are specific sequences located in RNA to which trans-acting elements bind and act upon. In the context of mRNA expression, a trans-acting element is a molecule, usually a protein, which regulates certain aspects of mRNA biology by interacting with the cis-acting elements in the mRNA. In the case of *MBP* mRNA, there are two essential cis-acting elements in the 3'untranslated region (3'UTR) relevant to transport: the RNA trafficking sequence (RTS) in which a sequence referred to as the A2 response element (A2RE) is located, and the RNA localization signal (RLS). A2REs are found in many mRNAs which are transported in granules to distal compartments of different cells for local translation (Kindler et al., 1996, Carson et al., 2008). The RLS is a region of the *MBP* mRNA which adopts a specific secondary structure that has been shown to be required for its location in the myelin compartment (Barbarese et al., 1999).

Regarding MBP, the RTS is a 21-nucleotide sequence in the 3'UTR of *MBP* mRNA which binds hnRNP-A2 via the 11-nucleotide A2RE. The RTS has been shown to be sufficient for RNA transport (Ainger et al., 1997). Binding of hnRNP-A2 to the A2RE seems to trigger the assembly of an mRNA-transport granule and the recruitment of translation repressors which will prevent ectopic translation of the *MBP* mRNA. The recruited molecules are shown in **figure 1.4** and will be discussed in the next section.



**Figure 1.4: Simplified current model of the transport granule.** A2RE: A2 response element; RLS: RNA localisation signal; A2, E1, F, K, A3, U: heterogeneous ribonucleoprotein A2, E1, F, K, A3 and U; 40S, 60S: ribosomal subunits 40S and 60S; CBF-A: CArG box-binding factor A; TOG: tumor overexpressed gene protein; EF1A: elongation factor 1a; ATS: arginyl-tRNA synthetase; TDP-43: transactive response-DNA binding protein of 43 kDa; FUS: fused in sarcoma/translocated in liposarcoma; sncRNA 715: small non-coding RNA 715; Arg2: Argonaute 2; UTR: untranslated region; START: initiation codon; STOP: stop codon; AAAAAAAA: polyadenine tail; 5'CAP: 5' 7-methylguanylate CAP. \*hnRNPs E1 and K compete for two RNA binding sites and only bind at certain stages of the transport.

# 1.7.3 The transport granule: Interactions between the components and their function during MBP mRNA transport

As shown in **figure 1.4**, the *MBP* mRNA transport granule is formed by the association of multiple proteins and RNA molecules, where some of them bind indirectly to the transported mRNA through those molecules that directly interact with it. This section describes the known roles for the molecules involved in the *MBP* mRNA transport granule and how they interact with each other to assemble and guide it towards the myelin compartment through the cytoplasmic microtubular network. This information can provide an idea of how the sequestration and mislocalisation of the different components of the granule, as sometimes observed in ALS, can affect the granule's transport.

hnRNP-A2 is the main molecule required for the coordinated assembly of the transport granule. It is likely that this function is performed by binding hnRNPs E1 and F, CBF-A and TOG. Knock-down of hnRNP-A2 in oligodendrocytes resulted in the confinement of MBP mRNA to the cell body, suggesting that hnRNP-A2 is essential for the initiation of transport (Laursen et al., 2011). A mutation in the G-rich C-terminus of hnRNP-A2 has been linked to multisystem proteinopathy (MSP) (Kim et al., 2013), which involves ALSlike pathology amongst other neurological problems (Benatar et al., 2013). hnRNP-A2 also has the intrinsic property of forming fibrils via the G-rich terminus, and this is exacerbated when the MSP-causing mutation is present (Kim et al., 2013). Additionally, the CGG repeat sequence that causes FXTAS binds hnRNP-A2 causing neurotoxicity, splicing dysregulation, and transport granule mislocalisation (He et al., 2014, Muslimov et al., 2011), and this protein is also part of the pathological protein inclusions present in that disease (Iwahashi et al., 2006). Therefore, there is the possibility that the ALScausing C90rf72 GGGGCC-repeat expansion causes hnRNP-A2 sequestration in ALS cases that harbour it. As explained above, C9orf72 mRNA containing the GGGGCC expansion does sequester a number of RNA-binding proteins of the hnRNP family. Additionally, C9orf72 binds hnRNP-A2 and mediates its shuttling between the nucleus

and the cytoplasm (Farg et al., 2014), so reduced levels of C9orf72 could potentially impair the function of hnRNP-A2 in the cytoplasm.

**CBF-A:** Raju et al. (2008) showed by protein-RNA interaction assays that CBF-A binds the RTS in *MBP* mRNA along with hnRNPs A2, U, and A3. They then demonstrated that the wild-type RTS, but not a scrambled RTS, binds hnRNP-A2 and CBF-A. In addition, RNAase treatment prevented the co-precipitation of hnRNP-A2 and CBF-A, meaning that they form part of the same complex but do not interact directly. Silencing the *CBF-A* gene resulted in fewer granules being transported. Instead, these remained in the cell body. They concluded that CBF-A plays a role in at least the initial steps of carriage of the granule.

**hnRNP-F:** White et al. (2008 and 2012) showed that hnRNP-F co-precipitates with hnRNP-A2 in an RNA-independent manner. They found that hnRNP-F is necessary for MBP synthesis upon arrival of the granule to the translation site, but whether or not hnRNP-F has any function during trafficking of the granule is yet to be discovered.

**hnRNP-E1** co-precipitates with hnRNP A2, even in the absence of RNA (suggesting that the interaction is direct with hnRNP A2 and not RNA-dependent) and inhibits the translation of A2RE-containing mRNA in a manner that is dependent on binding of hnRNP-A2 to A2RE (Kosturko et al., 2006). Furthermore, Torvund-Jensen et al. (2014) showed that overexpression of hnRNP-E1 leads to a delay in the translation of *MBP* mRNA, while its silencing causes premature translation of MBP and results in oligodendrocytes showing fewer and less extended processes, possibly due to the premature translation of *MBP* mRNA. This premature translation was previously demonstrated by Staugaitis et al. (1990), who showed that the inhibition hnRNP-E1 exerts upon *MBP* mRNA translation is crucial during transport.

**hnRNP-K** co-precipitates with hnRNP-A2 even in the absence of *MBP* mRNA (Laursen et al., 2011, Torvund-Jensen et al., 2014). hnRNP-K binds to three sites in the *MBP* mRNA, two of which are also binding sites for hnRNP-E1. hnRNP-K also co-localises with transport granules, but only in sections of the oligodendrocyte processes which are

proximal to the cell body but not in the distal parts. Furthermore, knockdown of hnRNP-K prevents the granules from being transported further than the first oligodendroglial branches, suggesting that its function has to do with the last part of the transfer rather than with the initiation (Laursen et al., 2011). Recent findings of Torvund-Jensen et al. (2014) propose that hnRNP-K replaces hnRNP-E1 to promote the transport of *MBP* mRNA from the oligodendrocyte branching points to the most distal part of the processes. They also showed that these two hnRNPs do not co-localise in differentiated oligodendrocytes, and that hnRNP-K is found co-localised with *MBP* mRNA transport granules only when differentiated oligodendrocytes begin producing myelin. Oligodendrocytes with more abundant and larger branches were observed upon silencing of hnRNP-K, which may be caused by the delayed translation of *MBP* mRNA that silencing causes.

**TOG:** Kosturko et al. (2005), using a two-hybrid system, demonstrated an interaction between the N-terminal of TOG and hnRNP-A2. Furthermore, Francone et al. (2007) have shown an association of TOG with MBP mRNA transport and translation. Ohkura et al. (2001) revealed that TOG promotes the elongation of microtubules in animal and cellular systems and that TOG interacts with kinesin-like and dynein-like proteins in RNA transport granules harbouring the A2RE sequence, to regulate the direction of transport. Therefore, they proposed that TOG might have a function in directing the granule along microtubules towards the distal processes. This was contradicted by Francone et al. (2007) in a rat oligodendrocyte culture system: They found that the number of granules in the myelin compartments did not change upon silencing of the TOG gene, although the silenced oligodendrocytes showed no myelin formation. This could mean that in humans this protein is essential for MBP mRNA translation in the myelin compartment but may not be as important for the regulation of granule transport as stated by Ohkura et al. (2001). Very recently, a study involving TOG-knockout mice showed that the absence of TOG leads to hypomyelination/dysmyelination and to motor deficits (Maggipinto et al., 2017). In the same study, they showed that MBP mRNA transport is impaired by TOG knockout in cultured spinal cord oligodendrocytes.

Small non-coding RNA 715 (**sncRNA-715**) precipitates with hnRNP-A2 and with *MBP* mRNA and probably acts as a trans-acting element by binding to a sequence of 21 nucleotides located in the 3'UTR of *MBP* mRNA and thereby silencing it (Bauer et al., 2012). Rat oligodendrocytes transfected with synthetic sncRNA-715 showed an abnormal morphology and lower levels of MBP compared to controls. Interestingly, multiple sclerosis lesions had much higher levels of sncRNA-715 compared to controls, this being one of the possible reasons why these lesions may fail to remyelinate in some circumstances. This direct inhibition of *MBP* expression by sncRNA-715 has also been observed in Schwann cells (Muller et al., 2015a).

**Ago2** is a protein of the Argonaute family. Argonaute proteins have an important role in RNA silencing via RNA interference: They bind to small, non-coding RNA molecules that silence a target RNAs (such as sncRNA-715). Ago2 co-localises and co-precipitates with hnRNP-A2, sncRNA-715, and *MBP* mRNA, suggesting thatAgo2 is involved in silencing *MBP* mRNA during transport (Muller et al., 2015b), especially as Ago2 is a target of Fyn kinase, a known *MBP* translation regulator in the myelin compartment. This means that Ago2 silencing effect could potentially be dependent on its phosphorylation state.

Other molecules which show no direct interaction with hnRNP-A2 but have been related to the *MBP* mRNA transport granule include kinesin motor protein **kiftb**, which has been demonstrated to be necessary for RNA granule trafficking along the microtubules, probably by interaction with TOG (Lyons et al., 2009). In order for *MBP* mRNA to be translated, the necessary translating proteins and RNA need to be transported too. Both fragments of the **ribosomal machinery** (40s and 60s), elongation factor 1a (**EF1a**) and arginyl-tRNA synthetase (**ATS**) (i.e. the necessary translation machinery) have been found to form part of *MBP* mRNA-containing granules by fluorescent *in situ* hybridisation (Barbarese et al., 1995). The way they are attached to the granule, however, remains uncertain.

# 1.7.4 The relationship of TDP-43 and FUS with mRNA transport granules

A number of other molecules have been found to be part of mRNA transport granules. Among these molecules are the proteins FUS and TDP-43. As above stated, both of them are RNA-binding proteins of the heterogeneous nuclear ribonucleoproteins (hnRNP) family and are usually found in cytoplasmic inclusion bodies in ALS cases. In the nucleus, TDP-43 and FUS regulate gene transcription, control the splicing of specific mRNAs in the nucleus in conjunction with other hnRNPs, and are involved in the processing of micro RNA and long, non-coding RNA (Ederle and Dormann, 2017). TDP-43 and FUS are most abundant in the nucleus, where they perform those functions. However, they are also found in small concentrations in the cytoplasm, especially clustered with other proteins and RNA molecules. Apart from being involved in mRNA stability there, TDP-43 and FUS are also necessary for mRNA trafficking as parts of kinesin-bound transport granules, and they co-localise with such structures in the dendrites and axons of neurones (Wang et al., 2008, Schoen et al., 2015, Belly et al., 2005). One key study revealed that ALS-causing mutations in TDP-43 caused TDP-43granules to not reach the distal part of the axons and show a net retrograde movement along the microtubules in Drosophila and human iPSC-derived neurones (Alami et al., 2014). Later, (Ishiguro et al., 2016) demonstrated that TDP-43 binds guanine-rich Gquadruplexes in vitro, and that this is essential for the TDP-43-dependent dendritic transport of mRNA in murine cultured neurones. This is very relevant for the 30% of dendritic mRNAs which share this pattern in their 3'UTR, which is sufficient for the localisation of mRNA in dendrites (Subramanian et al., 2011). Interestingly, C90rf72 mRNA GGGGCC expansions, as described above, form G-quadruplexes which can bind and sequester important RNA-binding proteins (Lee et al., 2013). Ishiguro et al. (2016) also showed that 8 GGGGCC repetitions are sufficient for TDP-43 to bind the expansion and that 60 repetitions, closer to the hundreds of repetitions found in C9ALS patients, are enough to impair mRNA dendritic transport, confining TDP-43 to the cell body.

In the light of the importance of TDP-43 and FUS as mRNA trafficking regulators, it would be relevant to ascertain whether TDP-43 and FUS are involved in the transport of *MBP* mRNA transport granules in oligodendrocytes. It has been shown that TDP-43 binds hnRNP-A2 when the latter is not bound to hnRNP-A1's target sequence, which happens when there is a shortage of hnRNP-A1, and hnRNP-A2 needs to assume its function (Buratti et al., 2005). The interaction between TDP-43 and hnRNP-A2 is also necessary for the splicing of particular mRNAs in the nucleus. Additionally, both TDP-43 and hnRNP-A2 are part of transport granules, but this does not mean that they necessarily work together while doing so. However, this remains to be ascertained. Therefore, it is likely that the pathological aggregation of TDP-43 in oligodendroglia and neurons seen in ALS patients and ALS models, could impair the transport of mRNA. This mRNA shortage could affect the myelin compartment of oligodendrocytes, but also the dendrites and spines or the distal part of the neuronal axons and dendrites (Fallini et al., 2012, Wang et al., 2008).

Thus, a shortage of TDP-43 and FUS caused by ALS-causing mutations or, in the case of TDP-43, by cytoplasmic aggregation in most cases of ALS, could result in an incorrect transport of the *MBP* mRNA in oligodendrocytes, which could cause the incorrect myelination seen in ALS. This is a key hypothesis for this thesis. Due to time limitations, this study was limited to the study of the effect of TDP-43, whose pathological aggregation is the most commonly seen in ALS –not only cases harbouring TDP-43 mutations-, while FUS aggregation is almost restricted to ALS caused by FUS mutations.

# 1.7.5 The transport granule: granule disassembly and MBP mRNA translation

Upon arrival to the myelin compartment, *MBP* mRNA must be released from the granule, especially from the translation inhibitors hnRNP-E1 and sncRNA-715 so localised translation can be initiated. It has been shown that proto-oncogene tyrosine-protein kinase (Fyn kinase) is crucial at this step because it phosphorylates hnRNP-F and hnRNP-A2, facilitating their release from the granule (White et al., 2008; see below, White et al., 2012). The current model for activation of Fyn kinase and initiation of *MBP* mRNA translation is explained in the next sections and can be seen in **figure 1.5**.

### 1.7.6 Axonal signalling

White et al. (2008) and Wake et al. (2011) found that electrical stimulation of the axon leads to increased axonal expression of the transmembrane cell adhesion molecule L1 and glutamate release from the axonal membrane. This activated Fyn kinase and MBP synthesis in the processes of nearby oligodendrocytes, most probably by inducing an accumulation of cholesterol-rich domains on the oligodendrocyte areas which responded to glutamate. The results suggest that myelination occurs preferentially on electrically active axons. This is also strongly supported by recent zebrafish studies (Mensch et al., 2015, Hines et al., 2015). They found that most of the myelin wrappings initially formed by oligodendrocytes do not fully wrap around their axons: Only those sheaths stimulated by neuronal activity –measured by synaptic vesicle release- extended the sheath completely, and axons with a decreased activity showed a significant loss of myelin sheaths. In other experiments, Laursen et al. (2009, 2011) found that oligodendrocyte expression of contactin F3 is essential for L1 signalling. They also showed that axonal laminin must interact with integrins  $\alpha 6$  and  $\beta 1$  to achieve oligodendroglial Fyn phosphorylation of hnRNPs F and A2.

Laminin, L1 and glutamate signalling is induced by electrical stimulation of the axon. Therefore, both series of experiments suggest that Fyn activation is dependent on electrical stimulation of the axon, which provides multiple coordinated signals (laminin, L1, and glutamate) to lead the oligodendroglial protrusion around the axon during myelination and to release *MBP* mRNA from the transport granule, as will be discussed below.

## 1.7.7 Granule breakdown and MBP synthesis

As previously stated, hnRNPs F and A2 are tyrosine-phosphorylated when Fyn is activated. hnRNP-K also seems to be tyrosine-phosphorylated, but whether or not this is due to the action of activated Fyn is still to be addressed. These phosphorylation events appear to lead to the disassembly of the transport granule and the lack of local inhibition of the translational machinery (White et al., 2008). The translation inhibitor sncRNA-715 is detached from *MBP* mRNA thanks to the action of Fyn kinase upon Ago2, as described above (Muller et al., 2015b).

Animal models have also been used to demonstrate the importance of Fyn during myelination. Czopka et al. (2013) showed that overexpression and depletion of Fyn upregulates or downregulates the number of myelin sheaths produced by oligodendrocytes in zebrafish. Sperber and McMorris (2001) showed that mice suffered hypomyelination that was not uniform in the CNS upon Fyn knockdown. This demonstrates the essential need of Fyn for MBP synthesis and myelination, and how this process is conserved between species.

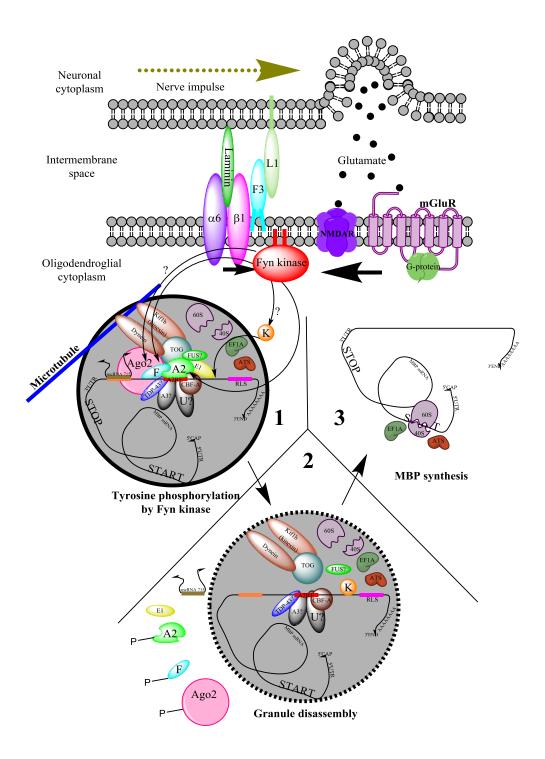


Figure 1.5: Current model in the initiation of *MBP* mRNA translation upon arrival to the myelin compartment. 1) The granule arrives at the distal end of the microtubule and hnRNPs A2 and F are tyrosine-phosphorylated by Fyn kinase once it is activated by a coordinated action of laminin-activated integrins  $\alpha 6$  and  $\beta 1$ , and glutamate receptors which are activated by glutamate released by the axon after stimulation by the nerve impulse. Phosphorylation of hnRNP-K might trigger its binding to its mRNA binding site, displacing hnRNP-E1 from it. 2) Phosphorylation of hnRNP-A2, hnRNP-F and Ago2 promotes their removal from the transport granule, which makes the translation inhibitory elements detach from *MBP* mRNA and allows 3) the translating machinery begins MBP synthesis.  $\alpha 6$ ,  $\beta 1$ : integrins  $\alpha 6$  and  $\beta 1$ ; F3: contactin F3; L1: contactin L1; NMDAR: glutamate NMDA receptor; mGluR: metabotropic glutamate receptor; Fyn kinase: proto-oncogene tyrosine-protein kinase.

## 1.8 Conclusion and aims of the project

Key aspects of ALS pathophysiology and MBP production have been discussed in this section. It is now hypothesised that impaired transport of *MBP* mRNA toward the end of the oligodendroglial processes is one of the main contributing factors to disease in ALS, with an increased relevance in cases bearing GGGGCC expansions in *C90rf72* (C9ALS). Zebrafish have also been discussed as an appropriate model organism to study myelin and ALS in vertebrates. Therefore, the aims this project pursued are the following:

- As stated in previous sections, cytoplasmic aggregation of ubiquitylated proteins in neurones and glia is a pathological hallmark of ALS. It was aimed to characterise the glial pathology of our cohort of ALS cases by quantifying the protein aggregates using IHC for p62, a ubiquitin-binding protein, in motor and extra-motor regions of the CNS. In most cases, phosphorylated TDP-43 (pTDP-43) is one of the main proteins aggregated in those p62-positive inclusions. Therefore, it was sought to quantify the pTDP-43 inclusion pathology using IHC to compare it to that found using p62 as the marker. In order to elucidate whether those glial p62/pTDP-43-positive inclusions were composed by dipeptide repeat (DPR) protein in C9ALS, IHC for the 5 different types of DPR would be performed as well.
- Moreover, TDP-43 is necessary for the splicing and transport of a number of mRNA molecules to neuronal dendrites whose transport depend on the binding of hnRNP-A2, so there is also the possibility that TDP-43 plays a role in the *MBP* mRNA transport granule, which also depends on hnRNP-A2. This would make TDP-43 aggregation directly detrimental to MBP production in ALS. It was, thus, hypothesised that ALS cases suffer a reduction in the quantity of produced MBP compared to other myelin proteins whose synthesis do not depend on mRNA transport, such as PLP. PLP transport to the myelin compartment does not follow the same mechanism as mRNA transport. It was also hypothesised that this selective reduction in MBP is not caused by a depletion of its mRNA. Thus, it was

aimed to compare the levels of MBP and PLP and their mRNAs in the lateral corticospinal tracts of C9ALS, sALS, and controls using Western blot and qPCR in extracts from human *post-mortem* tissue. To provide visual evidence of the trafficking impairment of *MBP* mRNA transport granules, it was also aimed to use *in situ* hybridisation to visualise the cellular localisation of *MBP* mRNA in human post mortem tissue. This would allow investigating whether *MBP* mRNA is mislocalised in oligodendrocytes pertaining to ALS cases.

- Additionally, TDP-43 binds hnRNP-A2, which is necessary for the transport of *MBP* mRNA. It is, therefore, possible that sequestration of TDP-43 into protein inclusions causes the mislocalisation of cytoplasmic hnRNP-A2 to these inclusions. hnRNP-A2 is mainly nuclear, but constantly shuttles between the nucleus and the cytoplasm. Sequestration of hnRNP-A2 in the cytoplasm might eventually cause its depletion in the nucleus. Therefore, it was aimed to quantify the number of nuclei which are positive for hnRNP-A2 using IHC in relevant areas of the CNS. Western blot and qPCR would also be used to test whether protein and mRNA levels were normal for hnRNP-A2.
- hnRNP-A2 sequestration by aggregated TDP-43 could be detrimental to the transport of *MBP* mRNA, but equally relevant is the known binding of hnRNP-A2 and other hnRNPs to the G-quadruplexes formed by *C9orf72* mRNA containing GGGGCC expansions. This could also impair MBP production and demyelination even further in C9ALS. It was then hypothesised that C9ALS cases suffer a greater lack of MBP in their myelin than sALS cases, presenting as well a greater oligodendroglial pathology and that this increased pathology is related to a decrease in myelin production. Part of this hypothesis would be tested using the Western blot for MBP and PLP mentioned above. Additionally, it was aimed to characterise and quantify the microscopic p62-positive and pTDP-43-positive pathology in our bank of C9ALS cases and contrast the findings to sALS cases and healthy controls. This includes the analysis of IHC for p62 and pTDP-43 mentioned above, and the quantification of oligodendrocyte precursor cells.

- It is yet unknown what first triggers motor neurone and glial degeneration in ALS. Neuronal failure and the degeneration of axons could cause the observed pathology of the related glia. However, oligodendroglial disfunction in the white matter would cause a shortage of trophic factors in the axons that could cause axonal degeneration. To test the hypothesis that oligodendrocyte dysfunction occurs before the degeneration of primary motor neurone axons in ALS, it was aimed to use IHC for axonal markers (SMI31) to quantify the axonal density of the dorsolateral motor tracts of spinal cord sections to compare it across the patient cohorts and to the glial pathology in the same region.
- As stated above, GGGGCC expansions in *C9orf72* have a deleterious effect in motor neurones through RNA-binding protein sequestration and the production of toxic RAN-translated dipeptide repeats. This has led to the production of an increasing amount of animal models expressing GGGGCC expansions in the CNS. In order to test whether *C9orf72* expansions are enough to cause ALS just through oligodendroglial dysfunction *in vivo*, it was aimed to generate and characterise a zebrafish model which expresses GGGGCC interrupted expansions exclusively in oligodendrocytes. In principle, the interruptions in the expansions should disable their propensity to give rise to dipeptide repeats and should only cause the sequestration of RNA-binding proteins. This could be detrimental to the synthesis of mbp, and therefore, this model would be useful to analyse how the production of mbp and the transport of its mRNA might be impaired in C9ALS.
- As stated before, TDP-43 ALS-causing mutations have the potential to cause ALS by the loss of function of the protein in the nucleus and the cytoplasm. This could impair *MBP* mRNA transport if TDP-43 were part of the transport granule. However, it is also known that mutant TDP-43 can sequester RNA-binding proteins and mRNA molecules into protein aggregates, and this could mislocalise other hnRNPs necessary for *MBP* mRNA transport. To elucidate whether any of these mechanisms can explain the oligodendroglial dysfunction observed in ALS,

it was aimed to characterise the development of the oligodendroglial cell line fate in two different zebrafish models. This would be done by using a whole-mount *in situ* hybridisation approach for markers of oligodendroglial fate at different stages of the zebrafish embryo development.

Initially, this would be analysed in an already generated zebrafish strain which does not express the *tardbp* gene. This way, it would be possible to test the hypothesis that TDP-43 loss of function impairs *MBP* mRNA transport and the full differentiation of oligodendrocytes. Additionally, it was planned to devise and test strategies using CRISPR/Cas9 technology to generate a zebrafish strain carrying *tardbp* point mutations. Those would be mutations that cause familial ALS when they occur in the same amino acids of the human *TARDBP* gene. This model could help explore the role that TDP-43 exerts in the transport of *MBP* mRNA and how ALS-causing mutations in this gene affect myelination in the adult individual.

**MATERIALS & METHODS** 

## **CHAPTER 2**

# Materials and methods

#### 2.1 Studies using human post-mortem tissue

#### 2.1.1 Patients

We had access to formalin-fixed, paraffin-embedded, and frozen CNS tissue blocks from 100 ALS patients who were selected following *El Escorial* revised criteria (Brooks et al., 2000) whose brains were donated at autopsy to the Sheffield MND Brain Tissue Bank with the consent of the next of kin. Of those 100 cases, 16 were neurologically healthy control cases, 67 did not carry any known ALS-causing mutation, and 17 carried a hexanucleotide-repeat expansion in *C90rf72*. All samples were anonymised. The Sheffield Brain Tissue Bank Management Board gave ethical approval for the use of tissue in this study under the provision to act as a Research Tissue Bank as approved by the Scotland A Research Ethics Committee (ref 08/MRE00/103). The analysed blocks include motor cortex, prefrontal cortex and spinal cord, although there was not available tissue from all three areas of all cases.

## Immunohistochemistry

The antisera and variable conditions for immunohistochemistry used are detailed in **table 2**.

### <u>2.1.1.1 p62 and pTDP43 immunohistochemistry</u>

Formalin-fixed, paraffin-embedded (FFPE) CNS sections had been previously stained using the anti-p62 Ick ligand antibody (BD Transduction Laboratories). This antibody labels p62: a small protein which binds to ubiquitin, and which is widely used as a marker for pathological ubiquitylated inclusions. Motor cortex and spinal cord sections from a sub-cohort of the cases stained for p62 were immunostained using an anti-pTDP43 antibody (BD Transduction Laboratories). This antibody labels specifically the pathological cytoplasmic hyperphosphorylated form of the TDP43 protein. This is the form of TDP43 that forms cytoplasmic inclusion bodies in the CNS tissue of most ALS cases. Our laboratory counted with optimised protocols for these two markers and, therefore, we decided to use them.

For both p62 and pTDP43 antibodies, antigen retrieval was performed in a pressure cooker (125°C for 30 s, A.Menarini Antigen Access Unit, A.Menarini Diagnostics, Berkshire, UK) at pH6. The MenaPath X-Cell-Plus HRP Detection Kit (A.Menarini Diagnostics, Berkshire, UK) was used to stain the CNS sections, applying the mouse anti-p62 antibody at a dilution of 1:200, or the anti-pTDP43 at a dilution of 1:4000. The detailed times for each step were:

- 1. Endogenous peroxidase block, 5 minutes.
- 2. Protein block, 5 minutes.
- 3. Primary antibody incubation, 60 minutes.
- 4. Universal probe, 10 minutes.
- 5. HRP polymer, 15 minutes.
- 6. DAB chromogen, 5 minutes.
- 7. Haematoxylin counterstain, 1 minute.
- HCM (Harry's haematoxylin, 2 min; Scott's tap water, until blue colour; rapid dehydration and clear through 70%/95%/2x100% ethanol-2x xylene; mount in DPX –i.e. Distyrene/ Plasticizer/Xylene).

All the staining steps were done at room temperature (RT).

# <u>Myelin basic protein immunohistochemistry (IHC)</u>

An anti-MBP antibody (Chemicon) was used to immunostain fixed motor cortex and spinal cord sections. MBP is the myelin-forming protein whose mRNA has to be transported in the hnRNP-A2-dependent transport granules.

Antigen retrieval was done in a microwave oven, soaking the samples in trisodium citrate (TSC) pH6.5 and heating them at 800 W for 10 minutes. The Vector ABC Elite kit was used afterwards, following these steps:

- 1. 3% H<sub>2</sub>O<sub>2</sub> in methanol, 20 min.
- 2. Serum, 30 min.
- 3. 1<sup>o</sup> antibody (1:500 dilution), 1h.
- 4. 2° biotinylated antibody, 30 min.
- 5. ABC reagent, 30 min.
- 6. Vector DAB reagent, 30 min.
- 7. HCM.

All the staining steps were done at RT.

# <u>2.1.1.2 Myelin proteolipid protein 1 IHC</u>

PLP is the major component of myelin. It is translated in the oligodendrocyte cell body and then transported to the myelin compartment. This contrasts with MBP, whose mRNA is transported in the cytoplasm before translation. Fixed motor cortex and spinal cord sections were stained with an anti-PLP antibody (Bio-Rad).

Apart from incubation time and antibody dilution (see table 2), the antigen retrieval and staining methods were otherwise identical to those used to stain p62.

# <u>2.1.1.3 hnRNP-A2 IHC</u>

hnRNP-A2 is the essential protein for the correct assembly and transport of the *MBP* mRNA transport granule. Most hnRNP-A2 is located in the nucleus. Expanded *C9orf72* mRNA and TDP-43 both bind hnRNP-A2 and can potentially sequester it, bringing its concentration below the detectable limits. Therefore, the aim was to quantify the number of oligodendroglial nuclei stained for hnRNP-A2 in the white matter below the motor cortex and in the dorsolateral motor tracts of the spinal cord. An available anti-hnRNP-A2 antibody (Abcam) whose working conditions had been previously optimised was used to stain fixed motor cortex and spinal cord sections.

Except for the antigen retrieval method –carried out at pH9- and the primary antibody dilution –see table 2-, the antigen retrieval and staining methods were otherwise identical to those used for p62.

## 2.1.1.4 Oligodendrocyte precursor cell (OPC) IHC

OPCs were stained in fixed motor cortex and spinal cord sections using the MAP2+13 antibody. This antibody was donated to us by Shafit-Zagardo et al. (1999 and, as they show, it labels OPCs which are ready to differentiate into myelinating oligodendrocytes. In the case of myelin loss, those OPCs normally take the place of the oligodendrocytes that have stopped myelinating. Our laboratory counted with an optimised method for this antibody and, thus, it was the one that was chosen for this study.

With the exception of the primary antibody incubation -1:100 incubation, 2h RT-, the antigen retrieval and staining methods were otherwise identical to those used for MBP.

## <u>2.1.1.5 Neurofilament IHC</u>

Mature, phosphorylated neurofilaments were stained in fixed spinal cord sections using an SMI31 antibody (Biolegend). Neurofilament protein is an important part of the neuronal cytoskeleton, especially in the axon. It becomes phosphorylated as a sign of maturation, and the SMI31 antibody recognises that form. There are three main types of neurofilament (i.e. light, medium and heavy). This antibody labels all three.

With the exception of the primary antibody incubation –see **table 2.1**-, the protocols for antigen retrieval and staining were otherwise identical to those used for p62.

## <u>2.1.1.6 Dipeptide repeat (DPR) IHC</u>

The five different forms of DPR (poly-AG, poly-AP, poly-GP, poly-GR, and poly-PR) that can be RAN-translated from the expanded *C90rf72* mRNA were immunostained in fixed motor cortex tissue. The five antibodies used (Proteintech) are specific to each of the DPR types and had been previously tested and optimised by other groups in our laboratories.

Apart from the primary antibody incubation –see table 2-, the antigen retrieval and staining methods for poly-AG and poly-PR were otherwise identical to those used for MBP.

For poly-AP. poly-GP and poly-GR, the Immpress HRP polymer detection kit was used as follows:

- 1. BLOXALL<sup>TM</sup> blocking solution 10 min.
- 2. RTU 2.5% blocking serum 20 min.
- 3. 1° antibody (time and temperature in table 2.1),
- 4. ImmPRESS<sup>TM</sup> reagent 30 min.
- 5. Brown peroxidase substrate solution 10 min.

6. HCM.

All the staining steps were done at RT.

Antibody	Source	Antigen retrieval	IHC kit	Primary antibody incubation
p62 Ick ligand	BD Transduction Laboratories	PC pH6	A.Menarini Intellipath	1:200 – 1h RT
Phosphorylated TDP-43	BD Transduction Laboratories	PC pH6	A.Menarini Intellipath	1:4000 – 1h RT
Myelin basic protein	Chemicon	TSC pH6.5 M/W	Vector ABC	1:500 – 1h RT
Nogo-A	Abcam	PC pH9	A.Menarini Intellipath	1:50 – 1h RT
Proteolipid protein	Bio-Rad	PC pH6	A.Menarini Intellipath	1:800 - 30min RT
SMI31	Biolegend	PC pH6	A.Menarini Intellipath	1:200 - 30min RT
hnRNPA2B1	Abcam	PC pH9	A.Menarini Intellipath	1:1000 – 1h RT
MAP2+13	Donation by Shafit-Zagardo et al. (1999)	TSC pH6.5 M/W	Vector ABC	1:100 - 2h RT
Poly-AG	Proteintech	PC pH6	Vector ABC	1:200 - O/N 4ºC
Poly-AP	Proteintech	PC pH6	Vector Impress	1:500 - O/N 4ºC
Poly-GP	Proteintech	PC pH6	Vector Impress	1:500 - O/N 4ºC
Poly-GR	Proteintech	PC pH6	Vector Impress	1:200 - O/N 4ºC
Poly-PR	Proteintech	PC pH6	Vector ABC	1:200 - O/N 4ºC

**Table 2.1: Sources and conditions used for the antibodies used in IHC.** TSC: Trisodium citrate. M/W: Microwave PC: Pressure cooker. RT: Room temperature. O/N: Overnight.

## 2.1.1.7 Oligodendrocyte-specific protein (OSP) immunohistochemistry

The clear labelling of oligodendrocytes cell bodies is a challenging task that different researchers approach differently. OSP is a protein which is primarily expressed by oligodendrocytes in the CNS (Bronstein et al., 1997). Initially, FFPE CNS sections, as well as frozen sections, were immunostained using rabbit anti-OSP antibodies ab7474 and ab53041 (Abcam, Cambridge, UK). Other classic oligodendroglial markers such as Olig2 had been unsuccessfully trialled in both frozen and FFPE tissue in our laboratories in the past. However, our laboratories counted with a working protocol for anti-OSP (ab7474) in frozen tissue and, thus, we decided to try it first for the co-staining of oligodendrocyte cell bodies and p62-positive inclusions. It was first attempted to optimise the ab7474 antibody for PPFE tissue. This antibody recognises the OSP C-terminus as it was raised against the 15 amino acids that compose it. The ab53041 antibody was optimised and used later, when ab7474 did not give the expected staining pattern when used along the anti-p62 antibody. The ab53041 antibody recognises the full human OSP protein rather than just the C-terminus, as it was raised against human OSP.

#### <u>2.1.1.8 Fixed tissue</u>

The protocol was similar to that used with the poly-GA antibody. The kit used was the VECTASTAIN ABC-AP KIT (Rabbit IgG; Vector Laboratories), and the coloured substrate was the VECTOR Red alkaline phosphatase (AP) substrate. The sections were not treated with hydrogen peroxide because the AP substrate does not detect endogenous peroxidase. Levamisole was instead used after the 1° antibody incubation to block endogenous phosphatase. After the antigen retrieval step and having been treated with the antibodies, they were incubated for 30 min with the avidin-biotin-AP complex (ABC), and 25 minutes with the AP red substrate, before HCM.

# <u>2.1.1.9 Frozen tissue (standard protocol)</u>

The sections were thawed at room temperature for 5 minutes and fixed in acetone at 4°C for 5 minutes, before proceeding to stain them following the same protocol as with the fixed tissue, omitting the antigen retrieval step.

# <u>2.1.1.10 Frozen tissue (rapid protocol)</u>

The reagents were the same as used in the frozen tissue standard protocol. The incubation times were as follows:

- 1. Thawing, 5 minutes;
- 2. Fixation in chilled acetone at 5°C, 5 minutes.
- 3. Serum block, 3 minutes.
- 4. Primary antibody, 5 minutes.
- 5. Biotinylated secondary antibody, 10 minutes.
- 6. Avidin-biotin-AP complex, 5 minutes.
- 7. AP red substrate, 25 minutes.
- 8. HCM.

With the exception of the fixation in acetone, all the staining steps were done at RT.

# <u>2.1.1.11 Double IHC of OSP and p62</u>

Multiple strategies were trialled to double stain sections with OSP and p62 in an attempt to co-stain oligodendrocytes and p62-positive inclusions, as motor cortex and spinal cord sections showing co-staining of both p62 and GFAP (a marker for astrocytes) or IBA1 (a marker for microglia) had already been counted. The first attempt was to use OSP and p62 antibodies, which became extremely challenging. The different strategies followed to optimise this double staining can be seen in

**table 2.2**. In our laboratories, DAB brown and VECTOR Red have become the gold standard for co-staining because they usually provide a good contrast between the two antigen locations when using brightfield microscopy. After this pair did not work as expected for the co-staining of OSP and p-62, different chromogens that usually provide good contrast were trialled. The aim of trialling all these different markers was to obtain a pair of chromogens that would provide enough contrast to distinguish the p62-positive inclusions from the oligodendroglial cell bodies. These are the protocols that were followed for each attempt, all in frozen motor cortex:

#### **OSP:** NovaRED and DAB

Brown (DAB) and alternative red staining (Nova Red) of OSP was performed following the same protocols as with the phosphatase Vector Red staining, using the VECTASTAIN Elite ABC Kit (Vector Laboratories, Peterborough, UK). The chromogens VECTOR DAB Peroxidase (HRP) Substrate and the VECTOR NovaRED Peroxidase (HRP) Substrate were used instead. The protocol was identical, but the peroxidase substrate incubation was only 10 minutes.

## **OSP:** BCIP/NBT

Blue staining of OSP was achieved using the same protocol as with Vector Red, only changing the chromogen to BCIP/NBT, included as part of the VECTOR Blue Alkaline Phosphatase (AP) Substrate Kit (Vector Laboratories).

#### **p62:** Ni-DAB

Black staining of p62 inclusions was done using a modified version of the DAB protocol, adding a nickel solution (included in the DAB kit) to the chromogen reaction mix, which turns the normal brown DAB colour into black. The incubation time was identical.

#### OSP and p62: FITC and TRITC

The rapid protocol was applied up to and including the incubation with the biotinylated secondary antibody, to visualise fluorescent oligodendrocytes and p62-positive inclusions. Then, the sections were incubated with a 1:100 dilution of either streptavidin conjugated to fluorescein isothiocyanate (Streptavidin:FITC, AbD Serotec) for OSP, or rhodamine conjugated to isothiocyanate (TRITC) for p62, for 60 minutes. The sections were then mounted with VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories), which stains the nuclei.

## **Methyl Green counterstaining**

For the protocols in which OSP was stained in blue, the counterstaining protocol was modified to show the nuclei in green rather than the usual haematoxylin blue. The counterstaining protocol was as follows:

- 1. Rinse slides in tap water.
- Submerge slides in preheated Vector Methyl Green solution (Vector Laboratories) at 60°C, 5min.
- 3. Wash in deionised water, 1min RT.
- 4. Dip in acetone containing 0.05% (v/v) acetic acid, 10 times RT.
- 5. Dehydrate through 95% and 100% ethanol, clear in xylene, and mount in VectaMount Permanent Mounting Medium (Vector Laboratories).

First primary antibody-Chromogen	Second primary antibody-Chromogen	Counterstain method	
p62-DAB (rapid)	OSP-Vector Red (rapid)	Haematoxylin	
OSP-Vector Red (rapid)	p62-DAB (rapid)	Haematoxylin	
p62-DAB	OSP-Vector Red (rapid)	Haematoxylin	
p62-DAB + double time avidin/biotin block	OSP-Vector Red (rapid)	Haematoxylin	
OSP-Vector Red (rapid) + double time avidin/biotin block	p62-DAB	Haematoxylin	
OSP-DAB	p62-Ni-DAB	Haematoxylin	
OSP-NovaRED	p62-DAB	Haematoxylin	
OSP-NovaRED	p62-Ni-DAB	Haematoxylin	
OSP-Vector Blue AP	p62-DAB	Vector methyl green	
OSP-Vector Blue AP	p62-Ni-DAB	Vector methyl green	

**Table 2.2: Attempts at the double staining of p62 and OSP.** Unless indicated (rapid), the protocols used standard IHC as described above.

#### <u>2.1.1.12 Nogo-A IHC</u>

We first tried to optimise this antibody using the same method used for MBP in fixed motor cortex, at primary antibody dilutions of 1:50/200/500/1500/5000. Then, we changed the method to that used for p62, trying antigen retrieval at both pH6 and pH9, and primary antibody dilutions of 1:50/100/150/200/500. Antigen retrieval at pH9, and 1:50 dilution were chosen as the option that provided the best balance of specificity and sensitivity. This will be later discussed in **chapter 3**.

# 2.1.1.13 Double IHC of Nogo-A and p62

We tried to optimise double immunostaining of Nogo-A and p62. To achieve this, the first step was to optimise staining of p62 using the pH9 antigen retrieval method, which works best for Nogo-A. We used p62 antibody dilutions of 1:20/100/200. The dilution we preferred was 1:200 using this antigen retrieval method.

For this double IHC, Nogo-A was stained using Vector Red as a chromogen instead of DAB, and p62 was stained using DAB to have a good contrast between the two. Two different protocols were used: In one, p62 was stained first, and in the second, Nogo-A was stained first.

# 2.1.2 IHC scoring

The areas where the different markers were quantified were the following:

**Layer V of frontal and motor cortices:** Layer V contains large pyramidal neurones that project to other brain areas. In the frontal cortex, layer V neurones synapse with the striatum and the thalamus. In the motor cortex, however, layer V neurones are the primary motor neurones that synapse with the lower motor neurones of the spinal cord. Investigating the pathology of the upper motor neurones and the surrounding glia in ALS could help explain any axonopathy found in the descending motor tracts of the spinal cord. Also, quantifying glial pathology in the motor cortex would be useful to discern whether it correlates with the glial and neuronal pathology of the spinal cord.

White matter underlying the frontal and motor cortices: the levels of glial pathology were quantified in the white matter directly beneath the cortices. Subcortical oligodendroglial pathology could be related to the above neuronal cortical pathology or, in the case of the precentral gyrus, even to that of the spinal cord descending motor tracts.

**Dorsolateral motor tracts of the spinal cord:** As mentioned above, upper motor neurones send their projections down the spinal cord to synapse with the lower motor neurones of the spinal cord. Most of those axons form the dorsolateral motor tracts of the spinal cord, in the most dorsal region of the lateral funiculi. Comparing the levels of glial pathology found in this area in ALS cases to those found in the primary motor cortex and underlying white matter could help elucidate whether they are correlated or independent events. Additionally, it was of interest to know whether the glial pathology in this region correlates with axonal counts for the same area.

**Ventral horns of the spinal cord:** This region harbours the large lower motor neurones that synapse with the muscles. These neurones degenerate in ALS and their pathology could be related to the surrounding glial pathology, or even that of the oligodendrocytes supporting the axons of the descending dorsolateral motor tracts, whose axons synapse onto the lower motor neurones.

# 2.1.2.1 p62-positive, pTDP43-positive and DPR inclusions

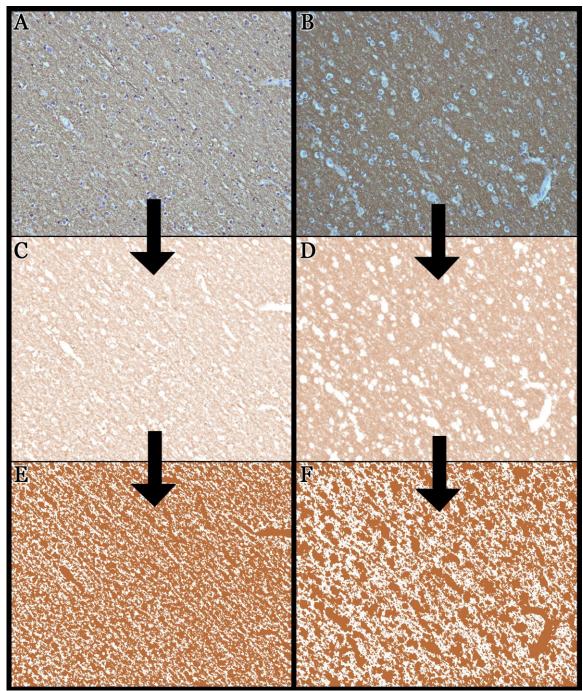
The total number of p62-positive and pTDP43-positive inclusions were counted in the ventral horn and the lateral motor tracts of spinal cord sections. In sections of the motor and frontal cortices, inclusions were counted in 10 fields at 25x magnification in layer V, and ten fields at 25x magnification in the underlying white matter.

In all types of tissue, for p62/pTDP43-positive inclusions, a distinction was made between inclusions present in neuronal cell bodies in the grey matter, those in glial cells in grey matter, and those present in glial cells in WM. DPR were only counted in the glia of the white matter under the motor cortex.

## 2.1.2.2 Percentage of MBP and PLP stained area

The proportion of area labelled for MBP and PLP in the white matter under the motor cortex, and the spinal cord dorsolateral motor tract was quantified by analysing 4 pictures from each section with ImageJ applying the following method:

- The images were taken using a Nikon DS Ri1 Eclipse microscope using a 20× objective.
- Each image was subjected to colour deconvolution (Ruifrok and Johnston, 2001) to separate DAB staining (brown) from hematoxylin staining (blue) (figure 2.1).
- 3. A Shanbhag threshold (Shanbhag, 1994) was applied to the DAB-only images, and the stained area fraction was measured in the pictures submitted to a threshold.
- **4.** The mean of the four values was used in the statistical analysis for each case.



**Figure 2.1: Processing of images to quantify the area labelled for MBP and PLP.** A and B are the original photographs. These example pictures are from fixed white matter under the motor cortex. C and D are the DAB images after using the colour deconvolution plug-in in ImageJ. E and F show the DAB images after subjecting them to a Shanbhag threshold, after which labelled area appears in white (quantified by ImageJ), and the unlabelled area appears in brown (not quantified by ImageJ).

#### <u>2.1.2.3 Axonal density using SMI31</u>

After staining neurofilaments in spinal cord with the SMI31 antibody in coronal sections of the spinal cord at the cervical level, axonal density was measured in the dorsolateral motor tracts using a protocol similar to that used by Highley et al. (1999) with some modifications:

- For each case, a total of four pictures at a 1280x1024 pixel resolution were taken from both tracts (two of each side) using a Nikon DS Ri1 Eclipse microscope with ×100 oil immersion objective.
- An 8x7 grid of identical rectangular counting frames (figure 2.3) of 160x146.18 pixels was placed on top of each image using the macro shown in figure 2.2.
- 3. Five counting frames were chosen from each image using the www.random.org random integer generator. Twenty frames were counted in total from each patient.
- 4. Section-spanning axons were counted according to standard stereology rules (Gundersen et al., 1988) in each chosen counting frame, discarding those axons touching the top and left sides and including the axons touching the bottom and right sides.
- 5. The approximate "axons per image" was calculated using the formula

$$Axons_{total} = \frac{\sum Axons_{rec}}{5} \times 56$$

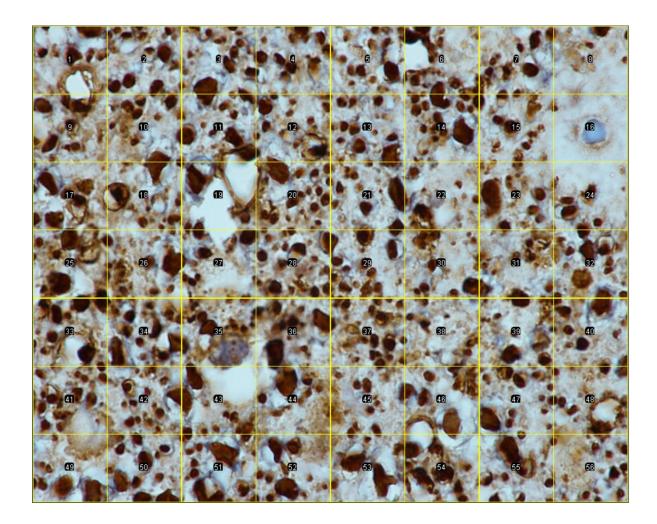
where  $Axons_{total}$  is the total number of axons in the picture, and  $\sum Axons_{rec}$  is the total number of axons counted in the five rectangles. The average number of axons per counting frame was multiplied by 56 because each image was divided into 56 rectangles.

6. The value for axonal density in each dorsolateral motor tract was calculated dividing *Axons<sub>total</sub>* by the total area represented in the picture (AREA):

$$Axon_{density} = \frac{Axons_{total}}{AREA}$$

```
x = 0;
y = 0;
width = 160;
height = 146.28;
spacing = 0;
numRow = 7;
numCol = 8;
for(i = 0; i < numRow; i++)</pre>
{
        for(j = 0; j < numCol; j++)</pre>
        {
                 xOffset = j * (width + spacing);
                 print(xOffset);
                 yOffset = i * (height + spacing);
                 print(yOffset);
                 makeRectangle(x + xOffset, y + yOffset, width, height);
                 roiManager("Add");
                 if (roiManager("count") > 100)
                         print("Maximum reached: 100 entries have been created.");
                         exit;
                          }
        }
}
```

**Figure 2.2: Macro to create a grid of rectangles.** This macro was used to overlay a grid of rectangles on an image. The variables *width* and *height* refer to the dimensions of each rectangle; *spacing* sets the space left between rectangles; *numRow* and *numCol* indicate the number of rows and columns in the grid respectively.



**Figure 2.3: Grid of rectangles.** This picture shows the result of applying the macro described in the previous figure. It was designed for the grid to cover the totality of the image. The numbers were assigned to select random rectangles from each image. This is a photography of a lateral corticospinal tract in a fixed spinal cord section.

## 2.1.2.4 Quantification of oligodendrocyte precursor cells (OPCs)

The number of OPCs labelled with the MAP2+13 antibody were counted in 20 fields at 20× magnification in the motor cortex grey matter (layer 5) and white matter. Regarding the spinal cord, total numbers of OPCs were counted in the ventral horn and the lateral motor tract of 4 sections. This number was divided by the number of half sections counted, to obtain the number of OPC per ventral horns or dorsolateral motor tracts.

## 2.1.2.5 Percentage of cells labelled for hnRNP-A2

hnRNP-A2 is mainly located in the nucleus. The relative number of nuclei labelled for hnRNP-A2 in the white matter under the motor cortex and the spinal cord lateral tracts was obtained by counting labelled and unlabelled nuclei in 4 images of these areas taken using the 20x objective in a Nikon DS Ri1 Eclipse microscope.

## 2.1.3 Quantitation of protein in human post-mortem tissue

In order to ascertain whether oligodendroglial mRNA transport is impaired in the motor tracts of ALS patients, MBP, whose translation is dependent on mRNA transport, was quantified in the dorsolateral motor tracts. A lack of MBP could be caused by a general malfunction of oligodendrocytes rather than a specific mRNA transport impairment. Therefore, PLP, a myelin protein that is translated in the cell body, whose translation does not depend on mRNA transport, was measured as well in the same area. This study also measured the same proteins in the dorsal column, an extra-motor area that would serve as an internal control. These and other proteins of interest and the reasons why we quantified them are as follows:

- **MBP**: The protein whose mRNA transport (and therefore, its translation) we hypothesised is impaired in C9ALS. If the hypothesis is correct, we would expect to see an acute reduction in the amount of MBP in C9ALS.
- PLP1: The most common protein found in myelin, which is translated in the cell body and transported to the myelin compartment as a protein. If there is a general myelin loss rather than specific MBP loss, PLP levels should be affected. Equally, if mRNA transport is affected specifically, PLP should be affected to a lesser degree than MBP. Thus, the MBP/PLP ratio will decrease.
- hnRNP-A2: The protein that is necessary for the correct transport of the *MBP* mRNA in oligodendrocytes. We need to know whether a hypothetical reduction in MBP is caused by depletion of this protein.
- Neurofilament heavy chain: This is an axonal marker, labelled by the SMI31 antibody. Whether axonal loss causes oligodendroglial degeneration or vice versa was still unclear. This marker would give some guidance on this issue: If MBP was affected disproportionately compared to neurofilament, it would provide evidence that degeneration of oligodendrocytes precedes axonal degeneration.
- β-tubulin: This is the control "housekeeping" protein of our choice to be used for normalisation, and it is not thought to be affected by the mechanisms under investigation.

#### 2.1.3.1 Spinal cord tissue dissection for protein and RNA extraction

Tissue from frozen dorsolateral motor tract and dorsal column was dissected from thoracic spinal cord blocks to measure their protein and RNA contents by a neuropathologist (JRH). Dorsolateral-motor-tract-enriched tissue pieces were excised from the dorsolateral area of the lateral funiculi, located in the immediate ventrolateral area from the grey matter posterior columns. Dorsal column tissue pieces were excised from the area between both grey matter posterior columns. Both the dissection surface inside the cryostat and the scalpel were sterilised between samples using 70% IMS in water.

#### <u>2.1.3.2 Protein extraction</u>

Tissue samples were homogenised in RIPA buffer, using an electric pellet pestle (Fisherbrand). The composition of the RIPA buffer was as follows: 50 mM Tris-HCl pH8, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl. Immediately before proceeding to homogenise the tissue, one tablet of Protease Inhibitor Cocktail (Roche) and 1 mM 4-benzenesulfonyl fluoride hydrochloride (AEBSF) were added to 50 ml of RIPA buffer. All the homogenisation steps were done with the samples on ice.

To homogenise the tissue, it was first weighed. Three times the weight in milligrams of the tissue was added of RIPA buffer volume in microliters. The tissue was then homogenised with the pellet pestle and centrifuged for 5 min at 4°C at 21000 g. The supernatant containing soluble protein was removed and kept in a separate tube. Both tubes containing soluble and insoluble proteins were kept at -80°C.

#### <u>2.1.3.3 Bicinchoninic acid (BCA) protein assay</u>

Due to the presence of EDTA in the RIPA buffer, the concentration of the extracted protein was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher). The protocol followed was the one indicated in the manual of the kit. A standard BSA curve was made using duplicate samples for the following serial concentrations of BSA (mg/ml): 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ ,  $\frac{1}{16}$ ,  $\frac{1}{32}$ ,  $\frac{1}{64}$ ,  $\frac{1}{128}$ . The absorbance of the solutions was measured using the PHERAstar FS plate reader (BMG Labtech).

#### <u>2.1.3.4 Western blot</u>

Western blotting was used to quantify the relative amounts of MBP, PLP, hnRNP-A2, neurofilament heavy chain, and  $\beta$ -tubulin in the protein extracts. To denature the protein, the necessary volume for 40 µg of protein, 3 µl of 6× denaturing protein loading buffer (0.375 M Tris pH 6.8, 12% sodium dodecyl sulfate, 60% glycerol, 0.6 M dithiothreitol, 0.06% bromophenol blue), and distilled water to 18 µl, were mixed and incubated at 95°C for 10min.

Polyacrylamide gels in which the samples were resolved were made as follows: 10% RESOLVING GEL: 7.9 ml distilled H<sub>2</sub>O; 6.7 ml 30% Acrylamide/Bis 37.5:1 Solution (Bio-Rad); 5 ml 1.5M Tris pH 8.8; 200  $\mu$ l 10% SDS; 200  $\mu$ l 10% APS; 8 $\mu$ l TEMED. *STACKING GEL*: 2.7 ml distilled H<sub>2</sub>O; 670  $\mu$ l 30% Acrylamide/Bis Solution; 500  $\mu$ l 1M Tris pH 6.8; 40  $\mu$ l 10% SDS; 40  $\mu$ l 10% APS; 4  $\mu$ l TEMED.

The samples were loaded on the gel along with the Precision Plus Protein Dual Color Standards (Bio-Rad) and were electrophoresed in electrophoresis buffer at 100v for approximately two hours until the blue dye reached the bottom of the gel.

*ELECTROPHORESIS BUFFER:* 3.5 mM SDS, 25 mM Tris, 0.192 M glycine, 2.5 mM Tris, 19.2 mM glycine, 2 mM Tris pH 7.5, 15 mM NaCl.

The protein from the gel was then transferred to a PVDF membrane which had been previously activated in 100% methanol. The transfer was performed in icecold transfer buffer, for 1h at 300mA.

TRANSFER BUFFER: 25 mM Tris, 190 mM glycine, 20% methanol.

Protein transfer was confirmed by staining the membrane with Ponceau S solution (0.1 % in 5% acetic acid in distilled water). The membrane was then

washed with distilled water and blocked for 1h in 5% reconstituted cow's milk/TBST. It was then washed 3x10min in TBST.

*TBST:* 20 mM Tris, 150 mM NaCl, 0.1% Tween 20.

All antibodies were diluted in TBST, and three 10min washes in TBST were done after each incubation. Image acquisition of the protein bands was performed with a G-Box (Syngene) after subjecting the membranes to treatment with an enhance chemiluminescence solution (Biological Industries). The image acquisition method is explained below. The antibody incubations were as follows: the whole membrane was incubated with 1:1500 anti-HNRNPA2B1 mouse antibody for 1h at RT, then 1:10000 anti-mouse IgG-HRP for 1h at RT. After obtaining an image, the membrane was incubated with 1:3000 anti-MBP rabbit antibody/0.02% sodium azide for 1h at RT, followed by 1:10000 anti-rabbit IgG-HRP for 1h at RT. After an image was acquired, the membrane was incubated with 1:10000 anti- $\beta$ tubulin mouse antibody/0.02% sodium azide for 1h RT, followed by 1:10000 antimouse IgG-HRP for 1h RT. This time, after the image was acquired, the membrane was cut to isolate the parts where neurofilament protein chains and PLP should be. Each of these sections of the membrane was incubated with either 1:1000 SMI31 mouse antibody (for neurofilament labelling) or 1:5000 anti-PLP mouse antibody for 1h at RT, followed by 1:10000 anti-mouse IgG-HRP for 1h at RT. Images were then acquired.

# 2.1.3.5 Image acquisition of Western blots using the G-Box

After treatment with a chemiluminescent reagent and incubation for 1 min, the blotted membrane was introduced in a transparent plastic bag to avoid evaporation of the liquid and dryness of the membrane. This bag was introduced in the G-Box chamber, and chemiluminescence was measured using GeneSnap software (Syngene). The program includes an algorithm called "Intelli-chemi", which determines the time the camera should be exposed to the chemiluminescent signal to avoid light saturation. This mode was used to acquire the images shown in the results.

# 2.1.3.6 Analysis of Western blots

Relative protein levels in gel bands were quantified using the GeneTools software (Syngene). Whole bands were selected as regions of interest (ROIs), and the optical density was measured for each ROI. This measured density is directly proportional to the amount of protein when the image is sub-saturated. All results were normalised to the average of the loading control to obtain a percentage related to the loading control.

# 2.1.4 Quantitation of RNA in human post-mortem tissue

mRNA encoding for the same proteins measured in the spinal cord was quantified in the same areas. This was done to confirm that any loss of a protein found in the Western blot had not been caused by a depletion of its corresponding mRNA.

## 2.1.4.1 RNA extraction from human post-mortem tissue

Tissue samples were homogenised in Trizol buffer using the Direct-zol MiniPrep Plus kit (Zymo Research). The RNA was prepared according to the manufacturer's instructions. It works similarly to a DNA miniprep kit, and the membrane-bound RNA was eluted in 50µl of nuclease-free water and stored at -80°C.

## <u>2.1.4.2 cDNA synthesis from total RNA</u>

cDNA was synthesised using a SuperScript II Reverse Transcriptase kit. Due to the multiple ways in which this kit can be used, the protocol used in our experiments is detailed here: On ice, 10  $\mu$ l of extracted total RNA was mixed with 1  $\mu$ l of random primer mix (3  $\mu$ g/ $\mu$ L in 3 mM Tris-HCl pH 7.0, 0.2 mM EDTA), and 1 $\mu$ l of dNTPs mix (each at a final concentration of 10 mM, in 10 mM Tris-HCl pH 7.5). The mixture was incubated for 5min at 65°C and put back on ice afterwards. After a rapid centrifugation step, 4 $\mu$ l of 5xFS Buffer and 2 $\mu$ l of 0.1M DTT were added. The mixture was incubated at 25°C for 2min. Then, 1 $\mu$ l of SuperScript II Reverse Transcriptase was added, and the mixture was incubated sequentially for 10min at 25°C, 50min at 42°C, and 15min at 70°C. The synthesised cDNA was stored at -20°C.

#### <u>2.1.4.3 Quantitative PCR</u>

Previously RT-synthesised cDNA was quantified using qPCR. A 5' nucleasequencher assay was used to generate fluorescence. In this type of assay, a probe specific to the region being amplified is attached to a fluorophore (6-FAM) and two quencher molecules (ZEN & Iowa Black FQ) which stop 6-FAM from producing fluorescence. When the polymerase starts synthesising a new DNA strand, it will reach the probe, and the 5' nuclease activity of the enzyme will cleave 6-FAM off the probe. This cleavage releases 6-FAM from the quenchers, allowing it to produce fluorescence, which is then measured by a detector. This fluorescence increases every PCR cycle as more probe molecules are cleaved, enabling us to measure the relative amounts of a certain cDNA. Predesigned assays were acquired from IDT (Integrated DNA Technologies). These include specific primers and probes to gauge the amount of certain mRNA isoforms. The assays used are described below.

*MBP*: Assay number Hs.PT.58.2684380 measures the amount of the isoform MBP-001 (RefSeq NM\_002385-2). This is the predominant isoform of *MBP* mRNA which is transported to the myelin compartment.

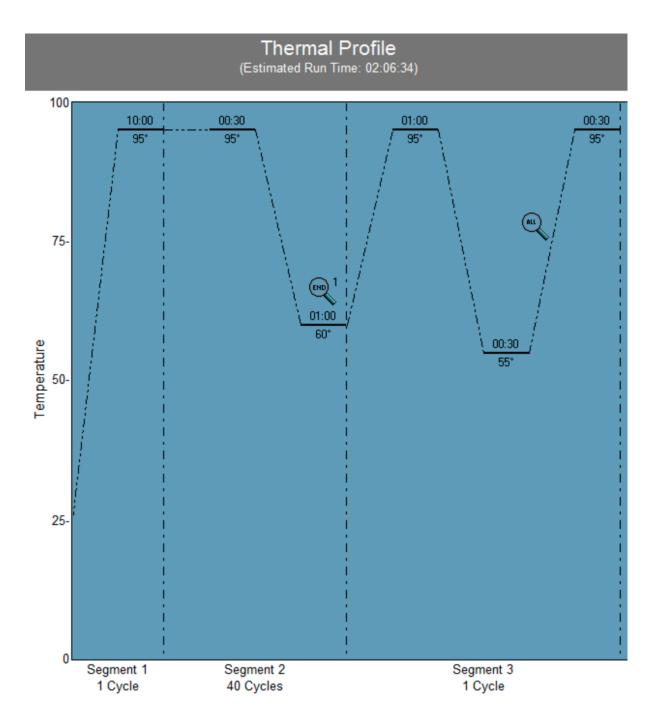
**PLP1**: Assay number Hs.PT.58.39005119 measures the amount of the different isoforms of PLP1 mRNA. No specific assays for different isoforms were available at the time of ordering.

*HNRNPA2B1*: Assay number Hs.PT.58.20218961 measures the amount of the different isoforms of the *HNRNPA2B1* gene. No specific assays for isoforms specific for hnRNP-A2 were available at the time of ordering.

**RNA18S5**: This was the control gene of choice. Assay number Hs.PT.39a.22214856.g measures the amount of 18s ribosomal RNA (rRNA) present in the sample.

The PCR protocol used was a standard 2-step protocol with the following steps (**figure 2.4**):

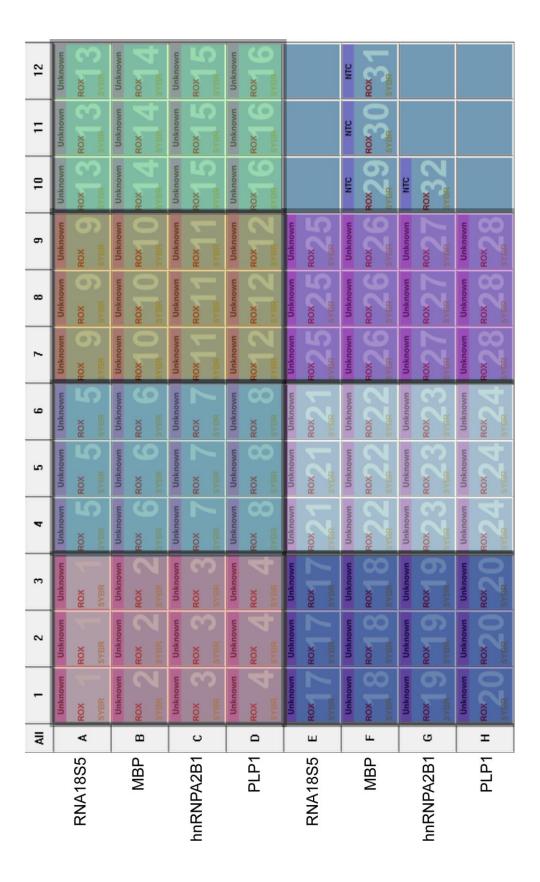
- 1. 10min initial denaturation step at 95°C.
- 2. 40 cycles (30 s at 95°C, 1min at 60°C, measuring fluorescence at 520nm).
- 3. 1min at 95°C.
- 4. Dissociation curve starting at 55°C and ending at 95°C with 0.5°C steps (the dissociation curve is not necessary for a 5'-nuclease-based protocol, but it was pre-set in the program because it is a protocol used for SYBR Green intercalating dye qPCR, which emits light at the same wavelength as 6-FAM, the dye we used).



**Figure 2.4: qPCR program.** Two-step programme for qPCR. END indicates the step after which a measurement is taken. Segment 3 generates a dissociation curve, usually used when DNA-intercalating dyes are used. We used a 5' nuclease-quencher assay which does not require this step, but it did not change our results, which were obtained during segment 2.

#### <u>2.1.4.4 qPCR setup</u>

Because the qPCR was done in triplicate for each sample and gene, all samples would not fit in a single 96-well plate. To be able to compare results from different runs, one of the samples was included in every run. This allowed for cross-comparison between runs. In **figure 2.5**, triplicates 1 to 4 are the ones done for our cross-referenced sample, while the four individually numbered ones (29-32) are non-template controls (NTC) to account for any DNA contamination in our samples. Contamination is particularly relevant when using ribosomal RNA as the reference gene, due to its ubiquitousness in all species. If our NTC presented a Ct value, and it was anywhere near or below the Ct values obtained for our samples, then the results obtained for those samples would not be reliable, since the contribution to the RNA measurement due to contamination would be indistinguishable from that due to our sample's RNA. If any of the samples did not reach a Ct value for one of the genes, then the protocol was repeated for that sample. If it did not reach a Ct value this second time, it was not included in the analysis for that gene.



**Figure 2.5: qPCR set-up.** Same numbers represent triplicates. Triplicates 1 to 4 are from the sample which was used as the inter-plate reference. Each colour represents one cDNA sample. Wells 29 to 32 are non-template controls.

## 2.1.4.5 Calculations for relative amounts of mRNA

The amount of mRNA in the original tissue homogenates is assumed to be directly proportional to the amount of cDNA synthesised during the reverse transcription reaction. Thus, measuring the relative quantities of a specific cDNA in the samples subjected to qPCR gives a direct correlation to the relative amount of specific mRNA in the homogenised tissue.

The following steps were followed to obtain a semi-quantitative result for the cDNA concentration of each gene from the Ct values provided by qPCR:

1. The Ct value for each triplicate was averaged.

2. The averaged Ct values were firstly normalised to the reference sample, present in all experiments. The obtained value is termed  $Ct_{eene}$ .

3. The Ct values obtained in step 2 for each gene different from *RNA18S5* were normalised to the *RNA18S5* Ct value obtained for the same sample ( $Ct_{RNA18S5}$ ). This value is termed  $\Delta Ct_{eque}$ .

$$\Delta Ct_{gene} = Ct_{gene} - Ct_{RNA18S5}$$

4. The average was calculated for the  $\Delta Ct_{gene}$  values of the healthy control samples obtained in step 3, for both dorsolateral motor tracts and DSSC samples. This average is called  $\Delta Ct_{control}$ .

5. For each spinal cord region, the  $\Delta Ct_{gene}$  values obtained in step 3 were used to calculate the concentration of cDNA relative to the control group ( $[cDNA]_r$ ), using the following formula:

$$[cDNA]_{gene} = \left(2^{\Delta Ct_{control} - \Delta Ct_{gene}}\right) \times 100$$

The 100 factor was added to get more intuitive numbers. It does not change the proportionality across the data so that the results will be the same.

The numbers obtained using this formula are the comparable mRNA copy numbers found in our samples, normalised to the average of the control group.

#### 2.1.5 Statistical analyses

All statistical analyses for significance were done using R (R Foundation for Statistical Computing, Vienna, Austria) and the  $\alpha$ -value was set at 0.05 for all tests. The normality of the data distribution was tested using the Shapiro-Wilk normality test. Due to the lack of normality presented by the data, and to the limited amount of cases, homoscedasticity was tested using the Brown-Forsythe Levene-type test. Stochastic dominance amongst the difference groups was tested with the Kruskal-Wallis test. Where a Kruskal-Wallis test showed significance, posthoc Mann-Whitney tests were performed. One-tailed Mann-Whitney tests were used when the hypothesis predicted change in a single direction and change in the other direction would be irrelevant to the study. Otherwise, 2-tailed tests were used. Statistical significance of correlations between variables was measured calculating the Kendall tau rank correlation coefficient. Post hoc statistical power analyses were performed with G-Power (version 3.1.9.2).

# 2.2 Generation of zebrafish transgenic and mutant models

## 2.2.1 General reagents

#### <u>2.2.1.1 LB agar solid medium</u>

Antibiotic-selective plates for growth of transformed bacteria were made using LB agar solid medium. LB agar powder (Fischer Bioreagents) was reconstituted with water at a concentration of 40 g/l, which was then sterilised by autoclaving. Antibiotics (carbenicillin 50 mg/ml, kanamycin 50 mg/ml, spectinomycin 50 mg/ml, and chloramphenicol 25 mg/ml) were added to the sterile agar for a final concentration of 50  $\mu$ g/ml, and the mixture was poured into sterile plastic Petri dishes near a flame and allowed to cool. Plates were stored inverted at 4°C if not used on that day.

## 2.2.1.2 LB broth liquid medium

This medium was used to grow bacteria for plasmid preparation. LB broth was dissolved in water at a concentration of 20 g/l, which was then sterilised by autoclaving, and hermetically closed for later use. It was stored at room temperature.

#### <u>2.2.1.3 TAE buffer</u>

Agarose gels were made using this buffer, and then electrophoresed immersed in it in a tank. A 1x TAE working solution contains 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA.

#### <u>2.2.1.4 TE buffer</u>

This buffer was used to extract plasmid DNA from pieces of filter paper. A 1x TE solution contains 10mM Tris, and 1mM EDTA, pH 8 (adjusted with HCl).

# 2.2.2 Methods for the generation of the Gateway construct

# 2.2.2.1 Polymerase chain reaction (PCR)

To amplify the mbpa promoter from the zebrafish (Danio rerio) genome, PCR was performed using primers.

Forward primer: 5'-GTCGACCAGATGCTGAGATGTGACTACTGCAAATGA-3'

Reverse primer: 5'-GGATCCGTTGATCTGTTCAGTGGTCTACAGTCTGGA-3'

Each PCR reaction contained the following: 1  $\mu$ l of diluted DNA, 1.25  $\mu$ l of forward primer (10  $\mu$ M), 1.25  $\mu$ l of reverse primer (10  $\mu$ M), 0.25  $\mu$ l of Q5 High-Fidelity DNA Polymerase (New England Biolabs), 5.0  $\mu$ l of 5x Q5 reaction buffer (New England Biolabs), 0.5  $\mu$ l of dNTPs solution (10 mM, 2.5 mM of each dNTP) (Bioline), and 15.75  $\mu$ l of sterile, nuclease-free water.

Two different thermal cycling programmes were utilised:

**-Standard PCR:** 5 minutes at 95°C followed by 35 cycles (10 seconds at 95°C, 30 seconds at 72°C, 90 seconds at 72°C) and 10 minutes at 72°C.

-Touchdown PCR: 5 minutes at 95°C followed by 15 touchdown cycles (1 minute at 95°C, 1 minute at  $65 \rightarrow 50°$ C decreasing 1 degree per cycle, 1 minute at 72°C), 25 standard cycles (1 minute at 95°C, 1 minute at 58°C, 1 minute at 72°C), and 10 minutes at 72°C.

#### 2.2.2.2 Plasmid extraction from filter paper

We obtained a small sample of a plasmid containing the *mbpa* promoter from Kim Lab (Jung et al., 2010). The plasmid was embedded in a piece of filter paper, so it needed to be extracted from it. To achieve this, the piece of paper was soaked in enough TE buffer to cover it and left overnight at room temperature to allow the extraction. The extracted plasmid was stored at 4°C for subsequent transformation.

#### 2.2.2.3 Plasmid DNA transformation

Three different commercially available strains of *E. coli* were used to grow up stock plasmids. Each of these required an independent protocol, both provided by the manufacturer:

## Subcloning Efficiency DH5α Competent Cells (Invitrogen)

This strain was transformed with the plasmid containing the zebrafish *mbpa* promoter, obtained from Jung et al. (2010) and plasmids containing probe templates for *in situ* hybridisation. This strain was used so that the concentration of plasmid obtained would be enough for the subcloning of the *mbpa* promoter.

# One Shot Mach1 T1 Phage-Resistant Chemically Competent *E. coli* (Invitrogen)

This strain was transformed with the plasmids necessary to construct zebrafish expression vectors. These plasmids are listed in **table 2.3**. Initially, this strained was selected for its high efficiency. However, knowing how unstable the *C9orf72* hexanucleotide repeats are, it would have been wiser to choose a strain that is better suited for cloning unstable sequences retrospectively. An example from the same brand would be the One Shot Stbl3 strain.

# One Shot ccdB Survival<sup>™</sup> 2 T1R Competent Cells (Invitrogen)

The Gateway destination vectors contain a *ccdB* gene, whose product is toxic to bacteria (Bernard and Couturier, 1992, Bernard et al., 1993). This strain is resistant to the ccdB product, making it useful to grow these vectors.

Transformed bacteria were spread on pre-warmed (30 min, 37°C) selective LBagar plates, and grown overnight at 37°C. The plates containing colonies of transformants were stored at 4°C.

#### 2.2.2.4 Plasmid DNA minipreparation

Competent bacteria which had been transformed previously were picked into 3 ml of sterile liquid LB medium containing 50 µg/ml of the appropriate antibiotic and grown at 37°C overnight on a rotating platform at 220 rpm. Plasmid DNA was then harvested using the NucleoSpin Plasmid kit (Macherey-Nagel) or the FastGene Plasmid Mini Kit (Nippon Genetics), following the manufacturer's protocol.

#### 2.2.2.5 Plasmid DNA midipreparation

A colony from each of the plates containing One Shot Mach1 transformants was picked into 3 ml of sterile liquid LB medium containing 50  $\mu$ g/ml of the appropriate antibiotic and grown at 37°C for 6-8 h on a rotating platform at 220 rpm. One hundred millilitres of autoclaved, fresh liquid LB medium containing 50  $\mu$ g/ml of the appropriate antibiotic, was then inoculated with the starter culture, and incubated overnight at 37°C as before. Plasmid DNA was then harvested using the Plasmid Midi Kit (Qiagen), according to the manufacturer's protocol.

# 2.2.2.6 Restriction endonuclease digestion of plasmid DNA

**Single digestion:** The reaction mix was composed of the following components: 10  $\mu$ g of diluted DNA (15-30  $\mu$ l), 5 $\mu$ l of either 10× B Buffer (Thermo Scientific) or 10× FastDigest Universal Buffer (Thermo Scientific) (according to the restriction enzyme requirements), 2.5  $\mu$ l of restriction enzyme (Thermo Scientific), and deionised water to a final reaction volume of 50  $\mu$ l. The mix was incubated at 37°C for 1 h.

**Double digestion:** The components were mixed in the following proportions: 10  $\mu$ g of DNA (15-30  $\mu$ l), 5  $\mu$ l of 10× FastDigest Universal Buffer (Thermo Scientific), 2.5  $\mu$ l of each enzyme (Thermo Scientific), and water to a final reaction volume of 50  $\mu$ l. The mix was incubated at 37°C for 1h.

# 2.2.2.7 Phenol: chloroform: isoamyl alcohol extraction of DNA

DNA was purified from digestion mixtures using a standard phenol:chloroform:isoamyl alcohol extraction: First, an approximately equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol was added to the digestion mix, and it was mixed briefly using a vortex to form an emulsion. The emulsion was centrifuged, and the upper aqueous phase pipetted into a fresh tube. The organic phase was back-extracted by repeating the previous steps and the aqueous phases pooled.

## 2.2.2.8 Ethanol precipitation of DNA

To precipitate DNA, 10% of the volume was added of 3 M sodium acetate pH 5.2. Two and a half volumes of that total volume was added of 100% ethanol to precipitate DNA. The mixture was incubated at -20°C for 1 h and then centrifuged at 4°C for 20min at a centrifugal force of 21000 g. The supernatant was removed carefully, and then the DNA pellet resuspended in 200µl of 70% ethanol. It was centrifuged at 4°C for 10 min at 21000g, and the supernatant was again removed. The DNA pellet was air-dried at room temperature until no visible traces of ethanol were present in the tube. The purified DNA was resuspended in an appropriate amount of deionised water. Half a microlitre of the resuspended DNA was analysed on an agarose gel to check the size and concentration.

# <u>2.2.2.9 Agarose gel electrophoresis</u>

DNA was electrophoresed on an agarose gel containing an appropriate concentration of agarose and 100 ng/ml ethidium bromide, at 120 or 80 V for enough time to allow clear resolution of the DNA bands. An appropriate DNA ladder was included in each run. DNA was visualised using a Geni Gel Documentation System (Syngene).

## 2.2.2.10 Purification of DNA from agarose gel slices

Bands were excised with a clean scalpel on a UV light transilluminator, weighed in a microcentrifuge tube, and then the DNA purified with the QIAEX II Gel Extraction kit (Qiagen) following the manufacturer's instructions.

## <u>2.2.2.11 5' dephosphorylation of digested DNA</u>

Dephosphorylation of the 5' ends of linearised plasmid DNA was performed to minimise vector recircularization during the ligation step. The reaction components were mixed in these proportions: 1  $\mu$ g of digested DNA (volume dependent on DNA concentration), 2  $\mu$ l of 10× CutSmart Buffer (New England Biolabs), 1 unit of Shrimp Alkaline Phosphatase (rSAP, New England Biolabs), and deionised water to a final reaction volume of 20  $\mu$ l (these volumes were scaled proportionally if a larger amount of DNA was to be dephosphorylated). The mix was incubated at 37°C for 30 min, and the reaction was then stopped by heat-inactivation at 65°C for 5 min.

## <u>2.2.2.12 DNA ligation</u>

T4 DNA ligase was used to clone purified inserts into their respective Gateway entry plasmids. The ligation components were mixed in the following proportions: 50 ng of vector (1-4  $\mu$ l), insert DNA giving a 1:3 vector:insert ratio

(1-4  $\mu$ l), 1  $\mu$ l of 10× T4 DNA ligase buffer (New England Biolabs), 1  $\mu$ l (400 units) of T4 DNA ligase (New England Biolabs), and deionised water to a final volume of 10  $\mu$ l. Ligation reactions were incubated overnight at room temperature.

## 2.2.2.13 Gateway recombination reaction

To recombine the inserts in the 5'E-[*mbpa* prom] (5' Entry-*mbpa* promoter), ME-10×/51xHNR (Middle entry-interrupted GGGGCC hexanucleotide repeats), and the 3'E-GFP (3' entry-Green fluorescent protein) plasmids with the pDestPA2 (Destination vector including a poly-adenine tail), 10fmol of each entry plasmid was added to 20 fmol of the pDestPA2 vector, to obtain a final volume of 8  $\mu$ l. To this mixture, 2 $\mu$ l of the Gateway LR Clonase II Plus (Thermo Fisher) was added, and the reaction was left at 25°C overnight.

#### <u>2.2.2.14 In vitro transcription of the Tol2 transposase mRNA</u>

The pCS2FA-transposase plasmid contains the transposon which encodes the Tol2 transposase, necessary for the Gateway construct to incorporate the desired genes in the chromosomal DNA of zebrafish embryos. The mRNA for this transposase needs to be co-injected with the DNA construct. To obtain this RNA, the pCS2FA-transposase plasmid was linearised with *Not*I, making it possible to obtain mRNA molecules for the transposase using the SP6 RNA polymerase. We used the mMESSAGE mMACHINE SP6 Transcription Kit (Thermo Fisher) to obtain this mRNA from 1µg of template DNA, according to the protocol provided in the kit. The template DNA was degraded using TURBO DNAse as indicated in the kit.

# 2.2.2.15 Alcohol precipitation of Tol2 transposase mRNA

To purify the generated mRNA, this is the protocol that was followed using the reagents included in the mMESSAGE mMACHINE SP6 Transcription Kit:

Nuclease-free water was added to the transcription reaction to a total volume of 135  $\mu$ l, then 15  $\mu$ l of Ammonium acetate solution and 300  $\mu$ l of 100% ethanol added, the mixture chilled for 15 min at -20°C, then centrifuged for 15 min at 4°C at 21000 g. The supernatant was removed, the pellet air-dried and resuspended in 10-20  $\mu$ l of nuclease-free water. RNA was stored at -80°C.

#### 2.2.2.16 Sequencing of DNA

The University of Sheffield Genomics Core Facility service uses the Applied Biosystems' 3730 DNA Analyser and BigDye v3.1 chemistry to analyse DNA sequences on demand. We sent 10  $\mu$ l of each purified DNA (about 100 ng/ $\mu$ l) to the UoS Genomics Core Facility for sequencing using the appropriate primers, which depend on the plasmid. When sequencing plasmids containing the HNR expansions, a sequencing reaction including betaine was requested. Betaine helps with the sequencing of GC-rich sequences.

## 2.2.2.17 Injection of zebrafish embryos with the Gateway vectors

The cell of one-cell stage zebrafish (Danio rerio) embryos was injected with a total of 30 pg of one of the generated *[mbpa prom]:10×/51xHNR:GFP:pDestPA2* constructs, 25 pg of Tol2 transposase mRNA, and phenol red to confirm the injection. The injection needed to be done directly in the cell for rapid integration of the constructed gene in the genome. Injected and uninjected (control) embryos were raised for five days in E3 medium containing methylene blue. The GFP reporter, which should hypothetically be expressed only by differentiating Mbpproducing oligodendrocytes in the CNS, should be visible by day 5.

## 2.2.2.18 Visualisation of GFP-fluorescent embryos

The embryos were anesthetised by adding 1 ml of 4 mg/ml tricaine methanesulfonate to each petri dish containing embryos to check for GFP fluorescence in the developing CNS. After cessation of movement, the embryos were visualised using a set of binocular lenses connected to a fluorescent light source, using filters to visualise GFP fluorescence.

Plasmid	Description	Resistance	Linearising enzyme	Double digestion enzymes
p5E-MCS	5'entry-Multiple- cloning site	Kanamycin	SalI	SacII/SalI
pME-MCS	Middle entry- Multiple-cloning site	Kanamycin	SalI	SmaI/SalI
p3E-EGFPpA	3'entry – Enhanced green fluorescent protein – Poly- adenine	Kanamycin	EcoRV	
pDestTol2pA	Destination vector – Poly- adenine	Ampicillin/Carbenicillin	<i>Eco</i> RI	
pGEM-T Easy- <i>mbpa</i> promoter	Plasmid containing the <i>mbpa</i> promoter, obtained from Jung et al. (2010)	Ampicillin/Carbenicillin		SacII/SalI
pcDNA 6.2 GW/emGFP- miR- HNRx10	Plasmid containing 10 interrupted hexanucleotide repeats (GGGGCC)	Spectinomycin	EcoRV	EcoRV/SalI
pcDNA 6.2 GW/emGFP- miR- HNRx51	Plasmid containing 51 interrupted hexanucleotide repeats (GGGGCC)	Spectinomycin	<i>Eco</i> RV	EcoRV/SalI
pcDNA 6.2 GW/emGFP- miR- HNRx102	Plasmid containing 102 interrupted hexanucleotide repeats (GGGGCC)	Spectinomycin	<i>Eco</i> RV	EcoRV/SalI

Table 2.3: Plasmids used to construct the Gateway zebrafish expression vectors, their integrated resistance gene, the restriction enzyme used linearise prior to electrophoresis, and the restriction enzymes used for cloning.

# 2.2.3 CRISPR/Cas9 experiment design

The aim of this part of the project was to use CRISPR/Cas9 technology to direct the mutagenesis of specific nucleotides in the zebrafish *tardbp* gene, the human *TARDBP* orthologue. The point mutations were planned to occur in conserved protein residues which are mutated in human fALS cases. A zebrafish *tardbp* point mutant model of ALS would be different from other zebrafish models involving the *tardbp* gene. For example, the *tardbp* knock-out strain generated by Hewamadduma et al. (2013) is useful to investigate the effects of a depletion of the *tardbp* protein product. This is different from investigating the effects produced by a defective protein, which includes a mutation found in humans in the same protein residue.

The CRISPR/Cas9 system, is based on a prokaryotic immune system which provides resistance against foreign segments of DNA, such as the ones contained in bacteriophage viruses. Cas9 nuclease recognises a small single-stranded RNA sequence called "guide RNA" (gRNA). This gRNA consists of a trans-activating scaffold region containing CRISPRs (tracrRNA) attached to a ~20-nucleotidelong sequence that is complementary to a genomic target sequence (spacer). Cas9 binds to the tracrRNA region of the gRNA, which brings Cas9 towards the target sequence (protospacer) of the genomic DNA, complementary to the spacer (**figure 2.6**). When the gRNA/Cas9 complex binds to the target sequence via the complementary region, Cas9 cleaves the DNA strand near the end of the target sequence. This cleavage only occurs if the protospacer is adjacent to a 3-5 nucleotide sequence named protospacer adjacent motif (PAM), which usually follows the sequence NGG (**figure 2.6**).

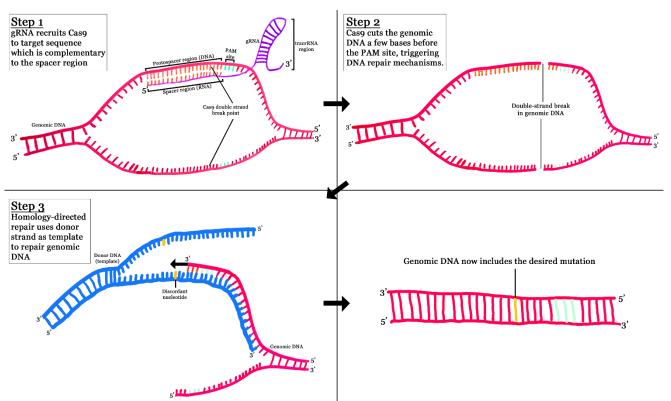
By designing a gRNA that matches a genomic sequence in which a missense mutation is desirable to be introduced, the CRISPR/Cas9 system could be used

to nick the genomic DNA strand close to the point in which the mutation should be introduced. This can trigger three different types of DNA repair mechanism: non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and homologous recombination (HR). NHEJ is prone to cause indels at the break point, where MMEJ causes deletions. HR recombines the cut DNA with an identical template for repair of both strands and causes no mutations in normal conditions.

A single-stranded oligodeoxynucleotide donor template (ssODN) is a DNA strand which is identical to the upstream and downstream sequence from the cleavage point except for the mutation we would like to introduce. Once the target sequence has been cleaved, the ssODN will serve as a template for the HR machinery in some events, thus introducing the desired mutation in the genomic DNA (**figure 2.6**).

The reason for selecting certain mutations amongst all the known ALS-causing *TARDBP* mutations, is because in the event of a successful mutagenesis, a restriction site would be lost, while a new restriction site would be generated. This would make screening for mutant zebrafish easier by using PCR and digesting the amplified DNA using specific restriction enzymes. There were two mutations that we selected to try and introduce in the zebrafish TARDBP protein: p.G347 $\rightarrow$ V and p.A379 $\rightarrow$ T. These mutations have been reported by (Budini et al., 2012) and (Orru et al., 2012) to cause ALS in humans. **Figure 2.7** shows that the targeted amino acids are conserved amongst the *TARDBP* orthologues of other species. To produce these mutants, the donor DNA strands needed to contain a mutation that changed the codons which encode those amino acids. In the case of the p.G347 $\rightarrow$ V mutation, the change would be GGT $\rightarrow$ GTC. For the p.A379 $\rightarrow$ T mutation, the change would be GCT $\rightarrow$ ACC. The DNA mutation to produce the

p.G347→V protein mutation would remove a *Cvi*QI restriction site and create a *Tsp*45I site in its place, whereas the mutation to obtain the p.A379→T protein change would eliminate a *Pvu*II site, generating a *Bsa*JI site instead.



**Figure 2.6: CRISPR/Cas9-directed mutagenesis.** First, a single-stranded gRNA binds to both Cas9 and to the genomic DNA region that is homologous to its spacer region, thus guiding Cas9 towards the desired cutting point. Then, Cas9 causes a double-strand break in the genomic DNA a few bases behind the PAM site. In some instances, the homology-directed repair machinery uses the donor DNA strand as the template to repair the DNA. This donor DNA includes the desired mutation, thus causing the repaired DNA to include it.

Zebrafish Xenopus Zebra Human Alpaca Pig	GGGGGSSSSLGNFGNFNLNPAMMAAAQAALQSSWGMMGMLAQ- PSSGALGNNQGGNMGGGGGMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQ GGGGGLGNNQGSNMGGGMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQ GGGAGLGNNQGSNMGGGMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQ GGGAGLGNNQGSNMGGGMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQ *** *:********************************	QNQSGPQG QNQSGPSG QNQSGPSG QNQSGPSG QNQSGPSG
Zebrafish	TSTSGTSSSRDQAQTYSSANSNYGSSSAALGWGTGSNSGAASAGFNSSFG	SSMESKSS
Xenopus	SNQGQGNQQRDQPQSFGSNN-SYGSNSGAIGWGSP-NAGS-GSGFNGGFG	SSMESKSS
Zebra	NNQPQGNMQREQNQGFSSGNNSYGGSNSGAAIGWGSASNAGS-SSGFNGGFG	SSMDSKSS
Human	NNQNQGNMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGS-GSGFNGGFG	SSMDSKSS
Alpaca	NNQSQGNMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGS-GSGFNGGFG	SSMDSKSS
Pig		SSMDSKSS

**Figure 2.7: Interspecies alignment of orthologous TDP-43 proteins.** The amino acids which were chosen for CRISPR/Cas9 mutagenesis in zebrafish are marked with a black arrow. The amino acid number indicates the position in the zebrafish sequence. The spacer sequence needs to be designed so that it is complementary to the genomic strand and targets the gRNAs to the target sequence. Additionally, the 5' end of the target sequence needs to contain a PAM site (NGG) for the Cas9 protein to effectively cleave both DNA strands three bases before the PAM site (**figure 2.8**). In general, there are five requisites for the choice of target sequences:

- It needs to be 20bp long.
- It must span the codon we intend to mutate.
- It needs to be adjacent to a PAM sequence which does not contain single nucleotide polymorphisms (SNPs) in the G nucleotides.
- It should not have a high degree of homology with other sequences in the genome of the target organism.
- The distance between the PAM sequence and the target codon has to be at least two bases, but they should not be too far from each other. This is the safe distance, so Cas9 does not cleave the site where our codon of interest is.

There are electronic tools to help choose target sequences that fulfil these requirements. We used the tool available at <u>http://crispr.mit.edu/</u> which scores multiple possible target sequences as a function of the degree of homology with other sequences in the genome of the organism of interest. The following sequence from *tardbp* exon 6 was introduced on the above website as a query (the red bases indicate the codons we aimed to mutate):

TTTGGGAATGGGTTCGGAGGTCAGGGTTTTGCAGGCAGCCGAAGCAACATGGGTGGT GGTGGTGGGGGGTAGCTCCAGCAGCTTGGGAAATTTTGGCAATTTCAATCTAAACCCG GCCATGATGGCTGCCGCCCAGGCTGCCTTGCAGAGTAGTTGGGGGTATGATGGGAATG CTAGCTCAGCAGAATCAGTCG**GGT**ACTTCAGGCACAAGCACAAGTGGCACCAGTTCC TCTCGAGACCAAGCCCAAACATATAGCTCGGCTAACAGCAATTACGGCAGCAGCTCAG

# CTGCTCTCGGCTGGGGGCACCGGCTCTAACTCGGGCGCTGCCAGTGCTGGCTTTAACT CCAGTTTTGGCTCTAGTATGGAGTCCAAGTCATCGGGGTG

The website proposed a significant number of plausible target sequences within the query sequence, and we selected the ones with the best scores that had all the requirements stated above. These are given below. The highlighted sequence is the PAM sequence, which is present in the genome, but which should not be included in the gRNA, and the red sequence is the codon of interest.

# p.G347→V

# GAATCAGTCGGGTACTTC AGG

p.A379→T

# GGCAGCAGCTCAGCTGCTCT CGG

# **Blue** = Target sequence; **Red** = codon to be mutated; **Yellow** = PAM site.

Thus, the full sequences for the gRNAs, including the scaffold sequence (common to all CRISPR gRNA) are as follows:

# p.G347→V

GAAUCAGUCGGGUACUUCGUUUUUAGAGCUAGAAAUAGCAAGUUAA AAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUU

p.A379→T

# GGCAGCAGCUCAGCUGCUCUGUUUUAGAGCUAGAAAUAGCAAGUU AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGU CGGUGCUUU

**Blue** = Target sequence; **Red** = codon to be mutated; **Black** = scaffold

To obtain these RNA sequences by in vitro transcription, a DNA template which included a T7 promoter was added at the end of the DNA reverse complement of the above sequences:

p.G347→V

# AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCC TTATTTTAACTTGCTATTTCTAGCTCTAAAACGAAGTACCCGACTGAT TCCTATAGTGAGTCGTATTACGC

p.A379→T

AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCC TTATTTTAACTTGCTATTTCTAGCTCTAAAAACAGAGCAGCTGAGCTGC TGCCCTATAGTGAGTCGTATTACGC

Blue = Target sequence; **Red** = codon to be mutated; Black = scaffold; Gold = T7 promoter p.G347→V

p.A379→T

GTTGCCGCCGCTTTGTGTGGAGAAGATCTGATCATCAAGGGCCGTCAG TGTGCATATCTCAAACGCTGAGCCCAAACACAATAACACTAGGCAGA TGATGGAGCGGGCAGGGCGC**IIITGGGAATGGGTITCGGAGG**TCAGGG TTTTGCAGGCAGCCGAAGCAACATGGGTGGTGGTGGTGGGGGGGTAGC TCCAGCAGCTTGGGAAATTTTGGCAATTTCAATCTAAACCCGGCCATG ATGGCTGCCGCCCAGGCTGCCTTGCAGAGTAGTTGGGGGTATGATGGG AATGCTAGCTCAGCAGAATCAGTCGGGTACTTCAGGCACAAGCACAA GTGGCACCAGTTCCTCTCGAGACCAAGCCCAAACATATAGCTCGGCT AACAGCAATTACGCCAGCAGCCCAAGCCCAAACATATAGCTCGGCT CTCTAACTCGGGCGCCGCCAGTGCTGGCTTTAACTCCA GTGTATGGAGTCCAAGTCATCGGGGTATGTAA

**Figure 2.8: CRISPR design for the exon 6 coding sequence. Blue** = Target sequence; **Red** = codon to be mutated; **Yellow** = PAM site; **Grey** = CviQI (p.G347 $\rightarrow$ V) or PvuII (p.A379 $\rightarrow$ T) restriction site, which disappears after successful mutagenesis, leaving a *Tsp*54I and a *Bsa*JI site respectively; **Green** = sequence to be added to donor DNA; **Black** = Primer target sequences

### 2.2.3.1 Testing for possible single nucleotide polymorphisms on the PAM sites

If the PAM sites contained common SNPs in any of the two compulsory G nucleotide residues, this technique would prove inefficient since Cas9 would be unable to cause the double strand break in the target sequence in the embryos where these SNPs were present.

A group of 2 days post-fertilisation (dpf) wild type zebrafish embryos were anesthetised to test for SNPs in exon 6, by adding 1 ml of 4 mg/ml tricaine methanesulfonate to each petri dish containing embryos, to extract their genomic DNA.

#### 2.2.3.2 DNA extraction of zebrafish embryos

To extract the genomic DNA of 2 dpf zebrafish embryos, each one was submerged in 30  $\mu$ l of DNA extraction solution containing 10 mM Tris pH 8, 2 mM EDTA, 0.2% Triton X-100, and 200  $\mu$ g/ml Proteinase K. The suspended embryos were incubated at 65°C for 2 h, followed by 98°C for 2 min.

The zebrafish lysate was subjected to PCR using a set of primers designed to amplify both target sequences (**figure 2.8**):

#### Exon6 forward primer: TTTGGGAATGGGTTTGGAGG

#### Exon6 reverse primer: CACCCCGATGACTTGGACTC

These primers will be called Exon6 primers henceforth. The following reagents were mixed for PCR: 13  $\mu$ l dH<sub>2</sub>O, 1  $\mu$ l extracted DNA, 1  $\mu$ l 10  $\mu$ M forward primer, 1  $\mu$ l 10  $\mu$ M reverse primer, 4  $\mu$ l 5x FIREPol Master Mix. The temperature settings

were the following: 94°C for 3 min, 30 cycles (94°C for 30 s, 58°C for 45 s, 72°C for 90 s), 72°C for 2 min, store at 10°C.

### <u>2.2.3.3 ExoSAP treatment of PCR-amplified DNA</u>

Primers and dNTPs, which would be a contaminant for sequencing, were degraded using an ExoSAP protocol, which includes the following reagents:  $5 \mu$ l PCR mix,  $3.95 \mu$ l dH<sub>2</sub>O,  $1 \mu$ l Shrimp Alkaline Phosphatase (New England Biolabs), 0.05  $\mu$ l Exonuclease I (New England Biolabs). This mixture was left at 37°C for 45 min, followed by 80°C for 15 min, and was performed in a thermocycler.

# 2.2.3.4 Sequencing of the tardbp exon 6 amplicon

The sequencing technique was as described in **section 2.2.2.16**, using the Exon6 primers described above.

### 2.2.3.5 Amplification of CRISPR templates

Template ultramers were obtained from IDT (Integrated DNA Technologies) in lyophilised form and were diluted to 0.01 mM. The ultramers were then amplified via PCR using these primers:

**Forward primer:** AAAGCACCGACTCGGTGCCA (targets the beginning of the scaffold sequence).

**Reverse primer:** GCGTAATACGACTCACTATA (targets the T7 promoter).

The PCR reaction was assembled mixing 73  $\mu$ l dH<sub>2</sub>O, 2  $\mu$ l template ultramer, 2.5  $\mu$ l forward primer, 2.5  $\mu$ l reverse primer, 20  $\mu$ l 5x FIREPol Master Mix (Solis Biodyne). The reactions and a negative control without template DNA were done in a thermocycler using the following PCR protocol: 1 min at 95°C, 40 cycles (15 s at 95°C, 30 s at 60°C, 20 s at 72°C), 5 min at 72°C, store at 4°C. The completed

reactions were analysed using a 3% agarose gel along with Hyperladder V (Bioline) to check for a band at 120 bp. The band was excised and purified using the PureLink Quick Gel Extraction Kit (Invitrogen).

#### <u>2.2.3.6 Synthesis of the gRNA</u>

A T7 reaction was assembled to synthesise the gRNA from the DNA templates, using these volumes: 4  $\mu$ l template DNA, 1  $\mu$ l 10× T7 reaction buffer, 1  $\mu$ l ATP 10 mM, 1  $\mu$ l CTP 10 mM, 1  $\mu$ l 10 mM UTP, 1  $\mu$ l 10 mM GTP, 1  $\mu$ l T7 enzyme mix. The mixture was incubated at 37°C for 4 h. One microliter of Turbo DNAse was added to degrade the DNA template, and the reaction was incubated at 37°C for 15 min.

#### <u>2.2.3.7 Purification of the gRNA</u>

Ethanol precipitation was used to purify the gRNA following these steps: 125  $\mu$ l of nuclease free water and 15  $\mu$ l ammonium acetate were added to the previous T7/Turbo DNAse reaction, then 302  $\mu$ l of ethanol added to precipitate the RNA, leaving it to chill at -20°C for 15 min. The RNA was centrifuged for 15 min at 4°C at 20000 g; the supernatant was discarded, and the RNA pellet was left to air dry, resuspended in 10  $\mu$ l of nuclease free water, and stored at -80°C. Half a microlitre of the purified gRNA was visualised in a 3% agarose gel, using the DNA Hyperladder V to give a rough approximate reference of the size (since single-stranded RNA migrates differently than double-stranded DNA in a gel).

#### 2.2.3.8 Injection of zebrafish using the gRNA and Cas9

Prior to injecting the gRNAs, they were individually mixed on ice with the following reagents: 1  $\mu$ l 0.5% phenol red (for visualisation of the injection), 0.5  $\mu$ l Cas9 protein (New England BioLabs) 2.5  $\mu$ l gRNA. Zebrafish embryos were harvested from pair matings, and 2 nl of the CRISPR mixture was injected into

the yolk mass of each zebrafish embryo at the 1-cell stage using a microinjector and pulled fine glass needles. Non-injected embryos were used as negative controls.

#### 2.2.3.9 Genotyping of injected zebrafish

When Cas9 cleaves genomic DNA, the DNA repair machinery will try to repair it using the error-prone non-homologous end joining pathway, typically introducing an insertion or a deletion. This makes it straightforward to test whether the injection has been successful using PCR primers flanking the target site and sequencing the amplified DNA.

# 2.2.4 Studying oligodendrocyte development in tardbp-deficient zebrafish

To investigate whether knocking out the *tardbp* gene causes defects in or delays the early myelination stages of zebrafish oligodendrocytes, heterozygous and homozygous mutants containing a nonsense mutation in *tardbp* (*tardbp*<sup>+/-</sup> and *tardbp*<sup>-/-</sup> respectively) were assessed for correct early oligodendrocyte differentiation and myelination. These mutants are described in detail by Hewamadduma et al. (2013). To assess them, OPCs were stained at different stages using different probes targeting mRNA molecules which are specifically expressed in these cells at various stages of development. Three dpf embryos were stained for the *sox10* transcription factor (Dutton et al., 2001), and 5 dpf for *mbpa* (Brosamle and Halpern, 2002) found in myelinating oligodendrocytes.

#### 2.2.4.1 Synthesis of the mbpa probe

A  $\lambda$ -ZipLox plasmid (Invitrogen) containing the template for the *mbpa* cDNA was available. It was amplified in OneShot chemically competent E. coli (using carbenicillin-selective LB plates and broth) and purified using a midiprep kit as described in **section 2.2.2.5** to obtain a concentration which allows for enough template to synthesise the probe efficiently. Ten micrograms of plasmid DNA was digested using *Sal*I by assembling the following reaction: 20 µl plasmid DNA (500 ng/µl), 5 µl 10× FastDigest digestion buffer, 20 µl nuclease free water (all water used for the synthesis and use of the probe was nuclease free, and will be referred as H<sub>2</sub>O henceforth), 5 µl FastDigest *Sal*I. The digestion was left at 37°C for 1.5 h, and the linearisation of the DNA was confirmed using a 1% agarose gel and Hyperladder 1Kb size standards.

The digested DNA was extracted from the reaction mix via phenol/ chloroform/isoamyl alcohol extraction and ethanol-precipitated as described in **section 2.2.2.7.** The concentration of the digested DNA was assessed using a Nanodrop spectrophotometer.

A transcription reaction was assembled to synthesise the desired antisense *mbpa* probe from 1 µg template as follows: 2.2 µl DNA (485 ng/µl), 2 µl 10× SP6 transcription buffer, 2 µl 10× DIG labelling mix, 1 µl RNAse inhibitor (murine), 10.8 µl H<sub>2</sub>O, 2 µl SP6 RNA polymerase. The transcription mix was incubated at  $37^{\circ}$ C for 2 h.

To degrade the template DNA, 2.5  $\mu$ l of 10× DNAse I buffer and 2.5  $\mu$ l of 1 U/ $\mu$ l DNAse I were added to the previous transcription reaction and incubated at 37°C for 30 min. The probe was then precipitated by adding 2.5  $\mu$ l of LiCl (4 M) and 75  $\mu$ l of ethanol and incubation at -80°C overnight. The pellet was then centrifuged

at 20000 g and 4°C for 20 min. The supernatant was discarded, and the pellet was washed with 75% ethanol and centrifuged at 20000 g and 4°C for 15 min. The supernatant was discarded, and the pellet was allowed to air dry until no traces of ethanol were visible. The pellet was then resuspended in 50  $\mu$ l H<sub>2</sub>O and allowed to rehydrate on ice for 15 min before resuspension and addition of 50  $\mu$ l of formamide prior to storage at -20°C.

#### 2.2.4.2 Production of zebrafish embryos

Adult *tardbp-/-* and wild type (*tardbp+/+*) zebrafish were either inbred or bred with each other to provide embryos with distinct genotypes: *tardbp-/-*, *tardbp+/-*, and *tardbp+/+*. Different groups of each phenotype were raised to either 2, 3 or 5 dpf in E3 medium with methylene blue.

#### 2.2.4.3 Fixation of zebrafish embryos

When necessary, the embryos were dechorionated before fixation. When the embryos reached the desired stage, they were anaesthetised by adding 1 ml of 4 mg/ml tricaine methanesulfonate to each petri dish containing embryos. They were transferred to 1.5 ml microfuge tubes (60 per tube) and were incubated in 1 ml PBS containing 0.15 mg/ml tricaine methanesulfonate for 5 min at room temperature on a rocker (all incubations were done on a rocker). Then, they were incubated in 1 ml Fish Fix solution (4% paraformaldehyde in PBS) overnight at 4°C. The following day, the embryos were washed with 1ml PBS twice, then 1 ml 50% methanol in PBS, for 5 min each at room temperature. The embryos were then submerged in 100% methanol and stored at -20°C.

# 2.2.5 Whole-mount in situ hybridisation of zebrafish embryos

# 2.2.5.1 General reagents

# PBS (Phosphate Buffer Saline)

Commercially available PBS tablets were dissolved to a final concentration of 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub> in distilled water. The solution was autoclaved.

# PTW (PBS-Tween)

A volume of 0.5 ml of 10% Tween-20 detergent was added to 50 ml PBS to a final concentration of 0.1%. Tween is a surfactant which prevents the embryos from sticking to pipette tips and other surfaces.

# PBT (PBS-BSA-Tween)

PBT is a PTW solution to which it has been added sheep serum to 2% and BSA (Bovine Serum Albumin) to 0.2%. It needs to be made just before use to prevent microbial growth.

# 20x SSC (Salt-Sodium Citrate)

A water solution containing 3 M NaCl and 0.34 M sodium citrate, whose pH has been set at 7.0 with NaOH.

# <u>2x/0.2x SSC</u>

1:10/1:100 dilution in distilled water of 20x SSC.

# Full hybridisation solution (HYB+)

A milli-Q H<sub>2</sub>O solution made in batches of 50 ml by mixing 25 ml formamide, 12.5 ml 20x SSC, 2.5 ml tRNA (from 10 mg/ml stock), 50  $\mu$ l heparin (from 50 mg/ml stock), 0.5 ml 10% Tween-20, 0.46 ml 1M citric acid, and 8.99 ml milli-Q  $H_2O$ .

# Incomplete hybridisation solution (HYB-)

A milli-Q  $H_2O$  solution made in batches of 50 ml by mixing 25 ml formamide, 12.5 ml 20x SSC, 0.5 ml 10% Tween-20, 0.46 ml 1M citric acid, and 11.54 ml milli-Q  $H_2O$ .

# Staining buffer

This solution was made by mixing 5 ml 1M Tris pH 9.5, 2.5 ml 1M MgCl<sub>2</sub>, 1 ml 5M NaCl, 0.5 ml 10% Tween-20, and 41 ml milli-Q  $H_2O$ .

### 0.1M PO buffer (Phosphate buffer)

This solution was made by mixing 20 ml 0.2M NaH<sub>2</sub>PO<sub>4</sub>, 80 ml 0.2M Na<sub>2</sub>HPO<sub>4</sub>, and 100 ml milli-Q H<sub>2</sub>O.

# <u>1x Fish fix</u>

This solution is used to fix zebrafish embryos. It was made by mixing 25 ml 0.1M PO buffer, 3  $\mu$ l 1M CaCl<sub>2</sub>, 1 g PFA (paraformaldehyde) powder, and 1 g sucrose. The mixture is brought to 70°C, aliquoted, and stored at -20°C.

#### **Bleaching solution**

This solution was used to clear the superficial pigments of zebrafish embryos and make the interior easier to visualise. It was made by mixing 8.9 ml distilled water, 0.25 ml 20x SSC, 0.5 ml formamide (5% final), and 0.33 ml 30% H<sub>2</sub>O<sub>2</sub>.

<u>Day 1</u>

All solutions were prepared using nuclease-free water. Fixed embryos from various stages (2, 3, and 5 dpf) and genotype ( $tardbp^{+/+}$ ,  $tardbp^{+/-}$ , and  $tardbp^{-/-}$ ) were washed once with 1 ml 50% methanol in PBS at RT and 4 times with 1 ml PTW at RT. The embryos were incubated with 1 ml 10 µg/ml proteinase K at RT to permeabilise them. The permeabilisation time depended on the embryonic stage due to the different sizes: 3 dpf for 30 min, and 5 dpf for 50 min. The embryos were then fixed in 1 ml Fish Fix for 20 min at RT and washed in 1 ml PTW 5 times for 5 min each at RT. After that, they were rinsed in 0.25 ml HYB-solution and were pre-hybridised in 300 µl HYB<sup>+</sup> for 2.5 h at 65°C. To hybridise the embryos, they were incubated overnight at 65°C with 0.3 ml preheated HYB<sup>+</sup> containing the DIG labelled probe at a 1:200 dilution. As mentioned above, the probes targeted *sox10* for 3 dpf and *mbpa* for 5 dpf.

#### <u>Day 2</u>

The probe was removed, and the embryos were washed in 0.5 ml 50% HYB<sup>-</sup> in 1x SSC for 20 min at 65°C, followed by another wash in 1 ml 2x SSC for 20 min at 65°C, and 2 washes in 1 ml 0.2x SSC for 60 min each at 65°C. The embryos were then washed in 1 ml 50% PBT in 0.1x SSC for 10 min at RT, followed by a wash in 1 ml PBT for 10 min at RT, and a blocking incubation in 1 ml PBT for 2.5 h at RT. The embryos were incubated overnight at 4°C with 1 ml of PBT including Anti-Digoxigenin-AP, Fab fragments (Sigma-Aldrich) in a 1:2000 dilution to label the previously hybridised RNA. The embryos were washed 6 times with 1 ml PTW for 20 min each time at RT. They were then equilibrated by washing them 3 times with 1 ml staining buffer without NBT and BCIP for 10 min at RT. The embryos were transferred to a 12well plate with 1 ml staining buffer containing  $3.5 \mu$ l/ml BCIP and  $4.5 \mu$ l/ml NBT per well. The embryos were stained in the dark for about 4 h at RT until the colour was fully developed. The staining reaction was stopped by washing the embryos 3 times with 1 ml PTW for 5 min at RT, and then they were fixed overnight in Fish Fix at 4°C.

#### <u>Day 4</u>

The embryos were rinsed in 1 ml PTW twice for 5 min at RT and bleached with a bleaching solution for about 2 h at RT until the endogenous pigment faded. This step was followed by four washes in PTW for 5 min at RT. To store the embryos, they were transferred to 75% glycerol through a series of glycerol dilutions in PBS:  $10\% \rightarrow 25\% \rightarrow 50\% \rightarrow 75\%$ .

#### 2.2.5.3 Visualisation of stained embryos

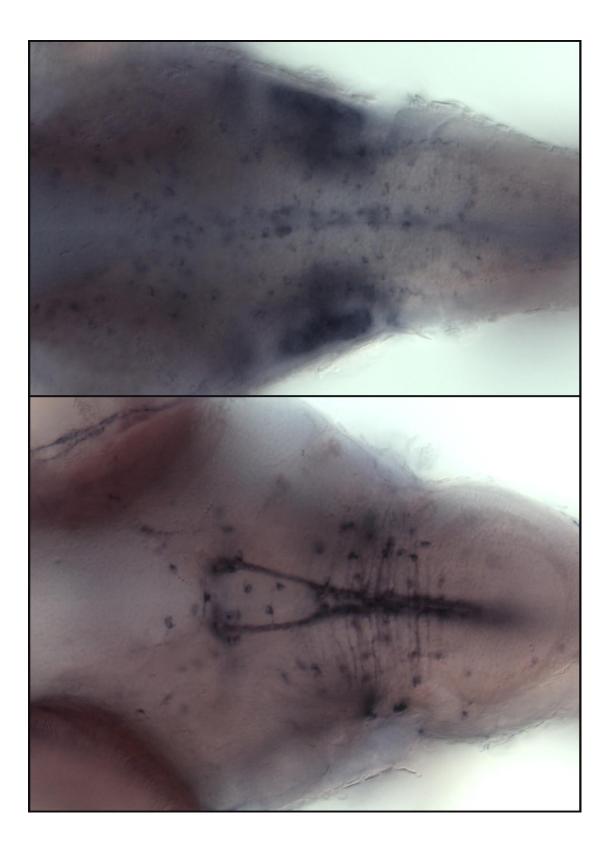
Six embryos of each embryonic stage and *tardbp* genotype were decapitated using two sharp syringes, and their heads mounted on glass slides which had a small receptacle loaded with 75% glycerol in PBS, and covered with a glass coverslip. The dorsal side was placed uppermost for easier inspection of the OPCs.

These mounted heads were photographed at different *z*-layers using an FV1000 confocal microscope using its associated software.

# 2.2.5.4 Quantification of OPCs and oligodendrocytes

For *mbp* (5 dpf), images where the myelinated axons were sharply in focus were used as a reference. One example can be seen in **figure 2.9 (bottom)**. On these, we counted the number of labelled myelinating oligodendrocytes and the number of myelinated axons.

For *sox10* (3 dpf), images where the midline of the hindbrain was sharply in focus were used as a reference. **Figure 2.9 (top)** shows an example of these. We counted the number of labelled OPCs in a fixed area of these pictures.



**Figure 2.9: Whole-mount in situ hybridisation.** Examples of reference pictures for 3 dpf *sox10* (top), and 5 dpf *mbp* (bottom).

**RESULTS: Part I** 

Study of oligodendroglial pathology and myelin loss in ALS

#### **CHAPTER 3**

# Protein aggregation in ALS

#### 3.1 Introduction

The first objective was to characterise the glial and neuronal pathologies in our cohorts of ALS cases which are positive for hexanucleotide expansions in *C9orf72* (C9ALS), sporadic ALS cases (sALS) with no known mutations of ALS, and neurologically healthy controls. The hypotheses underlying this characterisation were the following:

- C9ALS cases bear a higher degree of oligodendroglial pathology than sALS cases because aggregated expanded *C9orf72* mRNA can bind and sequester proteins relevant to mRNA transport, which adds to ALS protein aggregate pathology. To test this, p62 and TDP-43 stained and protein inclusions positive for these proteins were counted in the precentral gyrus, the prefrontal cortex and the spinal cord. Co-localisation with oligodendrocytes was aimed to be tested using double staining for p62 and oligodendroglial markers.
- Dipeptide repeat protein (DPR) caused by the RAN-translation of *C9orf72* mRNA hexanucleotide repeats is a component of those oligodendroglial p62-positive inclusions. Initially, this was tested using IHC for each of the five possible DPRs and quantifying the DPR-positive oligodendroglial inclusions in precentral gyrus sections.
- Aggregated cytoplasmic TDP-43 can trap hnRNP-A2 and will, therefore, cause its depletion from the cytoplasm and eventually from the nucleus in the oligodendrocytes supporting the primary motor neurones of ALS patients. To test this, hnRNP-A2-positive nuclei were counted in the white matter under the motor cortex and in the dorsolateral motor tracts of spinal cord sections. Total protein and mRNA amounts were also quantified for this protein.

- MBP synthesis is decreased in motor pathways of ALS patients, but PLP synthesis is not because MBP requires its mRNA to be transported to the myelin compartment to be translated, and mRNA transport is impaired in ALS, especially in C9ALS. To test this, MBP and PLP, and their respective mRNAs, were quantified in the dorsolateral motor tracts of the spinal cord.
- Oligodendroglial dysfunction is part of the etiology of ALS and leads to defects in the myelination of motor pathways, causing motor neurone axonopathy. This was tested by staining and calculating the density of axons panning the dorsolateral tracts of spinal cord sections, and the number of oligodendrocyte precursor cells available for re-myelination in both the spinal cord and the precentral gyrus.

The results presented in this and the next chapters provide evidence supporting a prevalence of glial pathology in relevant motor areas in C9ALS cases compared to sALS. Additionally, those levels of pathology and the *C9orf72* mutation were correlated with the loss of both myelin and oligodendroglial precursor cells.

#### 3.2 Subjects

Immunohistochemistry (IHC) was performed on a cohort of post mortem samples. The average demographic details of these cases can be found in **table 3.1** and the anonymised individual details are in **table 3.2**. The unbalanced female/male ratio for the control group is due to those tissue blocks being the only ones available to us at the time of the study. The samples used for each analysis depend on whether enough tissue was available and whether that cohort was relevant to the study -e.g. only the C9ALS group was relevant for the DPR analysis. The samples used for each analysis in this and the next chapters are detailed in **table 3.3**.

Group	N (female/male)	Post mortem delay* (h ± SD)	Age at death* (years ± SD)	Age at onset* (years ± SD)	Disease duration* (months ± SD)
C9ALS	17 (8/9)	$35 \pm 31$	$60 \pm 8$	$58 \pm 8$	$27 \pm 14$
sALS	67 (30/37)	$22 \pm 10$	64 ± 10	$62 \pm 11$	$36 \pm 13$
Controls	17 (4/12)*	$23 \pm 15$	$58 \pm 19$	N/A	N/A

**Table 3.1: Demographic data of the studied population.** \*Calculated using data from the patients for which that information was available at the time of the study.

Patient ID	C9ORF72 genotype	Sex	Age at onset	Duration of disease	Age at death	PM delay
1	C9ORF72	Female	58	7	58	2
2	C9ORF72	Female	59	40	62	63
3	C9ORF72	Female	50	28	52	
4	C9ORF72	Female	63	43	66	24
5	C9ORF72	Female	61	40	64	7
6	C9ORF72	Female	67	26	69	55
7	C9ORF72	Female	56	43	59	32
8	C9ORF72	Female	61	42	64	
9	C9ORF72	Male	56	13	57	
10	C9ORF72	Male	64	31	66	96
11	C9ORF72	Male	67	14	68	31
12	C9ORF72	Male	63	11	63	6
13	C9ORF72	Male	70	26	72	96
14	C9ORF72	Male	62	20	63	48
15	C9ORF72	Male	45	14	46	4
16	C9ORF72	Male	47	19	48	23
17	C9ORF72	Male	42	50	46	15
18	Control	Female	N/A	N/A	59	
19	Control	Female	N/A	N/A	69	
20	Control		N/A	N/A		
22	Control	Male	N/A	N/A	70	
23	Control	Male	N/A	N/A	27	
25	Control	Female	N/A	N/A		36
27	Control	Female	N/A	N/A	59	5
28	Control	Male	N/A	N/A	46	20
29	Control	Male	N/A	N/A	46	20
30	Control	Male	N/A	N/A	55	24
32	Control	Male	N/A	N/A	67	20
33	Control	Male	N/A	N/A	82	36
34	Control	Male	N/A	N/A	87	18
35	Control	Male	N/A	N/A	64	12
36	Control	Male	N/A	N/A	51	25
37	Control	Male	N/A	N/A	72	31
38	Control	Male	N/A	N/A	67	63
39	Sporadic	Female	55	56	59	
40	Sporadic	Female	81	24	83	44
41	Sporadic	Female	58	16	59	60
42	Sporadic	Female	73	31	75	48
43	Sporadic	Female	66	20	67	15
44	Sporadic	Female	45	20	46	20
45	Sporadic	Female	78	33	80	24
46	Sporadic	Female	67	33	69	

Patient ID	C9ORF72 genotype	Sex	Age at onset	Duration of disease	Age at death	PM delay
47	Sporadic	Female	39	28	41	10
48	Sporadic	Female	59	57	63	34
49	Sporadic	Female	59	46	62	36
50	Sporadic	Female	63	31	65	
51	Sporadic	Female	76	25	78	19
52	Sporadic	Female	66	44	69	24
53	Sporadic	Female	65	14	66	50
54	Sporadic	Female	57	48	61	36
55	Sporadic	Female	64	66	69	12
56	Sporadic	Female	57	38	60	18
57	Sporadic	Female	65	38	68	
58	Sporadic	Female	61	42	64	27
59	Sporadic	Female	68	26	70	35
60	Sporadic	Female	77	8	77	34
61	Sporadic	Female	72	104	80	50
62	Sporadic	Female	58	25	60	70
63	Sporadic	Female	58	121	68	40
64	Sporadic	Female	55	82	61	
65	Sporadic	Female	62	23	63	40
66	Sporadic	Female	49	23	50	24
67	Sporadic	Female	67	21	68	40
68	Sporadic	Female	71	50	75	24
69	Sporadic	Male	62	25	64	9
70	Sporadic	Male	63	26	65	
71	Sporadic	Male	47	13	48	8
72	Sporadic	Male	66	22	67	62
73	Sporadic	Male	49	29	51	
74	Sporadic	Male	63	53	67	6
75	Sporadic	Male	49	28	51	
76	Sporadic	Male	36	59	40	25
77	Sporadic	Male	56	38	59	
78	Sporadic	Male	69	47	72	18
79	Sporadic	Male	43	47	46	10
80	Sporadic	Male	75	6	75	28
81	Sporadic	Male	75	19	76	10
82	Sporadic	Male	60	40	63	32
83	Sporadic	Male	49	29	51	
84	Sporadic	Male	60	38	63	16
85	Sporadic	Male	68	17	69	11
86	Sporadic	Male	60	57	64	9
87	Sporadic	Male	31	66	36	35
88	Sporadic	Male	83	97	91	44

Patient ID	C9ORF72 genotype	Sex	Age at onset	Duration of disease	Age at death	PM delay
89	Sporadic	Male	38	32	40	96
90	Sporadic	Male	64	25	66	96
91	Sporadic	Male	63	43	66	30
92	Sporadic	Male	76	24	78	30
93	Sporadic	Male	48	22	49	48
94	Sporadic	Male	73	14	74	12
95	Sporadic	Male	66	36	69	34
96	Sporadic	Male	65	46	68	48
97	Sporadic	Male	67	12	68	
98	Sporadic	Male	56	27	59	60
99	Sporadic	Male	57	24	59	16
100	Sporadic	Male	64	21	65	46
101	Sporadic	Male	50	44	53	16
102	Sporadic	Male	71	9	71	53
103	Sporadic	Male	62	14	63	48
104	Sporadic	Male	49	29	51	22
105	Sporadic	Male	78	10	78	
106	Sporadic	Male	74	63	79	24

**Table 3.2: Anonymised individual demographic details for the** *post mortem* **study cases.** A blank cell indicates missing data at the time of the study. Control cases 21, 24, 26 and 31 are missing from the table due to their exclusion at the time of the study.

Study	Technique	Tissue	C9ALS cases	sALS cases	Control cases
		Spinal cord	1-8, 10-17	39, 44, 45, 47-49, 51, 52, 54-56, 58, 59, 61-68, 70, 73, 76, 78-82, 85, 90, 91, 95, 96, 98-103, 105, 106	25, 29, 30, 32, 33
p62-positive inclusions	IHC	Motor cortex	1-7, 10-17	39-42, 44, 45, 47-49, 51-59, 61-64, 66-73, 75, 76, 78, 80, 83, 84, 86-93, 95, 96, 98, 100-104, 106	27, 28, 34, 35, 38
		Frontal cortex	1-7, 9-17	39-46, 48-52, 55-57, 59-77, 79, 80, 82-85, 88, 89, 91-94, 96, 97, 99-106	22, 27, 28, 35, 37, 38
		Spinal cord	1-4, 6, 10, 12-16	56, 73, 90, 95, 105, 106	27, 30, 32,33, 36, 38
pTDP43 inclusions	IHC	Motor cortex	1, 2, 4-7, 10, 12-14, 16, 17	44, 55, 75, 78, 86, 96	18, 19, 22, 23, 35, 38
DPR inclusions	IHC	Motor cortex	4, 5, 13, 14, 16, 17	N/A	N/A
PLP vs. MBP (and other proteins)	Western blot	Spinal cord	4, 13, 14, 16	44, 55, 58, 96	20, 27, 35, 38
PLP vs. MBP (and other mRNAs)	qPCR	Spinal cord	6, 13, 14, 16, 17	44, 55, 58, 61, 64, 91, 96	20, 35, 38
		Spinal cord	1-4, 6, 10- 16	56, 73, 90, 95, 105, 106	27, 30, 33, 35, 36, 38
PLP vs. MBP	IHC	Motor cortex	1, 2, 4-7, 10-17	44, 55, 58, 86, 90, 96	19, 22, 23, 27, 35, 38
Axonal density (SMI31)	IHC	Spinal cord	1-4, 6, 10- 12, 14-16	56, 73, 90, 105, 106	27, 30, 33, 35, 36, 38
hnRNP-A2	IHC	Spinal cord Motor cortex	1-4, 6, 10- 16 2, 4-7, 10- 17	44, 56, 58, 73, 81, 90, 95, 99, 101, 106 44, 55, 56, 58, 86, 88-90, 96	27, 30, 33, 35, 36, 38 19, 22, 27, 30, 35, 38
OPC (MAP2-13)	IHC	Spinal cord Motor cortex	1-4, 6, 10- 16 2, 4-7, 10- 17	44, 56, 58, 73, 81, 95, 101, 105, 106 44, 55, 56, 58, 86, 88-90, 96	27, 30, 33, 35, 36, 38 19, 22, 27, 35, 38

Table 3.3: List of post mortem cases used for each experiment. Case numbers correspond to the patients on table 2.2.

#### 3.3 Quantification of the p62-positive cytoplasmic inclusions

Immunohistochemistry was performed for p62 for a large number of slides of human spinal cord, and motor and frontal cortices prior to this study. p62 is a well-characterised constituent of the ubiquitylated inclusion bodies which are a pathological feature of ALS (Neumann et al., 2006). Anterior frontal cortex sections were also quantified, as a representative extra-motor region which often shows pathological involvement in ALS (Brettschneider et al., 2013).

The total number of glial (**figure 3.1.2A**) and neuronal (**figure 3.1.2B**) p62positive inclusions were counted in the ventral horns and lateral corticospinal tracts of cervical spinal cord sections. Inclusions were also counted in 10 fields with a ×20 objective in layer V of the motor cortex –the neurones in this layer project their axons to the corticospinal tracts- and in white matter underlying the frontal and motor cortices. No difference was found between the density of inclusions counted in males and females ( $p \ge 0.1506$ ), so both sexes were pooled for subsequent analyses.

None of the data was normally distributed (Shapiro-Wilk normality test all p<0.05). Thus, differences between the three experimental groups were analysed using the Kruskal-Wallis (Kruskal-Wallis) test followed by *post hoc* Mann-Whitney U tests with a Bonferroni-Holm correction (only corrected p-values are shown henceforth). Kruskal-Wallis tests always have 2 degrees of freedom in these analyses.

# 3.3.1 C9ALS presents with a distinct glial p62-positive cytoplasmic protein inclusions pathology in the cortex, but not in the spinal cord

There were significant intergroup differences for glial inclusion counts in the grey matter and underlying white matter of both motor cortex (grey matter:  $\chi^2$ =29.42,

p= 4.08 × 10<sup>-7</sup>, **figure 3.1.1E**; white matter:  $\chi^2$ = 15.17, **figure 3.1.1F**) and prefrontal cortex (grey matter:  $\chi^2$ = 37.56, p= 6.96 × 10<sup>-9</sup>, **figure 3.1.1H**; white matter:  $\chi^2$ = 16.88, p=2.16 × 10<sup>-4</sup>, **figure 3.1.1I**), in the lateral corticospinal tracts of the spinal cord ( $\chi^2$ = 12.31, p=0.00213, **figure 3.1.1C**), and in the spinal cord ventral horns ( $\chi^2$ = 8.749, p= 0.0125, **figure 3.1.1B**).

*Post hoc* tests for both the <u>precentral gyrus</u> revealed a greater number of glial inclusions in both ALS cohorts compared to controls, and increased pathology in C9ALS cases compared to sALS cases. This was found for both the grey matter (C9ALS vs controls: W=0, p= 0.00474; C9ALS vs sALS: W=54.5, p=9.02 × 10<sup>-07</sup>; sALS vs controls: W=222, p=0.00481, **figure 3.1.1E**) and white matter (C9ALS vs controls: W=5, p=0.0201; C9ALS vs sALS: W=180.5, p=0.00235; sALS vs controls: W=221.5, p=0.00465, **figure 3.1.1F**).

*Post hoc* tests for the <u>anterior frontal cortex</u> gave the same pattern to the motor cortex in the grey matter (C9ALS vs controls W=96, p=0.00121; C9ALS vs sALS W=869.5, p= $6.894 \times 10^{-8}$ ; sALS vs controls: W=66, p=0.0310, **figure 3.1.1H**) and the underlying white matter (C9ALS vs controls W=92.5, p=0.00307; C9ALS vs sALS W=692, p=0.00403; sALS vs controls: W=310, p= 0.00652, **figure 3.1.1I**).

*Post hoc* Mann-Whitney U tests for the <u>spinal cord</u> revealed greater numbers of glial inclusions in both C9ALS and sALS cases compared to controls in both the ventral horns (controls vs C9ALS: W=101.5, p=0.00394; controls vs sALS: W=222.5,

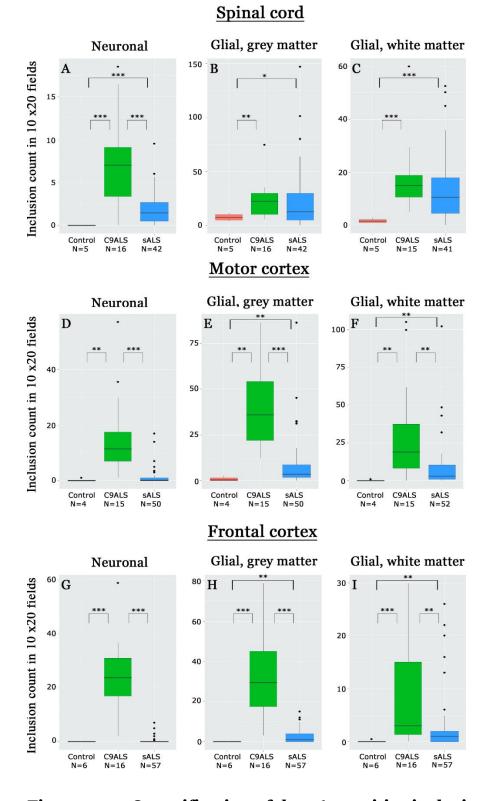
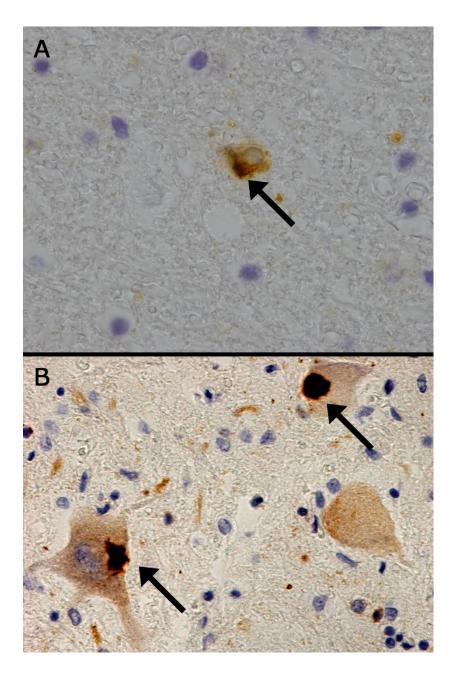


Figure 3.1.1: Quantification of the p62-positive inclusion pathology in C9ALS, sALS, and controls. Neuronal inclusion pathology is consistently greater in C9ALS in the spinal cord ventral horns (**A**), motor cortex (**D**) and frontal cortex (**G**). The amount of glial pathology is larger in ALS than in controls in the motor and frontal cortices (**E**, **H**), and the associated subcortical white matter (**F**, **I**), but C9ALS cases bear greater pathology in the same areas. In the spinal cord, both ALS cohorts show more inclusions than controls, but no differences can be seen between them (**B**, **C**). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 by Mann-Whitney U test.



**Figure 3.1.2: Representative images of p62-positive cytoplasmic inclusions.** On the top image (**A**), a glial p62-positive cytoplasmic inclusion (arrow) is shown in the dorsolateral motor tract of a C9ALS patient's FFPE spinal cord section. The bottom image (**B**) is a photograph of the motor cortex (layer V in a FFPE section) showing two neuronal p62-positive cytoplasmic inclusions (arrows).

p=0.0319, **figure 3.1.1B**) and the corticospinal tracts (controls vs. C9ALS: W=0, p=0.00369; controls vs. sALS: W=18, p=0.00904, **figure 3.1.1C**).

# 3.3.2 C9ALS cases show increased counts for neuronal p62-positive cytoplasmic inclusions in both the motor cortex and the spinal cord

Neuronal inclusion pathology was assessed to investigate how this related to the glial pathology seen. For neuronal inclusion counts, there were significant intergroup differences in all three CNS regions (spinal cord:  $\chi^2$ = 26.31, p= 1.937 × 10<sup>-6</sup>, **figure 3.1.1A**; motor cortex:  $\chi^2$ = 29.67, p= 3.60 × 10<sup>-7</sup>, **figure 3.1.1D**; frontal cortex:  $\chi^2$ = 51.0, p= 8.25 × 10<sup>-12</sup>, **figure 3.1.1G**).

*Post hoc* Mann-Whitney U tests for the <u>spinal cord</u> revealed greater numbers of neuronal inclusions in the ventral horns in both C9ALS and sALS cases compared to controls (C9ALS vs controls: W=77.5, p=  $1.92 \times 10^{-4}$ ; sALS vs controls: W=195, p= $2.81 \times 10^{-4}$ , **figure 3.1.1A**) and significantly greater numbers of neuronal inclusions in C9ALS compared to sALS (W=579.5, p=  $3.34 \times 10^{-5}$ , **figure 3.1.1A**). *Post hoc* Mann-Whitney U tests for the <u>motor cortex</u> showed that the number of neuronal inclusions in the C9ALS group was significantly higher than in the controls (W=0.5, p=0.00137, **figure 3.1.1D**) and sALS groups (W=49, p= $2.45 \times 10^{-07}$ , **figure 3.1.1D**). There was no difference between controls and sALS cases (p=0.0902). The <u>prefrontal cortex</u> showed the same pattern as the motor cortex: the number of neuronal inclusions in the C9ALS group was significantly higher than in the controls (W=96, p=0.000168, **figure 3.1.1G**) and sALS groups (W=905.5, p= $1.16 \times 10^{-11}$ , **figure 3.1.1G**). There was no difference between controls and sALS groups (W=905.5, p= $1.16 \times 10^{-11}$ , **figure 3.1.1G**). There was no difference between controls and sALS groups (W=905.5, p= $1.16 \times 10^{-11}$ , **figure 3.1.1G**). There was no difference between controls and sALS groups (W=905.5, p= $1.16 \times 10^{-11}$ , **figure 3.1.1G**).

### 3.3.3 Summary of glial and neuronal inclusion counts

In summary, p62-positive glial pathology was greater in both C9ALS and sALS than controls in the lateral corticospinal tracts and the ventral horns of the spinal cord, and both cortical areas and the subjacent white matter. C9ALS cases had significantly more glial pathology than sALS cases in the cortical and subcortical regions.

The C9ALS cases had a greater burden of neuronal p62-positive inclusion pathology than both controls and sALS cases in all three regions. As expected, sALS cases also had more neuronal pathology than controls in the spinal cord ventral horns.

# 3.3.4 C9ALS cases show different patterns of p62-positive inclusion pathological dependency when compared to sALS cases

It was next sought to determine whether there was a relationship between the levels of p62-positive inclusion pathology shown within CNS regions in both C9ALS and sALS cases. Firstly, the relationship between neuronal and glial inclusions within the three CNS regions (spinal cord, motor cortex and anterior frontal cortex) was studied using Kendall correlation tests. The results of this study are presented in **table 3.4** and **figures 3.2** and **3.3**.

Analysis of the <u>spinal cord</u> inclusion counts in *C*9ALS cases consistently revealed positive correlations between neuronal and glial inclusion pathology and between both anterior horn and corticospinal tract glial inclusion counts (**figure 3.2A-C**). However, sALS cases only showed a significant relationship between grey matter and white matter glial cell counts, but no relationship was found between motor neurone and glial inclusions (**figure 3.3A-C**). Evaluation of the <u>motor cortex</u> glial inclusion counts again showed a significant correlation between the counts in grey matter and white matter in both disease groups (**figure 3.2F** and **figure 3.3F**). For the sALS cases, there was a significant correlation between neuronal and glial inclusions in both the white and grey matter (**figure 3.3D**,**E**). In contrast, neuronal inclusion pathology was not correlated with glial pathology in the motor cortex of C9ALS cases (**figure 3.2D**,**E**).

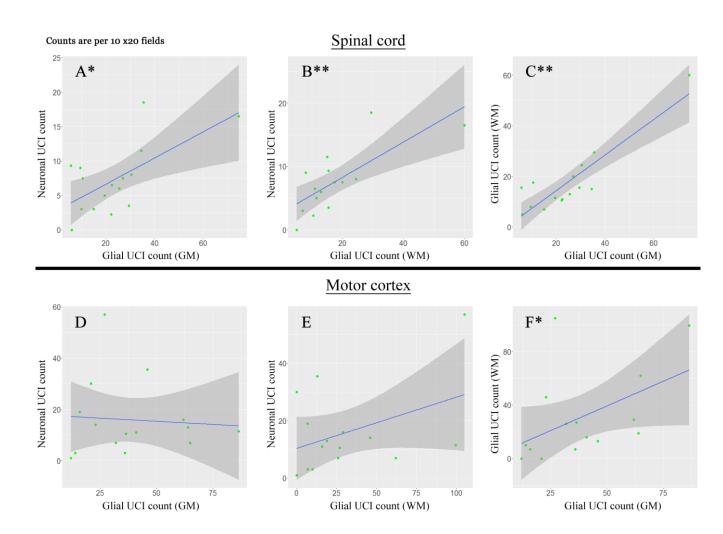
The <u>anterior frontal cortex</u>, as with the spinal cord and motor cortex, showed positive correlations for the white and grey matter glial inclusion pathology in both C9ALS and sALS cases (**figure 3.2I** and **figure 3.3I**). The neuronal pathology in the frontal cortex of both C9ALS and sALS groups was positively correlated with glial pathology in the grey matter (**figure 3.2G** and **figure 3.3G**) but not in the white matter (**figure 3.2H** and **figure 3.3H**).

Area	Tested variables	Z	p	Tau
	C9orf72 /	ALS		
	SC neuronal vs. SC glial GM*	1.985	0.04714	0.369761
	SC neuronal vs. SC glial WM**	2.8277	0.004689	0.5480769
	SC glial GM vs. SC glial WM**	2.9729	0.00295	0.5741693
ANT IN	MCx neuronal vs. MCx glial GM	0.5457	0.5853	0.1057741
	MCx neuronal vs. MCx glial WM	0.9449	0.3447	0.184466
	MCx glial GM vs. MCx glial WM*	2.2324	0.02559	0.4327123
	FCx neuronal vs. FCx glial GM**	2.8873	0.003886	0.5378341
	FCx neuronal vs. FCx glial WM	1.5453	0.1223	0.293147
	FCx glial GM vs. FCx glial WM**	2.9938	0.002755	0.5642881
	MCx neuronal vs. SC glial WM	-0.2745	0.7837	-0.05555556
	MCx glial GM vs. SC glial WM	-0.4386	0.6609	-0.08839914
	MCx glial WM vs. SC glial WM	-0.11	0.9124	-0.02234672
	Sporadic /	ALS		
	SC neuronal vs. SC glial GM	1.8917	0.05854	0.2111265
	SC neuronal vs. SC glial WM	1.8934	0.05831	0.214516
	SC glial GM vs. SC glial WM***	5.9384	2.878 × 10 <sup>-9</sup>	0.6601866
ST.	MCx neuronal vs. MCx glial GM***	4.0755	4.591 × 10 <sup>-5</sup>	0.4491098
	MCx neuronal vs. MCx glial WM***	3.3018	9.605 × 10 <sup>-4</sup>	0.3677013
	MCx glial GM vs. MCx glial WM**	2.6521	0.007999	0.2733926
	FCx neuronal vs. FCx glial GM***	3.3799	7.252 × 10 <sup>-4</sup>	0.3792862
	FCx neuronal vs. FCx glial WM	1.1753	0.2399	0.1342031
	FCx glial GM vs. FCx glial WM***	3.9674	7.265 × 10 <sup>-5</sup>	0.414105
	MCx neuronal vs. SC glial WM	1.7054	0.08812	0.225486
	MCx glial GM vs. SC glial WM*	2.1912	0.02843	0.2724669
	MCx glial WM vs. SC glial WM	0.5139	0.6073	0.06439714

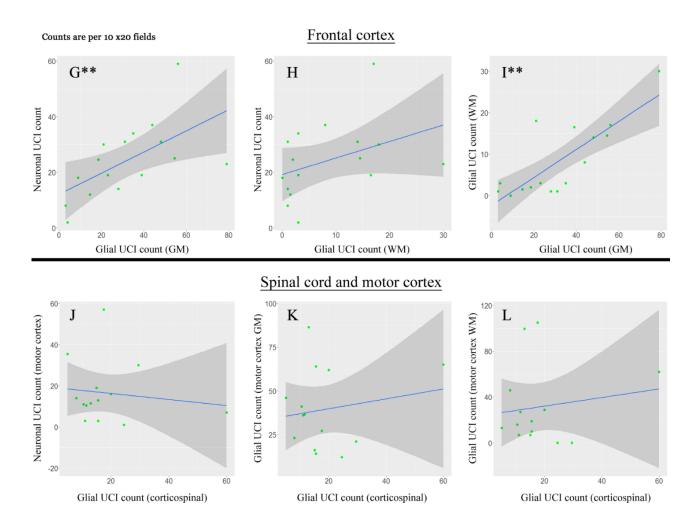
Table 3.4: Correlations between p62-positive inclusion pathology of different areas. SC=spinal cord, MCx=motor cortex, FCx=frontal cortex, GM=layer V of the grey matter (for MCx and FCx) or ventral horns (for SC), WM=white matter under the cortex (for MCx and FCx) or corticospinal motor tracts (for SC). p, tau statistic, and z values calculated using the Kendall tau rank correlation coefficient. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

Cell populations	Interpretation of results
	C9orf72 ALS
Neuronal vs. grey matter glia	A positive correlation exists between the p62-positive pathology of the <b>ventral horn</b> motor neurones and that of the surrounding glia in the grey matter, suggesting that a common factor is affecting all cell types equally. A similar result was seen in the <b>prefrontal cortex</b> , but not in the <b>motor cortex</b> . This could mean that the pathology in the precentral gyrus of C9ALS cases has a different origin for neuronal and glial cells.
Neuronal vs. white matter glia	No correlations could be found between these in the examined <b>cortical areas</b> , meaning that neuronal and subcortical glial (myelinating the proximal part of their axons) pathologies occur independently of each other, maybe because of different origins. However, a positive correlation was found between <b>ventral horn</b> neurone pathology and that of the glia in the descending <b>dorsolateral motor tracts</b> of the spinal cord. This suggests that the pathology levels of the glia supporting the axons of the upper motor neurones could directly affect lower motor neurone degeneration or vice versa.
White matter vs. grey matter glia	The levels of pathology between glia in the grey and the white matter correlated in <b>all the studied areas</b> , probably meaning that there is a common factor that causes all glia in a given area to degenerate.
Cortical vs. spinal cord	No relationship could be seen between the pathology levels seen in the <b>precentral gyrus</b> and those of the supporting glia in the <b>spinal cord motor tracts</b> , meaning that they might happen independently of each other.
	Sporadic ALS
Neuronal vs. grey matter glia	Sporadic cases show positive correlations between <b>cortical</b> neuronal pathology and that of the surrounding glia. Most of these cases have no p62-related pathology in the <b>cerebral cortex</b> , but where it can be found, it affects neurones and glia at the same level. However, the <b>ventral horns</b> show no correlation between motor neurone and glial pathologies unlike C9ALS cases, where glial pathology is more common.
Neuronal vs. white matter glia	Amongst the studied areas, only the <b>precentral gyrus</b> of sALS cases shows a correlation between pyramidal neurone pathology and that of the proximal glia in the white matter below. Added to the above, these results might indicate that the motor pathways of the few sALS cases with high pathology in this area share a common affection between neurones and glia.
White matter vs. grey matter glia	As seen in the C9ALS cases, the levels of glial pathology correlate within <b>each</b> <b>of the three studied areas</b> . It seems that what affects glial degeneration in a particular area does not distinguish grey matter from white matter glia and all are affected in the cases where pathology is present.
Cortical vs. spinal cord	The only correlation that reached significance was that between <b>motor cortical</b> glia and the supporting glia in the <b>dorsolateral motor tracts</b> of the spinal cord. As the latter did not correlate with the white matter glia under the <b>motor cortex</b> too, it is hard to find a logical reason for this correlation, which might be a false positive.

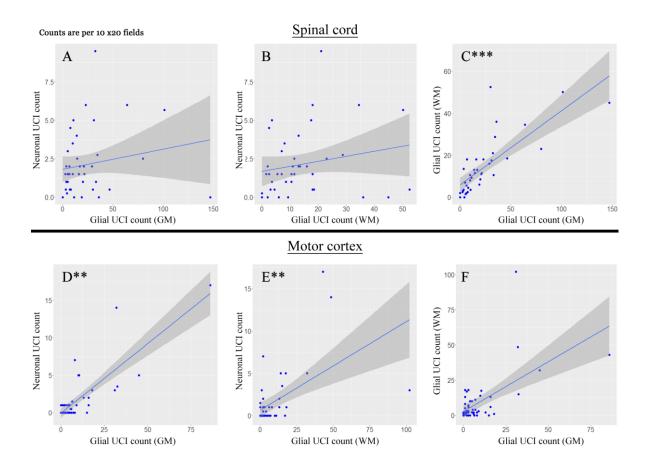
Table 3.5: Interpretation of the correlation data for p62-positive pathology.



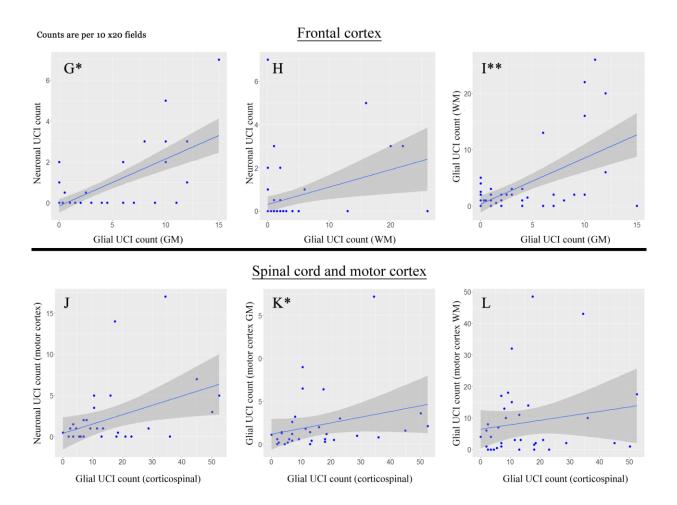
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**Figure 3.2: Pathological correlations in C9ALS.** Significant correlations were observed in the spinal cord between glial p62 inclusion pathology in the ventral horns and the corticospinal tracts, and that on ventral horn neurones (**A**, **B**). The pathology found between those glial cells themselves also significantly correlated (**C**). Only glial cell pathology significantly correlates in the motor cortex, between that on the grey matter and that on the underlying white matter (**F**). The same is seen in the frontal cortex (**I**), where neuronal inclusion pathology also correlates with grey matter glial pathology. No correlation is seen between the pathology in the motor cortex and that observed in the corticospinal tracts. \*/\*\*p < 0.05/0.01. p values calculated using the Kendall tau rank correlation coefficient. The shadowed area shows the 95% confidence interval.



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**Figure 3.3: Pathological correlations in sALS.** Glial p62 inclusion pathology in the ventral horns of the spinal cord correlates with that found in the corticospinal tracts (**C**), but none of the pathology on those areas correlates with ventral horn neuronal pathology. Conversely, motor cortex neuronal pathology is correlated to glial pathology in the neighbouring grey matter (**D**) and in the subcortical white matter (**E**). However, glial pathology in the motor cortex and the underlying white matter do not show a correlation (**F**). In the frontal cortex, neuronal pathology is correlated to grey matter glial pathology (**G**), but not to that in the white matter (**H**). Glial pathology in these two locations correlates significantly (**I**). Only a weak significant correlation was found when comparing grey matter glial pathology in the motor cortex with corticospinal pathology (**K**), but no relationship in pathology between these areas was generally found (**J**, **L**). \*/\*\*/\*\*\*\***p** < 0.05/0.01/0.001. **p** values calculated using the Kendall tau rank correlation coefficient. The shadowed area shows the 95% confidence interval.

Thus, overall, for the spinal cord, motor cortex and frontal cortex glial pathology, there was a correlation in both groups between the grey and white matter within each region. The relationship between neuronal and glial pathology within these regions is more complex: for the spinal cord, a relationship between neuronal and glial pathology was seen only in the C9ALS but not sALS cases in spite of greater statistical power in the latter group. However, for the anterior frontal and motor cortices, the sALS cases showed positive correlations between neuronal and grey matter glial pathology. This was not seen in C9ALS cases potentially due to less statistical power or due to more than one type of p62-positive protein pathology caused by expanded *C9orf72* mRNA.

We also investigated the relationships between pathology levels in different CNS regions (**figure 3.2J-L** and **figure 3.3J-L**). Firstly, we examined whether there was a relationship between inclusion pathology present in motor cortex neurones and the glia that support their axons in the spinal cord dorsolateral spinal tracts. Secondly, we investigated whether there was a relationship between the inclusion counts in glia of those dorsolateral spinal tracts and the glial pathology in the motor cortex grey matter or white matter. There was very little evidence for any of these relationships save for a correlation between the number of layer V glial inclusions in motor cortex and corticospinal glial inclusions in the spinal cord of sALS but not C9ALS cases (p=0.02843, tau=0.2724, z=2.1912). In general, glial pathology levels showed greater correlations within CNS regions than between them, indicating that the p62-related pathology burden in the 2 different motor areas is independent of each other.

## 3.3.5 Relationship of p62-positive inclusion pathology to disease severity

We wished to assess the possibility that the different levels of glial p62-positive inclusion pathology found above were related to the progression of the illness or to the different age of the patients at death. To assess this, correlation tests were performed between the levels of pathology found in each area, and the duration of the disease and the age at death.

No correlations were found between glial p62-positive inclusions and the duration of the disease ( $p \ge 0.08912$ , tau=-0.145, z=-1.7001) or the age at death ( $p \ge 0.2593$ , tau=-0.1158, z=-1.1279) in any of the studied areas in either disease group.

#### 3.3.6 Summary of p62 findings

At this point, a specific, more severe glial p62-related pathology has been defined for our cohort of C9ALS cases, especially in the cortical areas. Additionally, even though the spinal cord glial pathology levels do not differ between sALS and C9ALS, only the latter shows a significant dependency of that pathology and that of the motor neurones in the ventral horns. Although the morphology of the affected glial cells indicates they are likely to be oligodendrocytes, it was necessary to show the co-localisation of oligodendroglial markers and the observed p62-positive inclusions. This was attempted and is explained in the next section.

### **3.4 Optimisation of oligodendrocyte specific protein + p62 immunohistochemistry in CNS tissue**

After counting the inclusions in the sections stained with the anti-p62 antibody, the next step was to determine which type of glial cell (oligodendrocytes, astrocytes or microglia) contained the inclusions in C9ALS and sporadic cases. Morphologically, it appeared that the affected cells were mainly oligodendrocytes. However, double staining techniques were used to label both p62 and one of either oligodendrocytes, astrocytes or microglia in order to confirm the affected cell types.

In the CNS, oligodendrocyte specific protein (OSP) is specifically produced by oligodendrocytes (Bronstein et al., 1997). For this reason, an antibody which specifically binds to this protein was thought to be a good option for double staining with p62. Other classic markers of oligodendrocytes such as the transcription factors SOX-10 or OLIG2 had either not been trialled or had been unsuccessfully trialled respectively in our laboratories, whereas an optimised working protocol for OSP existed, which made it preferable for this project. Our laboratory also has a well-established method to label p62 with 3,3'-diaminobenzidine (DAB), so a method which stained OSP in a contrasting colour was sought.

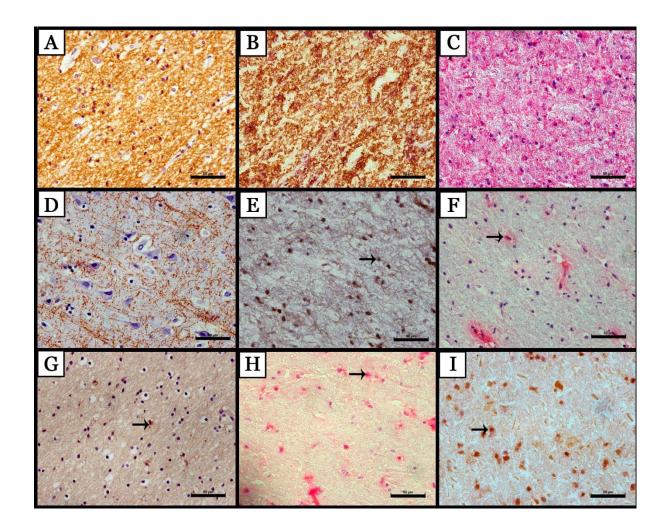
#### 3.4.1 OSP single-staining IHC

Firstly, the anti-OSP 53041 antibody (Abcam) was trialled because it had already been successfully used in our laboratories. This antibody is raised against the fulllength OSP protein. The first step was to repeat the standard IHC protocol using the 53041 antibody, using several dilutions within the range recommended by the manufacturer, and the peroxidase-red chromogen: the tissue showed a level of background staining that was too high for oligodendrocytes to be identified (**figure 3.4A**). The next approach was to try a rapid protocol that we had used successfully in the past, using the peroxidase (**figures 3.4B**) and phosphatase red chromogens (**figure 3.4C**). It was found that both similarly gave excessive levels of background staining.

As the last approach for this antibody, formalin-fixed, paraffin-embedded tissue was stained with multiple dilutions of the 53041 antibody, within the range recommended by the supplier, and the peroxidase-red chromogen. **Figure 3.4D** shows a representative example of how fixed tissue appeared after this treatment: an intricate web of processes was seen in grey matter, whereas white matter showed another extremely unclear pattern which consisted of a large amount of processes and cell bodies (not shown).

The next step was to try a different anti-OSP antibody (7474). This antibody is raised against the 15 C-terminal amino acids of the OSP protein. A rapid staining protocol was applied to frozen brain tissue, using a high concentration of primary antibody (1:10) and DAB (the existing protocol for this antibody). Many positively-stained oligodendrocytes were visible, which suggested the antibody worked well for IHC (**figure 3.4E**). Since this method showed that the anti-OSP 7474 antibody stained oligodendrocytes effectively, a standard, non-rapid, IHC protocol was performed on frozen tissue, using different dilutions of primary antibody (1:50/100/200/500/1000), and the alkaline phosphatase (AP)-Red chromogen. This method did not show the same oligodendrocyte staining pattern, staining mainly the blood vessels, due to the endogenous phosphatase activity within vessels, as well as a very small number of oligodendrocytes (**figure 3.4F**). It was hypothesised that the AP kit did not work well with this antibody, so a peroxidase-based red chromogen was tried in a standard IHC protocol using frozen CNS tissue, which showed the same staining pattern (**figure 3.4G**).

The above protocols did not allow the identification of individual oligodendrocytes, so the next step was to try the 7474 antibody with a rapid staining protocol, using the three different chromogens: the two red ones, and DAB as a positive control. All three methods gave clear labelling of individual oligodendrocytes, but the AP-red chromogen (**figure 3.4H**) was chosen to use in further experiments since the peroxidase-red condition (**figure 3.4I**) had a colour closer to that produced by DAB (**figure 3.4E**), which would lead to less contrast when performing the double-staining experiments.



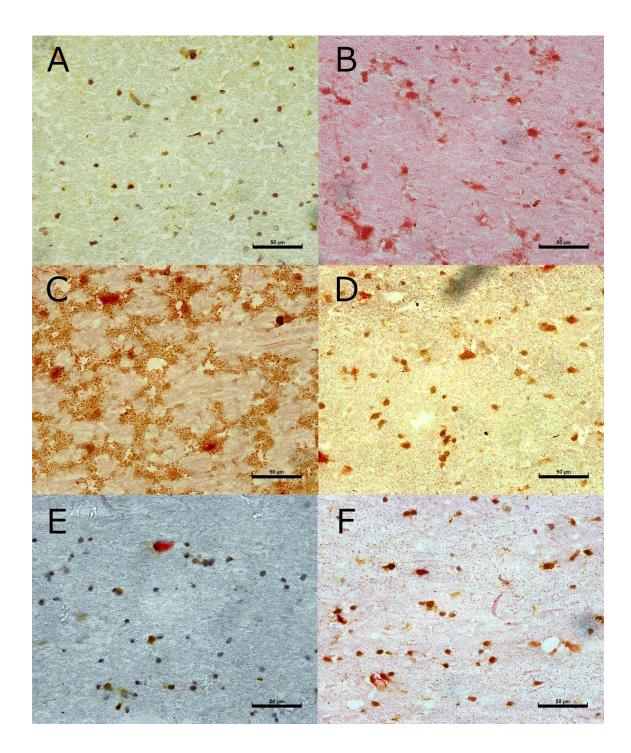
**Figure 3.4: Anti-OSP antibody optimisation. A, B, C**: Anti-OSP 53041 antibody produces too much background staining for any individual oligodendrocyte to be identified, whether standard peroxidase-red (**A**), rapid peroxidase-red (**B**), or rapid AP-red (**C**) staining is used. **D**: Anti-OSP 53041 antibody labels oligodendrocyte processes in formalin-fixed, paraffin-embedded tissue, which does not allow for the identification of individual oligodendrocytes. **E**: The rapid DAB (brown) staining protocol with the anti-OSP 7474 antibody confirms it labels oligodendrocytes in frozen tissue. **F, G**: Standard AP-red (light red) and peroxidase-red (darker red) staining with the anti-OSP 7474 antibody labels few oligodendrocytes in frozen tissue. **H, I**: Rapid AP-red and rapid peroxidase-red protocols worked well with the anti-OSP 7474 antibody, but the rapid AP-red protocol was chosen for double staining because the colour it provides contrasts better with the one DAB provides. Black arrows point toward positively stained oligodendrocytes. The scale bars represent 50 μm.

All these images were taken from frozen precentral gyrus sections coming from the same block.  $\mathbf{D}$  shows layer V of the cortex, while the rest show the underlying white matter.

#### 3.4.2 OSP + p62 double-staining IHC

Since the rapid IHC protocol had worked for both anti-p62 and anti-OSP 7474 antibodies individually, this was the first approach taken toward double staining of frozen tissue sections, using a 1:10 dilution for each primary antibody. A case with a high degree of p62 pathology was chosen to test the technique since it should show a large number of p62-positive inclusions. p62 DAB IHC was performed before OSP AP-red IHC, and the resultant staining can be seen in figure 3.5A. No traces of red OSP staining could be found, and while some of the brown staining had the appearance of standard inclusions, others resembled the oligodendroglial staining seen in figure 3.4I. It was decided that the experiment would be repeated following the double-staining standard protocol, starting with a rapid OSP AP-red IHC (1:10 dilution) followed by a standard p62 DAB incubation (1:100 dilution) overnight -thus, reversing the order of the antibody incubations. In figure 3.5B it can be appreciated that the result of this was the opposite of what happened before: lots of red-labelled oligodendrocytes could be seen, but no inclusions were visualised. The same procedure was tried, but the order in which the primary antibodies were applied was reversed, leading sections in which there appeared to be some positively-stained to oligodendrocytes, but these appeared in an unusual red/brown colour (figure **3.5**C). It was hypothesised that this could be caused by cross-reactivity between staining steps, which was initially thought to be unlikely because a 15 min avidin & biotin-blocking step was always done in between both IHC steps. Thus, the avidin-biotin complex added in the first IHC should not interact with the chromogen added in the second step. Therefore, the avidin & biotin-blocking step was increased to 30 min, to reduce cross-reactivity. This rendered sections in which OSP again seemed to be labelled by DAB in brown, but this time, a few red dots could be appreciated, unlike before (**figure 3.5D**,**E**). This looked promising, so the same approach was tried (30 min avidin & biotin block), but with reversal of the order of the IHC steps again, doing OSP IHC before p62. The resulting sections (**figure 3.5F**) still showed the unusual red/brown colour, but a few oligodendrocytes were apparent with a redder colour than in the previous experiments, although the staining obtained was not enough to differentiate oligodendrocytes from inclusions, so a different approach was needed.

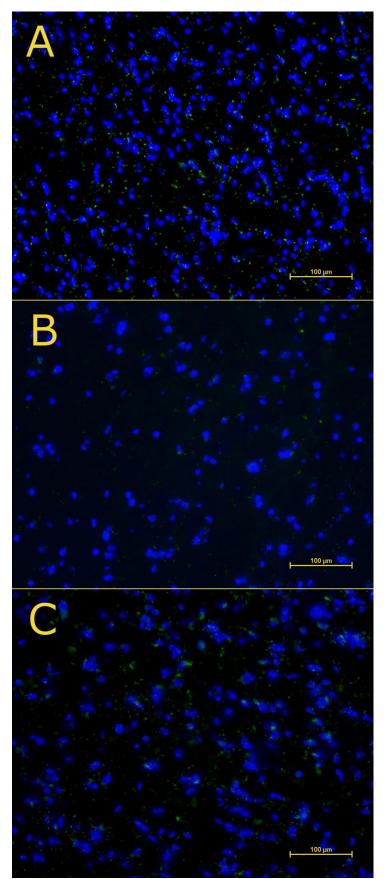
In summary, attempts of the double labelling of OSP and p62 with Vector Red and DAB resulted in a likely cross-staining which did not allow distinguishing between oligodendrocytes and inclusions.



**Figure 3.5: Initial optimisation of the OSP and p62 double-staining IHC.** Representative images of the unsuccessful double-staining IHC protocols attempted are shown. OSP should be labelled in light red (AP-Vector red), and p62 should be labelled in dark brown (DAB). In all of them, speckles in colour between light red and brown can be seen, without the possibility of distinguishing OSP labelling from p62 labelling in most labelled cells. **A**: p62 1:10 followed by OSP 1:10, rapid IHC. **B**: OSP 1:10 rapid IHC followed by p62 1:100 standard IHC. **C**: p62 1:100 standard IHC followed by OSP 1:10 rapid IHC. **D**: p62 1:100 standard IHC followed by OSP 1:10 rapid IHC, double avidin & biotin-blocking time. **E**: p62 1:200 standard IHC followed by OSP 1:10 rapid IHC, double avidin & biotin-blocking time. **F**: OSP 1:10 rapid IHC followed by p62 1:100 standard IHC, double avidin & biotin-blocking time. All images show subcortical precentral gyrus white matter from frozen tissue.

#### 3.4.3 OSP fluorescent staining

The next method attempted was to label OSP with a fluorescent marker, using an FITC-labelled secondary antibody, along with the traditional DAB peroxidasebased IHC to label p62. This should show brown p62 inclusions in brightfield and green-fluorescent oligodendrocytes. It was first necessary to confirm the antibody worked using the immunofluorescence protocol, so the rapid IHC protocol was performed. All the tissue sections stained presented with a green granular background, even the negative control (**figure 3.6A,B**), for which no anti-OSP antibody was used. This was possibly caused by autofluorescence and non-specific binding of the secondary antibody. However, the clear oligodendroglial staining pattern seen when using chromogenic IHC methods was not seen (**figure 3.6C**). The next step involved using Sudan black to mask autofluorescence. This resulted in darker sections with no visible fluorescence (not shown).



#### Figure 3.6: Unsuccessful OSP 1:10 fluorescent IHC. A:

Negative control for which no primary antibody was used. Green puncta were seen, which may represent autofluorescent lipofuscin or non-specific binding of the secondary antibody. B: Case with C9orf72 expansions. C: Non-ALS, healthy control. No noticeable differences were found among the three tissue sections. All images show subcortical precentral gyrus white matter from frozen tissue.

#### 3.4.4 Other attempts at double staining OSP and p62

A series of other chromogens with high contrast were used to co-stain OSP and p62. These are detailed in table 3.3. The first chromogen pair was DAB for OSP first, then Nickel-DAB (Ni-DAB) for p62. Ni-DAB is a modified version of DAB that labels using black colour. This resulted in a mixed black/brown overall colour again, which did not allow for a clear differentiation of OSP and p62 labelling. After that, NovaRED for OSP, then DAB or Ni-DAB for p62 was pursued. NovaRED and DAB provided the clearest co-staining we had seen so far for these two antigens (figure 3.7), but many oligodendrocytes still showed an amalgam of red and black. Following that, we attempted to stain oligodendrocytes in blue using the Vector Blue alkaline phosphatase kit, and then p62 using DAB or Ni-DAB. Nuclei would be stained in green using Vector Methyl green, for contrast with the blue colour (not shown). Neither of the two techniques provided the desired results, showing unclear p62 protein deposits and inconsistent oligodendrocyte staining. After all these failed attempts, it was decided not to pursue this double staining any longer, and to use another marker for oligodendrocytes.



**Figure 3.7: Best attempt at double staining of OSP and p62.** The black arrow highlights acceptable contrast in an oligodendrocyte (OSP-NovaRED) with a p62-positive protein deposit (p62-DAB). Staining was not generally as clear as this, showing oligodendrocytes with poor contrast between the two chromogens. This image shows subcortical precentral gyrus white matter from frozen tissue.

#### 3.4.5 Nogo-A staining of oligodendrocytes

Neurite outgrowth inhibitor (Nogo-A) is a protein which was first found to be expressed by neurones as an inhibitor of neurite growth during brain development and after axonal injuries (Chen et al., 2000). The apparition of mature oligodendrocytes expressing Nogo-A also impairs axonal recovery after a demyelinating event in the CNS. Nogo-A is expressed predominantly by oligodendrocytes and some neuronal subpopulations after the development of the brain (Kuhlmann et al., 2007, Buss et al., 2005, GrandPre et al., 2000). As it can be seen in those studies, Anti-Nogo-A antibodies only label differentiated oligodendrocytes in the white matter of the mature CNS. In the grey matter, oligodendrocytes can be distinguished from Nogo-A-expressing neurones due to the much stronger staining presented by oligodendrocytes (Kuhlmann et al., 2007) and, in the areas studied in this analysis, due to the larger size of the motor neurones.

The Nogo-A antibody (Abcam) was optimised using serial dilutions, first performing a microwave antigen retrieval method. This resulted in a small subset of oligodendrocytes being stained. Serial dilutions were also performed using a pressure cooker for the antigen retrieval step at either pH 6 of pH 9. It was decided that a dilution of 1:50 for the Nogo-A antibody and antigen retrieval using a pressure cooker at pH 9 were optimal for the staining of oligodendrocytes, presenting distinct cell bodies and low levels of non-specific background staining (**figure 3.8A**).

The next step was to optimise the p62 antibody to work using this antigen retrieval method. For this, serial dilutions of the p62 antibody were used, and it was determined that a dilution of 1:100 worked best at pH 9. Using these conditions plus the Nogo-A optimal conditions, a final attempt was made for the double staining of oligodendrocytes (Nogo-A-NovaRED) and inclusions (p62-DAB). This resulted in a clear staining of inclusions, but all of them had some red 'aura' surrounding them, which made it impossible to distinguish whether the inclusion was in an oligodendrocyte or not (**figure 3.8B,C**).

After this, it was decided to stop pursuing the co-staining of oligodendrocytes and p62-positive inclusions because too much effort and time was put into different approaches that produced no results. The use of anti-Olig2 antibodies -a classic marker for oligodendrocytes-, to stain oligodendrocytes was not trialled due to the fact that nobody got it to work in our laboratories after repeated attempts.

The next step would be explaining the protein components of the p62-positive inclusions using markers for proteins usually found in this kind of inclusions in most cases of ALS and in C9ALS specifically.

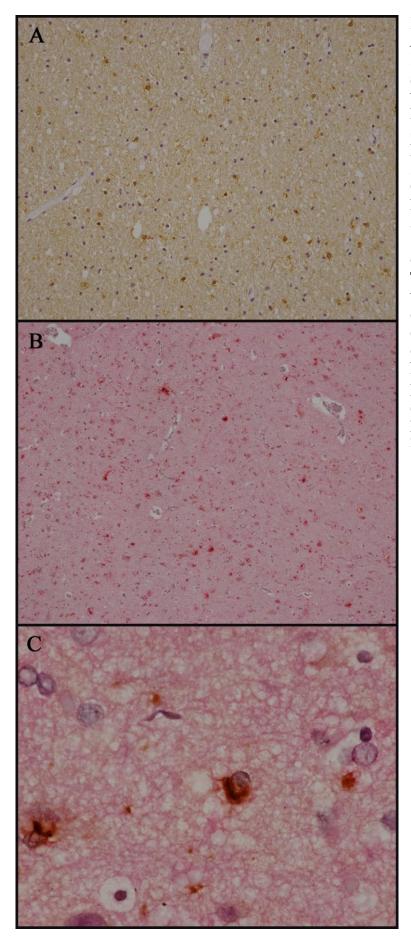


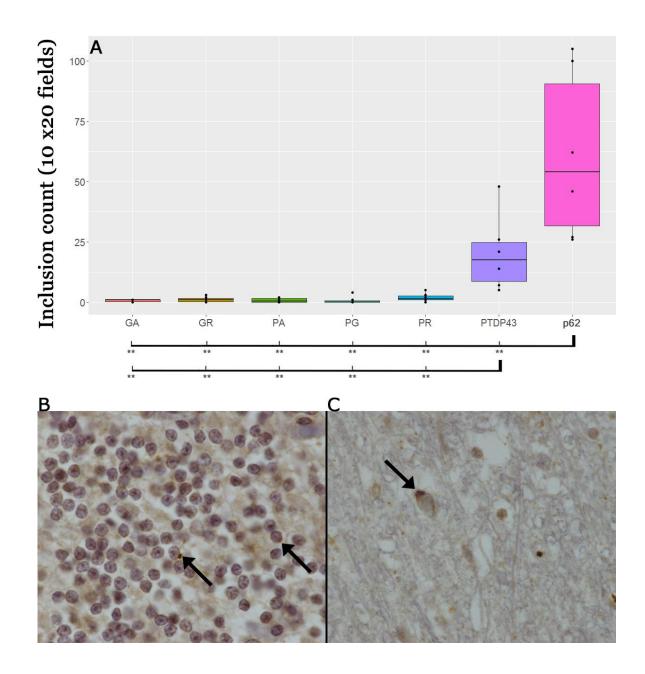
Figure 3.8: Nogo-A/p62 double IHC.

Representative images showing the results of the attempt at double Nogo-A/p62 staining. A: Single staining using Nogo-A antibody and DAB as chromogen. **B**, **C**: Double staining of Nogo-A (Vector Red) and p62 (DAB) using  $20 \times (\mathbf{B})$  and  $100 \times (\mathbf{C})$ objectives respectively. The contrast between the two chromogens is often not completely clear. This lack of contrast allows for a high chance of false positives. All images show subcortical precentral gyrus white matter from fixed tissue.

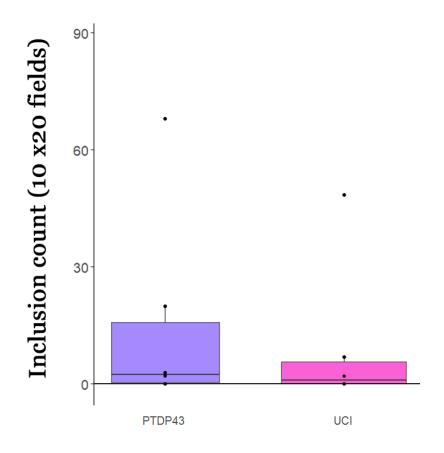
#### 3.5 Elucidating the components of glial p62-positive inclusions

Next, it was decided to try to elucidate principal protein components of the glial p62-positive cytoplasmic inclusions. The most likely possibilities were TDP43 and dipeptide repeats. DPR are a product of repeat-associated non-ATGtranslation of the expansion in the C9orf72 mutant mRNA. There are five possible DPR polypeptides from the different sense and antisense reading frames that can be read in the expansion: poly-AP, poly-GP, poly-GR, poly-AG, and poly-PR. The literature shows that most neuronal TDP43-negative inclusions in C9ALS motor cortex contain DPR, but glial DPR are usually not found in the grey matter or the white matter of these cases (Mackenzie et al., 2013). To confirm this finding in our cohort, the 6 C9ALS cases with the greatest number of glial inclusions in the precentral gyrus white matter were selected. The amount of glial DPR and pTDP43 inclusions present in this area was quantified using the same counting protocols and compared to the glial p62 inclusions. Representative inclusions can be seen on **figure 3.9B,C.** This revealed that the burden of p62 pathology was, on average, 3 times larger than that of pTDP43 (Mann-Whitney p=0.02472, W=3.5, **figure 3.9A**) and that the amount of glial DPR in this area is negligible compared to the amount of p62/TDP43-containing inclusions (figure 3.9A). Therefore, we confirmed previous findings that glial inclusion pathology cannot be explained by DPR in the precentral gyrus (Mackenzie et al., 2013) and additionally established that it could not be accounted for by pTDP-43 pathology. The sALS group, however, shows a similar amount of pTDP-43 inclusions compared to the total p62-positive inclusions (p=0.618, figure 3.10).

Having established a marked excess of p62-positive inclusions in C9ALS cases in the precentral gyrus and the prefrontal cortex, and that this pathology could not be accounted for by DPR or pTDP43, we next sought to ascertain the burden



**Figure 3.9: Quantification of glial DPR inclusions in the precentral gyrus white matter in C9ALS.** It is evident from these results (**A**) that the inclusion pathologic inclusions in the precentral gyrus white matter do not contain DPR, which is negligible in this area. *Post hoc* tests done using the Mann-Whitney U test, comparing the scores of p62-positive and TDP43-positive inclusions and each different type of DPR inclusion (indicated by the bars underneath). The number of p62-positive inclusions was also significantly higher than the number of TDP-43-positive inclusions. \*\*p<0.01. **B** shows the positive control tissue used for the poly-GR antibody, which is representative of all the positive controls for the different DPR. The positive controls were cerebellar FFPE sections of a C9ALS case with particularly elevated levels of DPR inclusions in the granular layer of the cerebellum (arrows). **C** shows a glial poly-AP DPR cytoplasmic inclusion (arrow) in the subcortical white matter of a prefrontal gyrus FFPE section.



**Figure 3.10: Comparison of glial TDP43 and p62-positive cytoplasmic inclusions in the precentral gyrus white matter in sALS.** The number of p62–positive cytoplasmic inclusions (UCI) in the sALS group is similar to that of pTDP-43 cytoplasmic inclusions in this area.

of phosphorylated TDP43 (pTDP43) inclusions in the motor cortex and the spinal cord of our cohort. pTDP43 aggregation was quantified using an anti-pTDP43 antibody (BD Transduction Laboratories) and the same counting protocols used for p62-positive inclusions. This was done for spinal cord and motor cortex sections. pTDP43-positive inclusions were also counted in sALS and control tissue to compare the burden of pTDP43 pathology between C9ALS and these groups.

#### 3.5.1 pTDP43 inclusions vs. p62-positive inclusions

Two types of analyses were used to assess to which extent glial pTDP43 pathology was related to glial p62 pathology in C9ALS cases. Firstly, the 6 C9ALS cases from the study cohort harbouring the highest burden of glial p62 pathology in the white matter under the motor cortex were selected.

Then, using a larger cohort (n=12), correlation tests were performed between the numbers of both types of glial inclusions, to ascertain whether a dependency could exist between them in the spinal cord or the motor cortex. No relationship was found in any of the studied areas (ventral horns: p=0.2743, tau=-0.2568915, z=-1.0932; corticospinal lateral tracts: p=0.08581, tau=-0.4036867, z=-1.7179; motor cortex: 0.6304, tau=0.1068733, z=0.48114; precentral gyrus white matter: p=0.1682, tau=0.3076923, z=1.3779). Therefore, it was concluded that p62-positive glial inclusion pathology was mostly independent of pTDP43 glial pathology in the spinal cord and in the precentral gyrus.

#### 3.5.2 Group comparison of glial pTDP43 inclusion counts

Having established that TDP-43 could not account for the full burden of p62positive glial pathology, it was none the less of interest to know if there were intergroup differences in the burden of TDP-43 inclusion pathology similar to that seen for p62. Kruskal-Wallis tests revealed that there were inter group differences in glial pTDP43 inclusions were found in the grey matter ( $\chi^2$ = 6.2902, p=0.04306, **figure 3.11E**) and the underlying white matter ( $\chi^2$ = 9.1922, p=0.01009, **figure 3.11F**) in the precentral gyrus. In the spinal cord, differences between groups were found in the glial pTDP43 inclusion scores in the ventral horns ( $\chi^2$ = 9.4053, p=0.009071, **figure 3.11B**) and the lateral corticospinal tracts ( $\chi^2$ = 6.1422, p=0.04637, **figure 3.11C**)

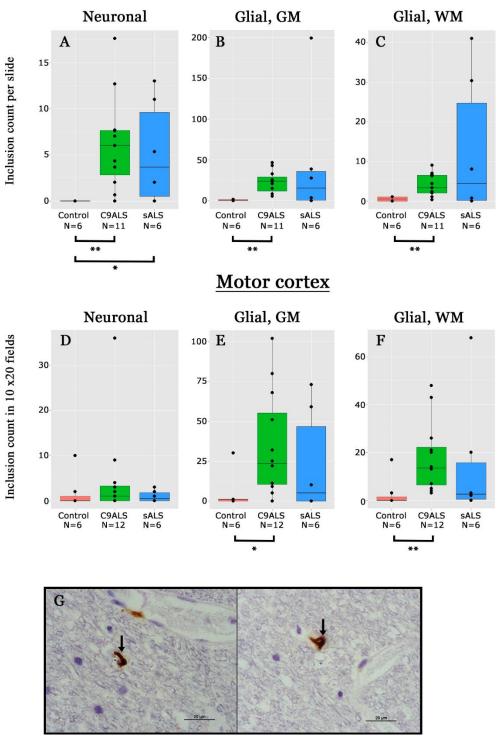
Mann-Whitney *post hoc* tests confirmed that the glia showed a significantly greater number of pTDP43 inclusions in the C9ALS group when compared to controls in the grey matter (W=72.5, p=0.0107, **figure 3.11E**) and white matter (W=76.5, p=0.003712, **figure 3.11F**) of the precentral gyrus. This difference between the glial scores of C9ALS and controls was also seen in the ventral horns (W=66, p=0.001044, **figure 3.11B**) and the lateral corticospinal tracts (W=61.5, p=0.004507, **figure 3.11C**) of the spinal cord. However, the sALS group did not show any differences in the glial pTDP43 inclusion count when compared to C9ALS (ventral horns: p=0.6507; corticospinal tracts: p=1; motor cortex grey matter: p=0.2583; precentral gyrus white matter: p=0.1457, **figure 3.11B,C,E,F**) or to controls (ventral horns: p=0.1574, corticospinal tracts: p=0.2133, **figure 3.11B,C,E,F**).

#### 3.5.3 Group comparison of neuronal pTDP43 inclusion counts

Kruskal-Wallis tests showed that there were intergroup differences in the neuronal pTDP43 score in the ventral horns ( $\chi^2$ =9.6095, p=0.008191, **figure 3.11A**). *Post hoc* M-W tests revealed significant differences between the neuronal pTDP43 scores of both ALS groups and controls (C9ALS vs controls: W=3,

p=0.002125; sALS vs controls: W=30, p=0.02844, **figure 3.11A**) in the ventral horns. Conversely, there were no differences in the number of motor cortex neuronal pTDP43 inclusions amongst the three study groups (p=0.4149, **figure 3.11D**).

In summary, there seems to be a greater glial pTDP43 pathology in C9ALS cases compared to controls, but no significant difference between C9ALS and sALS cases in either area. This pattern is different from that shown by inclusions on p62 immunohistochemistry, in which clearly a greater pathology was seen in the C9ALS cohort in the precentral gyrus compared to the sALS group. This confirms that both ALS groups present glial pTDP43 aggregation, which could potentially affect the processing and transport of certain mRNA molecules in oligodendrocytes.



**Figure 3.11: Scoring of cytoplasmic pTDP43 aggregates.** A-C: neuronal and glial pTDP43 inclusion counts in ventral horns (A-B) and corticospinal tracts (C). D-F: neuronal and glial inclusions in the motor cortex grey matter (D-E) and in the underlying white matter (F). G: representative examples of pTDP43 inclusions. Both images show subcortical precentral gyrus white matter from fixed tissue. GM=grey matter, WM=white matter. *Post hoc* p-values calculated with the Mann-Whitney's U test are shown. \*p<0.05, \*\*p<0.01.

# 3.6.1 Glial p62-positive inclusion pathology is more prevalent in the cortex of C9ALS and cannot be completely explained by DPR or pTDP43.

Here, we present a series of quantitative pathological findings in our cohort of familial C9ALS and sALS cases. First, we analysed the post mortem pathology concerning the p62-positive cytoplasmic protein deposits (inclusions) found in the relevant regions of the spinal cord, precentral gyrus, and anterior prefrontal cortex. The results of the study show how C9ALS cases present a common glial and neuronal inclusion pathology in both the prefrontal lobe and the precentral gyrus when compared to both sALS and control cases. The sALS group also showed significantly more p62-positive inclusions than the controls in the same regions. However, no difference could be found between the ALS groups in the spinal cord regarding glial inclusion pathology, even though both ALS groups showed more glial and neuronal inclusions than the control group. This confirms that glial inclusions in the prefrontal cortex and the precentral gyrus is a common feature of the ~90% of ALS cases which are sporadic and the ~5% of C9ALS cases, but it is far more prevalent in C9ALS. This is consistent with previous findings (Cooper-Knock et al., 2012). Additionally, glial inclusion pathology in the corticospinal tracts and the ventral horns, which is typically composed of pTDP43 (Neumann et al., 2006), is relevant in both types of ALS.

Then, it was established that glial inclusion pathology in the grey matter is correlated with the inclusion pathology of the glia in the adjacent white matter, in the precentral gyrus, the spinal cord, and the anterior prefrontal cortex, for both sALS and C9ALS cases. A direct correlation was not found between the inclusion pathology in the precentral gyrus and that of the spinal cord or prefrontal lobe. This suggests that glial inclusion pathology in these three areas occurs independently.

The current study also confirmed that the glial inclusion pathology found in the precentral gyrus white matter could not be explained by C9ALS-related DPR protein aggregates. This supports the findings of Mackenzie et al. (2013), who used double labelling immunofluorescence to describe the lack of DPR inclusions in any of the three main types of glial cells in the grey/white matter of different cortical areas. Their study confirmed this, not only for C9ALS but also for cases harbouring the *C9orf72* mutation with other disease phenotypes, such as FTD.

The results also show that the levels of glial pTDP43 pathology found in the precentral gyrus of C9ALS cases, but not of sALS cases, are quantitatively smaller than those of glial p62-positive inclusions in the same area. This means that pTDP43 alone cannot fully explain p62-related pathology. Leaving the question as to precisely what proteins are aggregating in this cell type in C9 cases.

Repeated attempts at double-labelling to confirm the oligodendroglial lineage of the cells affected by inclusions were not successful. However, p25 has recently been used successfully as a suitable oligodendrocyte marker for this purpose. According to the emerging literature, about 80-90% of the pTDP43immunoreactive cells in the grey matter and precentral gyrus white matter are oligodendrocytes (Fatima et al., 2015, Rohan et al., 2014). The consistency of an increased glial pTDP43 pathology in the motor cortex of our C9ALS cases combined with the fact that most of them are very likely localised to oligodendrocytes suggest that there is likely to be a particular mechanism by which oligodendrocytes degenerate in C9ALS as compared to sALS. Neuronal pTDP43 aggregation is especially prevalent in the ventral horn neurones of all ALS cases, but not in the neurones populating the motor cortex. The high levels of p62-positive inclusion pathology combined with low levels of pTDP-43 pathology in the motor cortex of C9ALS cases are consistent with the existing literature (Troakes et al., 2012).

In summary, the attempts to explain what the protein components of most of the p62-positive inclusions are by using DPR and pTDP-43 antibodies, and whether those inclusions co—localise with oligodendrocytes specifically, did not work as expected. Therefore, it was desirable to ascertain whether there was any evidence for mRNA transport deficiency in oligodendrocytes in the motor pathways of the CNS in sALS and C9ALS patients, and this was attempted in the next chapter.

#### **CHAPTER 4**

#### Analysis of myelination and MBP mRNA transport in ALS

#### 4.1 Introduction

Thus far, pathological studies have established that there is a significant burden of glial pathology –in the form of cytoplasmic p62-positive inclusions- in ALS and, on morphological grounds, this appears to affect oligodendrocytes. Specifically, this was observed to be most prominent in the corticospinal tracts of the spinal cord of both ALS groups and the white matter of the precentral gyrus of the C9ALS group.

It was thus hypothesised that whatever causes glial p62-positive and pTDP-43 pathology would also affect mRNA transport, which is a key biological process in oligodendrocytes, which needs to transport *MBP* mRNA. This would, therefore, be expected to affect *MBP* expression in the corticospinal tracts in sALS and C9ALS.

The first aim was to quantify the expression of a number of proteins related to myelination and axons using Western blot (**figure 4.1A**). To control for the degradation of the protein in the tissue due to post-mortem delay, an internal non-motor control area was included by quantifying the same proteins in the dorsal columns (somatosensory) of the same cases (**figure 4.1B**). The correlation between the relative protein amounts to the post-mortem delay was assessed for all the measured proteins, and none were found to be statistically significant (all  $p \ge 0.48$ ). Additionally, no significant differences were found amongst the three cohorts in terms of post mortem delay (p=0.4317).

IHC was also performed for the same proteins to help understand whether there were any differences in the localisation of the expression in either C9ALS or sALS cases. Thus, these two techniques would complement each other, one providing spatial characterisation (IHC), while the other would assess the relative levels of expression (Western blot). qPCR was also performed in order to assess overall levels of mRNA from the genes encoding the proteins measured using Western blot. After the total RNA had been purified from homogenised corticospinal dorsolateral motor tract and dorsal somatosensory tract samples, a quality check was performed to ensure that its integrity was sufficient for qPCR analysis. This was achieved by using an RNA electrophoresis pico-chip (**figure 4.2A**), which determined that the RNA integrity number (RIN) for the tested RNA samples was between 3.5 and 5.1 out of 10 (**figure 4.2B**). These RINs are within the acceptable range for qPCR of brain tissue with control genes (Koppelkamm et al., 2011). Only four random mRNA samples were tested for quality control as representative samples of the cohort. Retrospectively, all the samples should have been tested for quality assurance, but it was not possible anymore at the time of writing this thesis.

The measured mRNAs were *MBP* and *PLP* (components of myelin); *HNRNPA2B1* (splice variant that encodes hnRNP-A2) which is involved in *MBP* mRNA transport; and 18s rRNA, that was used to normalise the data. 18s rRNA was also used to check whether the DNA quantity was acceptable for qPCR (**figure 4.3**). One sample reached the Ct value for 18s rRNA after the 35<sup>th</sup> qPCR cycle and was disregarded in the statistical calculations.

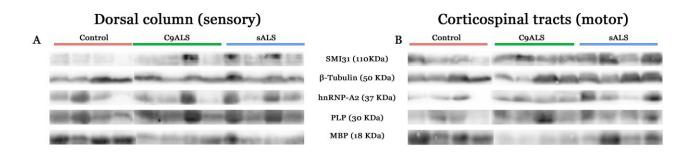
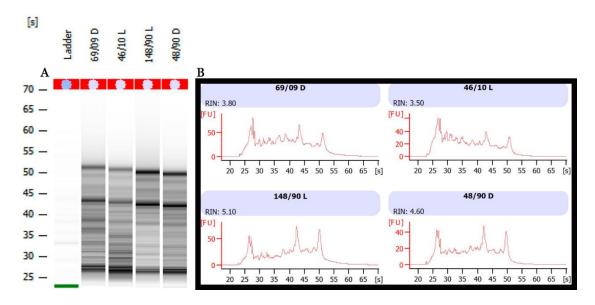
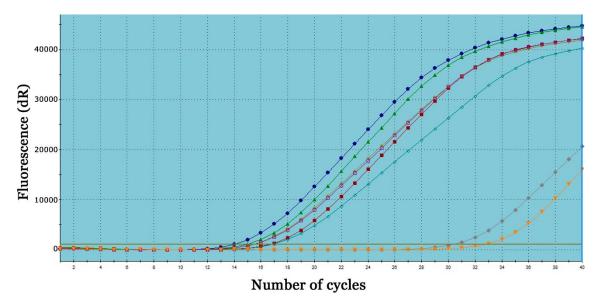


Figure 4.1: Analysis of protein levels in spinal cord tissue. A and B show Western blots performed using protein extracts from the dorsal sensory columns and the lateral motor tracts of ALS patients and controls. They were conducted for neurofilament heavy chain protein,  $\beta$ -tubulin, hnRNP-A2, PLP, and MBP. This image shows bands of single membranes that were used for the statistical analyses shown in the next sections. The results for the control group have been repositioned on the left for easier interpretation. These results were analysed using Kruskal-Wallis tests. Where Kruskal-Wallis tests showed significance (p < 0.05), Mann-Whitney tests were used to compare the cohorts in pairs. Full-membrane images can be found in **Appendix 1**.



**Figure 4.2: Tissue-extracted RNA pico-chip electrophoresis. A** shows the results of RNA electrophoresis using a pico-chip using the extracted RNA from a small subgroup of the full cohort. **B** shows densitometry spectra of the fluorescence emitted by different sizes of RNA. These four samples were the only samples that were analysed. With such a small number, no statistical methods were used to compare the RIN of the different groups. RIN=RNA integrity number (out of 10). [s]=RNA size. FU=Fluorescence units.



**Figure 4.3: qPCR for 18s rRNA.** Representative 18s rRNA qPCR showing that the synthesised cDNA was sufficiently concentrated for levels to be measured using qPCR. To get accurate and reproducible results and in order for that RNA sample to be considered,  $C_t$  for 18s rRNA had to be reached before the 35<sup>th</sup> cycle. dR: baseline-subtracted fluorescence reading.

#### 4.2 Protein transport (PLP) vs mRNA transport (MBP)

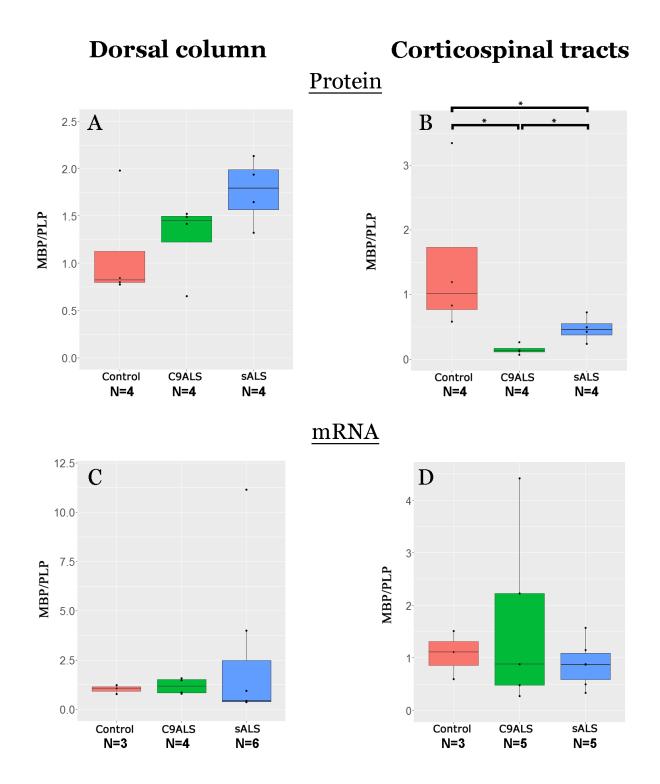
In the introduction, it was described how MBP depends on mRNA transport, while PLP depends on protein transport for myelination. To measure this relationship, we calculated the MBP/PLP ratio and found out that in the dorsal columns there are no differences between the groups (Kruskal-Wallis p=0.2319,  $\chi^2$ =2.9231, **figure 4.4A**). However, the lateral corticospinal tracts of C9ALS cases presented a 4-fold difference in the MBP/PLP ratio compared to controls (Kruskal-Wallis p=0.0132,  $\chi^2$ =8.6538; C9ALS vs controls: p=0.0285, W=0, **figure 4.4B**), as hypothesised. Just a 2-fold drop in the MBP/PLP ratio was found in sALS cases (sALS vs controls: p=0.0285, W=1, **figure 4.4B**), which was significantly higher than in the C9ALS group (p=0.0285, W=1, **figure 4.4B**).

One possible cause of the lower MBP/PLP protein ratios shown by the Western blot in ALS cohorts could be a reduction in the amount of *MBP* mRNA or an increase in the amount of *PLP* mRNA rather than issues pertaining to mRNA transport per se.

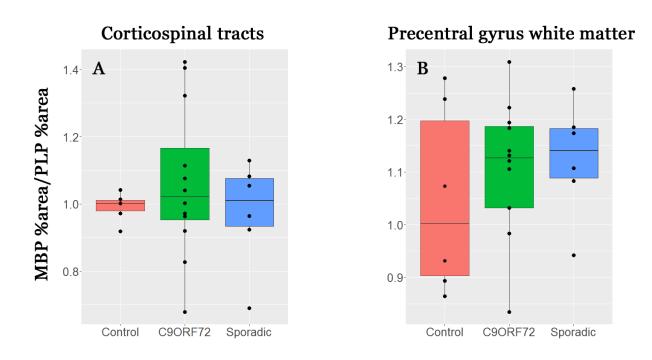
The ratio of [*MBP* mRNA]/[*PLP1* mRNA] was measured using qPCR to establish if there was a difference in their transcription. No differences were found in the regions analysed (Kruskal-Wallis <u>corticospinal tracts</u>: p=0.484,  $\chi^2$ =0.0638, **figure 4.4D**; <u>dorsal columns</u>: p=0.601,  $\chi^2$ =1.01, **figure 4.4C**).

Using immunohistochemistry for MBP and PLP on blocks of motor cortex and spinal cord from control, C9ALS, and sALS cases, the ratio of area stained for MBP to that stained for PLP was then calculated. The idea was to see whether the effects observed using Western blot could be replicated using a less labor-intense technique such as IHC, even though IHC was less likely to have the same sensitivity than Western blot for this matter. Images from the precentral gyrus white matter and from the lateral corticospinal tracts were captured, and using an image analysis method involving colour deconvolution, the percentage of the image that was positive for MBP or PLP was quantified.

It can be observed in **figure 4.5C**, **D**, that the percentage area stained for MBP was very similar to the percentage area stained for PLP in both the lateral corticospinal tracts (Kruskal-Wallis p=0.8557,  $\chi^{2=0.31167}$ , **figure 4.5C**) and the precentral gyrus white matter (Kruskal-Wallis p=0.6642,  $\chi^{2=0.81833}$ , **figure 4.5D**) for all groups.



**Figure 4.4: Intergroup comparison of MBP protein and mRNA levels normalised to PLP.** The MBP/PLP protein ratio (assessed by Western blot) did not vary between any groups in the dorsal sensory tracts of the spinal cord (**A**), while the C9ALS group presented a dramatic decrease of this ratio in the dorsolateral motor tracts (**B**). At the mRNA level (assessed by qPCR), none of the groups showed a significant difference in the *MBP/PLP* ratio in any of the two regions (**C** and **D**). \*p < 0.05.



**Figure 4.5: Relationship between MBP- and PLP-stained areas. A** (p=0.8557,  $\chi^{2=}0.31167$ ) and **B** (p=0.6642,  $\chi^{2=}0.81833$ ) show there are no differences amongst groups in the MBP/PLP ratio regarding the stained areas in either region using IHC.

# **4.3 Analysis of MBP and PLP in the precentral gyrus and the spinal** cord.

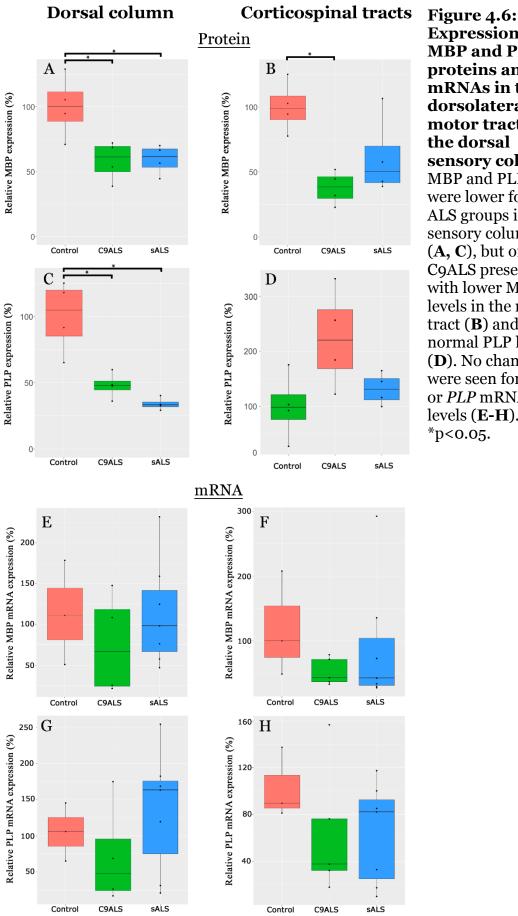
To further elucidate the MBP/PLP protein ratio alterations seen in C9ALS, the expression of MBP and PLP were analysed separately across the study groups.

After normalising MBP levels using  $\beta$ -tubulin, a Kruskal-Wallis test indicated differences amongst the groups for the amount of MBP in both the <u>corticospinal</u> <u>motor tracts</u> ( $\chi^{2=}6.04$ , p=0.0488, **figure 4.6B**) and the <u>dorsal columns</u> ( $\chi^{2=}6.5$ , p=0.0388, **figure 4.6A**). A consistent greater than 50% reduction in the total amount of MBP was found in the <u>corticospinal tracts</u> of the C9ALS cases (W=0, p=0.0286, **figure 4.6B**). Both the C9ALS and sALS groups showed a significant ~40% reduction in the <u>dorsal column</u> MBP compared to controls (C9ALS vs. controls: W=1, p=0.0310; sALS vs. controls: W=0, p=0.0429, **figure 4.6A**). No statistical difference was found between sALS and controls in the <u>dorsolateral motor tracts</u> or between the two ALS groups in either area (p ≥ 0.343, **figure 4.6A,B**).

Regarding PLP expression, it was not found reduced in the <u>corticospinal tracts</u> of the ALS cohorts (Kruskal-Wallis p=0.06904,  $\chi^2$ =5.35, **figure 4.6D**). However, it was surprising to find that the total amount of PLP in both ALS groups was reduced by about a half when compared to the control group in the <u>dorsal</u> <u>columns</u> (Kruskal-Wallis p=0.00971,  $\chi^2$ =9.27; C9ALS vs controls: p=0.0286, W=0; sALS vs controls: p=0.0286, W=0, **figure 4.6C**).

The above results indicate that both MBP and PLP are affected in the dorsal columns of ALS patients, while only MBP is affected in the corticospinal tracts.

Regarding the mRNA levels, no differences were found between the groups after quantifying *MBP* or *PLP1* mRNA in either the <u>corticospinal motor tracts</u> (all  $p \ge$  0.351, **figures 4.6E-H**).



**Expression of MBP and PLP** proteins and mRNAs in the dorsolateral motor tract and the dorsal sensory column. MBP and PLP levels were lower for both ALS groups in the sensory columns  $(\mathbf{A}, \mathbf{C})$ , but only **C9ALS** presents with lower MBP levels in the motor tract (B) and normal PLP levels (D). No changes were seen for *MBP* or PLP mRNA levels (E-H). \*p<0.05.

# 4.4 MBP and oligodendroglial pathology vs axonal density in the corticospinal tracts

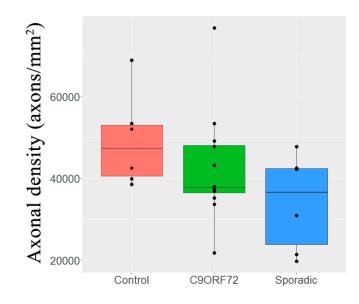
We hypothesised that oligodendrocyte dysfunction precedes and causes axonal degeneration in C9ALS, due to impairment of *MBP* mRNA transport and thus myelination. However, the possibility of *MBP* expression being affected by reduced axonal density could not be disregarded. A way to normalise the myelination data is therefore needed so that it reflects whether myelination levels in the examined tissue could have been caused by axonal loss. The SMI31 antibody was used to stain phosphorylated neurofilament protein and thereby visualise axons in spinal cord sections. The axonal density was assessed in the lateral corticospinal tracts in controls, sALS, and C9ALS cases.

A Kruskal-Wallis test showed no significant intergroup differences in axonal density in the <u>dorsolateral motor tracts</u> (p=0.121, **figure 4.7**) between control, C9ALS and sALS cases. However, a significant negative correlation was found between the number of p62-positive glial inclusions and the pooled axonal densities (p=0.00815, z= -2.65, tau= -0.399, **figure 4.8**). This indicates that axonal loss in the lateral corticospinal tracts could be associated with the glial pathology found in that area. However, the causal relationship remains to be elucidated - axons may degenerate due to the lack of oligodendroglial support, or oligodendroglia may be affected if axons degenerate.

Using Western blot, Kruskal-Wallis tests showed intergroup differences in the MBP/Neurofilament ratio in both the <u>lateral corticospinal tracts</u> ( $\chi^{2=}9.27$ , p=0.00971, **figure 4.9B**) and the <u>somatosensory dorsal columns</u> ( $\chi^{2=}7.539$ , p=0.0231, **figure 4.9A**). We found that in both C9ALS and sALS cases, this ratio was reduced to about 25-50% of that found in controls in both the <u>corticospinal</u>

motor tracts (C9ALS vs. controls: W=0, p=0.0286; sALS vs. controls: W=0, p=0.0286, figure 4.9B) and the dorsal columns (C9ALS vs. controls: W=0, p=0.0286; sALS vs. controls: W=0, p=0.0286, figure 4.9A). Moreover, C9ALS cases showed a significant ~50% reduction in the MBP/Neurofilament ratio compared to the sALS cohort in the corticospinal tracts (C9ALS vs sALS: W=1, p=0.0286, figure 4.9B) However, as was seen using immunohistochemistry, the amount of neurofilament protein was not reduced in either area for the ALS cohorts (dorsal columns:  $\chi^2$ =5.81, p=0.0548, figure 4.9D; corticospinal tracts:  $\chi^2$ =2.42, p=0.298, figure 4.9C).

Thus, the evidence from both immunohistochemistry and Western blotting implies that ALS cases do not show axonal loss in the corticospinal tracts at the level of the cervical cord to anywhere near the degree seen for loss of MBP. As the myelin loss is disproportionately greater than any axonal loss (indeed, the latter was not detectable by our measures), this implies that the myelin loss is not secondary to axonal degeneration in this area. It would be of interest to replicate the experiment using lumbar sections of the spinal cord to elucidate whether the rate of degeneration of more distal axons shows the same results. The cervical axonal density data shows a considerable variance, and it shows that axonal loss might be dependent on oligodendroglial pathology in the corticospinal tracts regardless of whether the patients have ALS.



**Figure 4.7: Comparison of the axonal density in the corticospinal tracts.** No differences were identified between the three groups regarding axonal density, using a Kruskal-Wallis test. A representative image of the analysed pictures can be found in the Materials and Methods chapter, **figure 2.3**.

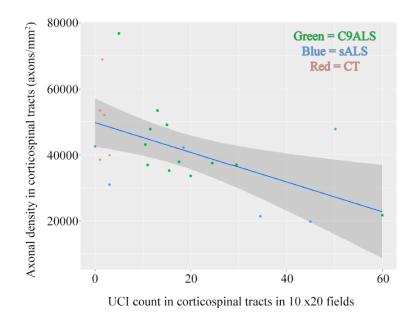
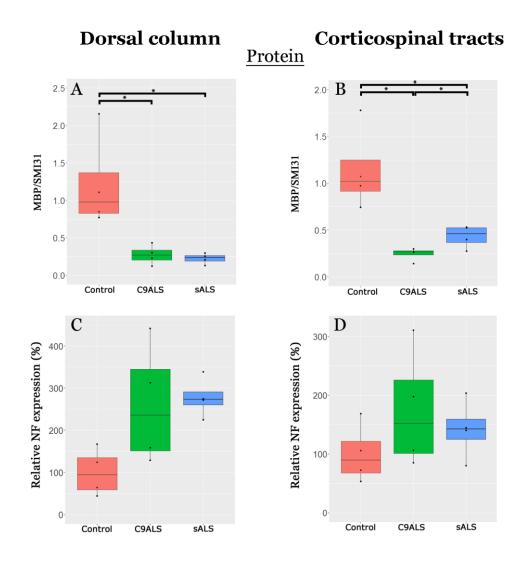


Figure 4.8: Correlation between glial inclusion counts and axonal density in the corticospinal tracts at the cervical spinal cord level. A significant correlation was found between these two variables in the overall population (Kendall tau= -0.399, p= 0.00815, z= -2.61).



**Figure 4.9: Western blot results for neurofilament protein heavy chain.** A and B show the expression of neurofilament protein (NF) relative to the control group in the dorsal columns and the corticospinal tracts respectively. C and D show the relative expression of PLP in the dorsal sensory columns and the corticospinal tracts respectively. \*p  $\leq$  0.05 for Mann-Whitney *post hoc* tests.

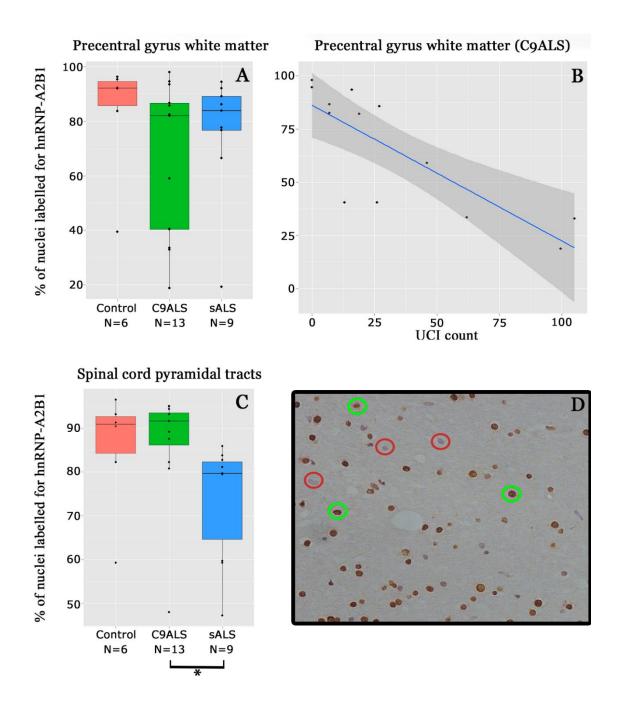
#### 4.5 Quantification of hnRNP-A2

We examined the expression of hnRNP-A2, a key component of *MBP* mRNA transport granules, in the lateral corticospinal tracts of the spinal cord and in the precentral gyrus white matter by immunohistochemistry: hnRNP-A2-positive and negative glial nuclei were counted to calculate the proportion of glial nuclei that were positive in these areas

#### (figure 4.10D).

As noted above, we had hypothesised that oligodendrocyte pathology, as indexed by p62positive inclusions which are most pronounced in the precentral gyrus white matter, may also cause mRNA transport deficits. It was therefore hypothesised that there would be a negative correlation between the percentage of hnRNP-A2-positive glia (**figure 4.10A**) and the burden of glial inclusions in the C9ALS cases in this region. This negative correlation was indeed statistically significant (p=0.0114,  $\tau$ =-4.39, **figure 4.10B**).

Regarding the corticospinal tracts of the spinal cord, Kruskal-Wallis analysis revealed significant differences amongst the groups ( $\chi^2$ =8.9579, p=0.01135, **figure 4.10C**). *Post hoc* tests showed that the sALS group had a smaller number of hnRNP-A2-positive nuclei in the lateral corticospinal tracts of the spinal cord compared to the C9ALS group (p= 0.012348). No statically significant differences were found when the sALS cases were compared to the control group (p=0.0559). This could be due to a low number of cases in our control group, which confers a low statistical power ( $\beta$ =0.54).



**Figure 4.10: Quantification of nuclei positive for hnRNP-A2. A** and **C** show the group comparison of the percentage of nuclei which were labelled for hnRNP-A2 in the corticospinal tracts (**A**) and the precentral gyrus white matter (**C**). **B** shows the significant negative correlation found between the p62-positive inclusion score and the percentage of nuclei stained for hnRNP-A2 in the white matter under the motor cortex in individual C9ALS cases (p=0.0114). **D** shows a representative picture for hnRNP-A2 staining: Red circles indicate nuclei below the threshold (negative), and green circles indicate positive nuclei. This image shows subcortical precentral gyrus white matter from fixed tissue. \* p < 0.05.

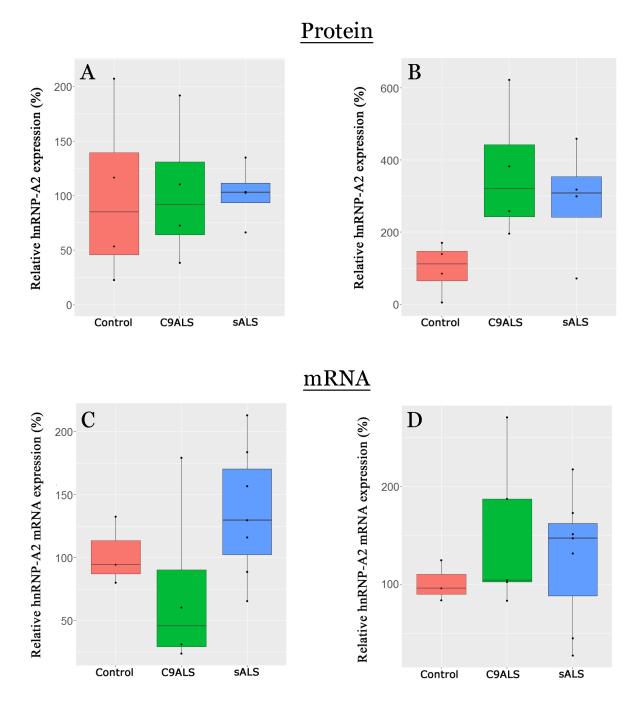
The lack of MBP relative to PLP in the corticospinal tracts of C9ALS cases described above could be due to a depletion of the hnRNP-A2 protein. When the quantity of this protein was measured using Western blot in the <u>dorsolateral</u> <u>corticospinal tracts</u>, ALS cases showed no significant reduction of hnRNP-A2 (Kruskal-Wallis p=0.0775,  $\chi^2$ =5.12, **figure 4.11B**). No differences could either be observed in the <u>dorsal columns</u> of our groups (Kruskal-Wallis p=0.981,  $\chi^2$ =0.0385, **figure 4.11A**). This indicates that hnRNP-A2 is not reduced in either the dorsolateral corticospinal tracts or the dorsal somatosensory columns of the C9ALS cases.

The relative levels of hnRNP-A2 mRNA were also measured to determine whether any difference in the levels of hnRNP-A2 protein could be caused by a reduction in mRNA. No differences were found amongst the groups regarding the levels of this mRNA molecule (Kruskal-Wallis <u>corticospinal tracts</u>: p=0.594,  $\chi^2$ =1.04, **figure 4.11D**; <u>dorsal column</u>: p=0.189,  $\chi^2$ =3.34, **figure 4.11C**).

The conclusions that we can derive from these data is that the lower amount of MBP protein found in the spinal cord of our C9ALS cohort is not caused by a lack of hnRNP-A2 protein or mRNA.



Motor lateral tract



**Figure 4.11: hnRNP-A2 expression levels.** The protein and mRNA expression levels (quantified using Western blot and qPCR respectively) did not vary in the different study groups in either the dorsal column (**A**, **C**) or the corticospinal tracts (**B**, **D**).

### 4.6 Quantification of oligodendrocyte precursor cells (OPCs)

OPCs are the progenitor cells which differentiate into mature oligodendrocytes for oligodendrocyte turnover and remyelination of axons after events incurring a loss of myelin. Having shown that greater glial inclusion pathology loads are associated with a reduction of glia expressing hnRNP-A2 in the white matter of the precentral gyrus, it was hypothesised that there would be a commensurate alteration in the numbers of OPCs. This alteration would be shown as a negative correlation between the number of OPCs and p62-positive inclusions in the precentral gyrus. OPCs were immunostained (**figure 4.12F**) using the MAP2+13 antibody (Shafit-Zagardo et al., 1999) and counted in the spinal cord ventral horns and lateral corticospinal tracts, the motor cortex grey matter, and the precentral gyrus white matter.

As seen before with hnRNP-A2-positive cells, there was a significant negative correlation between the number of stained OPCs and the number of p62-positive inclusions in the precentral gyrus white matter, with fewer OPCs in the tissue which had more inclusions (p=0.0429, z=-2.02, tau=-0.431, **figure 4.12E**).

There were no significant differences in the number of OPCs between the groups in either the spinal cord <u>ventral horns</u> (Kruskal-Wallis  $\chi^{2=1.11}$ , p=0.575, **figure 4.12A**), <u>lateral</u> <u>corticospinal tracts</u> (Kruskal-Wallis  $\chi^{2=2.49}$ , p=0.288, **figure 4.12B**), or the <u>precentral</u> <u>gyrus white matter</u> (Kruskal-Wallis  $\chi^{2=3.04}$ , p=0.219; **figure 4.12D**).

In the motor cortex, a significant alteration in the number of OPCs was found (Kruskal-Wallis  $\chi^{2=}6.66$ , p=0.0359, **figure 4.12C**). *Post hoc* tests revealed that this corresponds to an increased number of OPCs in the C9ALS group compared to controls (W=71, p=0.00558). The sALS group was not statistically different from the C9ALS group (p=0.663) or the controls (p=0.344).

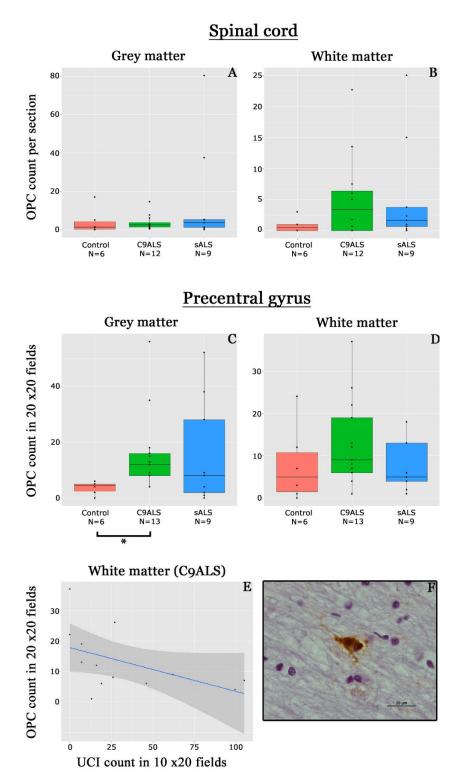


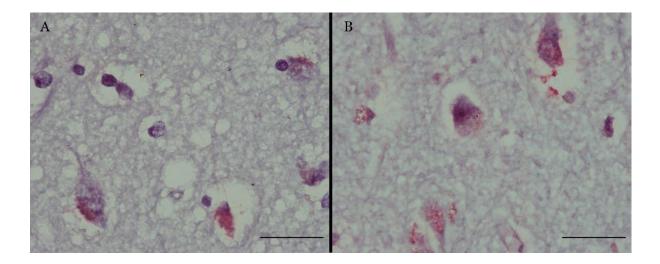
Figure 4.12: Quantification of OPCs. A and B show that there were no differences amongst the three groups regarding OPC counts in the ventral horns (A) and the corticospinal tracts (B). C and D demonstrate the comparison between the groups in the motor cortex grey matter (C) and the precentral gyrus white matter (D). A significant difference between OPCs in control and C9ALS was found in the grey matter. E shows a significant negative correlation in the precentral gyrus white matter, between the inclusion score and the OPC counts. F shows a representative picture of an OPC in the motor cortex stained for MAP2+13. This image shows subcortical precentral gyrus white matter from fixed tissue. *Post hoc* analyses were performed using the Mann-Whitney test. \*p<0.05.

# **4.7** Attempt at *MBP in situ* hybridisation to address a possible dysfunction in *MBP* mRNA transport

*In situ* hybridisation was attempted in order to label *MBP* mRNA in human tissue. It had been hypothesised that *MBP* mRNA transport is impaired in ALS. This technique would help visualise *MBP* mRNA transport in the oligodendrocytes of ALS cases to determine whether there was any evidence of such an impairment. To accomplish this, both an antisense probe and a sense probe –as a negative control- were used.

Unfortunately, the multiple trials at staining *MBP* mRNA in human tissue did not show the distinct puncta staining pattern surrounding oligodendroglial bodies. Instead, the sense probe –which should not stain the tissue- produced nonspecific staining as can be seen in **figure 4.13B**, which did not differ much from what it could be observed when using the antisense probe **figure 4.13A**.

Therefore, this technique could not be used to assess *MBP* mRNA transport in our human *post mortem* tissue.



**Figure 4.13:** *In situ* hybridisation of *MBP* mRNA. A: Antisense probe complementary to *MBP* mRNA's sequence to enable its staining. B: Sense probe matching the sequence of *MBP* mRNA, which should not provide any staining. In both cases, both neurones and glia showed unspecific cytoplasmic and nuclear staining. These images show ventral horns from fixed cervical spinal cord sections. Scale bar corresponds to 25 µm.

#### 4.8 Discussion

### 4.8.1 mRNA levels do not differ, but the levels of MBP do

Quantitative PCR revealed that there were no differences between controls and the ALS groups regarding the levels of the mRNA encoding MBP, PLP1 and HNRNPA2B1. The differences in levels of any of these proteins are, therefore, not caused by differences at the point of transcription. It was hypothesised that there would be a reduction in the MBP protein -which is dependent on mRNA transport- when normalised to PLP levels -which is not- in the motor tracts of ALS cases. Western blots primarily revealed that the MBP/PLP ratio was dramatically lower in the corticospinal tracts of C9ALS cases compared to controls. This was not seen for sALS cases. This loss could have potentially been caused by a reduced translation of hnRNP-A2 (for MBP mRNA transport), or by a degeneration of the axons passing through the section. The latter was considered unlikely since the amount of neurofilament heavy chain protein present in the region was not different from that of controls, and neither was the axonal density. We found no evidence for reduced hnRNP-A2 levels; these were in fact generally higher in C9ALS cases. The fact that the MBP/PLP ratio was so dramatically reduced for the C9ALS group supports our hypothesis that MBP mRNA transport and translation are impaired in C9ALS.

It has been argued by one group that the rate of degradation of MBP post mortem is too high for it to provide a reliable measurement of protein levels (Barker et al., 2013). These researchers incubated fresh deep brain white matter tissue lysates at room temperature and at 4°C for variable amounts of time to simulate postmortem delay. Myelin proteins were quantified using ELISA, which revealed that MBP (but not PLP) was significantly degraded with time in this way. While ELISA is a more sensitive technique than Western blotting, the main difference with our approach is that the proteins in post-mortem tissue are in a very different environment compared to proteins in tissue lysate –as they were used in their study. Proteins in lysate are surrounded by detergents which very likely affect the structure, binding pattern, and stability of proteins, which may lose the protection conferred by their local environment in the tissue. Additionally, room temperature is reasonably higher than the 2-4°C at which bodies are kept in the morgue before dissection and autopsy. Furthermore, an older study showed that unfrozen brain tissue does not lose MBP when left at room temperature overnight (Ansari et al., 1976). They showed the same for frozen tissue. They suggested that although MBP is an excellent substrate from brain acid proteinases, it is protected from them as long as the myelin lamellae are not disrupted. The results shown above (MBP levels vs post mortem delay) show no correlation between postmortem delay and MBP levels in the specimens used and not significant differences amongst the three groups in our study.

# 4.8.2 Different myelination markers reveal possible relationships between C9ALS and myelination defects in the precentral gyrus.

In the previous section, it was stablished that the C9ALS cohort has a distinct greater pathology in the precentral gyrus in the form of p62-positive protein inclusions, and a spinal cord inclusion pathology similar to that of sALS cases. Then, markers related to myelination and *MBP* mRNA transport were quantified to test the hypothesis that *MBP* mRNA transport is impaired in ALS. A negative correlation was found between p62-positive inclusion counts and the percentage of glial cells labelled for hnRNP-A2 in the white matter of the precentral gyrus. This correlation was not seen in the corticospinal motor tracts in the spinal cord, which suggests that the pathogenic action of mutant *C90rf72* favours the forebrain.

The number of available oligodendrocyte precursor cells in the white matter of the precentral gyrus was also reduced with higher inclusion counts in C9ALS, thereby further supporting the idea that the pathology in glial cells caused by mutant *C9orf72* might cause oligodendroglial dysfunction. It is also clear from our results that the p62-tagged proteins aggregated in glia in the precentral gyrus are not primarily constituted of pTDP43 or any of the *C9orf72* DPR. Further investigation may elucidate which proteins are aggregated and tagged with p62 in the oligodendrocytes and whether they are involved in their degeneration. There are some potential candidate proteins based on co-localisation studies:

- RNA Binding Motif Protein 45 (RBM45) is an RNA-binding protein which has been found to co-localise with ubiquitylated inclusions in the spinal cord of ALS patients, with a higher abundance of RBM45-positive inclusions in C9ALS (Collins et al., 2012).
- Rho guanine nucleotide exchange factor (RGNEF) regulates the GTP/GDP exchange in Rho GTPases, some of which are expressed by oligodendrocytes (Erschbamer et al., 2005). RGNEF has been found to co-localise with p62-positive inclusions in motor neurones of C9ALS cases, and it would be of interest to investigate their co-localisation in oligodendrocytes as well.
- TDP-43 missing the C-terminus. The antibody used in this study binds to the C-terminus of TDP-43, which leaves the possibility for the N-terminus to be part of those protein inclusions.

# 4.8.3 Loss of myelin proteins in the corticospinal tracts is not C9ALSspecific, but a common feature of ALS.

sALS cases showed a reduced number of hnRNP-A2-expressing cells in the corticospinal tracts of the spinal cord. This difference was not seen in C9ALS. However, MBP is greatly reduced in both ALS cohorts compared to PLP in this area. The only study to compare both MBP and PLP in health and ALS to date is

a short paper from nine years ago, which studied the lipid and protein composition of myelin in groups of three SOD1<sup>G93A</sup> mice at different ages (Niebroj-Dobosz et al., 2007). The authors showed a reduction in both MBP and PLP protein compared to controls, and electron micrographs showed degeneration of myelin sheaths in the spinal cord.

Axonal loss in the corticospinal tracts was then quantified to investigate the relationship between glial pathology in the pyramidal tract and the axons they support. Interestingly, our study shows a negative correlation between the number of glial inclusions and the density of axons in the corticospinal cords of the pooled cohort. Funfschilling et al. (2012) and Lee et al. (2012) have both found that axonal metabolism is critically dependent upon the provision of glucose and lactate by oligodendrocytes. Thus, pathology in human glia might hinder metabolite export to axons in ALS.

# 4.8.4 Final remarks

The C9ALS group showed changes indicative of altered *MBP* mRNA translation and thus myelination in the precentral gyrus. The spinal cord, however, presented a generalised loss of MBP, and several myelination markers were related to the aggregation of proteins in oligodendrocytes, accompanied by axonopathy. The approaches here described, however, have the limitation of using human *post mortem* tissue. Therefore, these results indicate the state of the brain at the time of death, not providing much data as to how the disease develops at the cellular level, and when and how the loss of myelin is initiated in relationship to neuronal degeneration. This should then be addressed further by analysing models of C9ALS and ALS caused by mutations in *TARDBP*, which help understand the development of the disease. In the following chapters, this is addressed by attempting to generate transgenic strains of zebrafish that should help understand how mRNA transport might impair myelination in ALS. **RESULTS: Part II** 

Development of in vivo systems for investigating the

molecular basis of glial pathology in ALS

# **CHAPTER 5**

# Generation of C9orf72

# hexanucleotide repeat transgenic zebrafish

#### 5.1 Introduction

IHC studies using human *post-mortem* tissue showed that cases harbouring expanded *C9orf72* presented a severe glial pathology which correlated with loss of expression of myelination markers in the precentral gyrus compared to sporadic cases of ALS with non-expanded *C9orf72*. To investigate this further and establish developmental cause-effect relationships, which cannot be achieved using only human *post-mortem* tissue, zebrafish models containing CCCCGG-expanded mRNA were desirable.

The aim of this part of the project was to make vectors which allowed the oligodendrocyte-specific transcription of versions of the *C9orf72* hexanucleotide repeat expansion (C9HNR) containing 5-6 bp interruptions every 10-13 repeats. These vectors would theoretically allow the toxic phenotype caused by GGGGCC expansions, except for the dipeptide repeat (DPR) toxic proteins, products of the RAN-translation of pure repeats – thanks to the interruptions. However, a recent study shows that these RNA sequences also cause the formation of toxic DPRs (Stopford et al., 2017). This was not known at the beginning of our study. The zebrafish *mbpa* gene is only expressed by oligodendrocytes in the CNS, making its promoter a desirable one to regulate the transcription of the C9HNR expansions. Ideally, the expression vector used should also produce a green fluorescent protein (GFP) expression marker.

# 5.2 Construction of the zebrafish expression vector

The strategy chosen to make the expression vectors was to excise the *mbpa* promoter and the interrupted C9HNR sequences from existing plasmids, and then to clone those fragments into linearised Gateway 5'-entry and middle entry vectors (figure 1A, B). The vectors containing our desired inserts, and the destination vector – containing a poly-adenylation (poly-A) signal and Gateway recombination sites, would then be recombined in a Gateway recombination reaction, which should generate the desired expression vector in a single-step reaction. This would render a vector with the HNR sequences downstream of the *mbpa* promoter, followed by the gene for GFP and a poly-A tail (**figure 1C**). This mbpa promoter was the same used by Jung et al. (2010) successfully and reliably labelling myelinating oligodendrocytes. Interrupted C9HNR repeats have previously been used in drosophila (Mizielinska et al., 2014) although in their case, the interruptions added stop codons every 12 repeats, totally blocking DPR translation. The use of Gateway vectors produced using the Tol2 kit provided by Kwan et al. (2007) has proven to be a useful tool to integrate transgenes into the zebrafish genome. As an example, Don et al. (2017) have created a triple transgenic line which expresses 3 different fluorescent proteins regulated by different promoters. Motor neurones express blue fluorescent protein under the regulation of the motor neurone-specific -3mnx1 promoter, TDP-43 expression is accompanied by the expression of GFP, and the cell death reporter Annexin V has mCherry (red fluorescence) as a reporter. These studies support our choice of expression vector for the study of the effect that C9HNR cause in oligodendrocytes.

### 5.2.1 Cloning the zebrafish mbpa promoter

A pGEM plasmid construct containing the *mbpa* promoter was obtained from Kim Lab (Jung et al., 2010). The plasmid was extracted from the filter paper that it was sent in, and the extract was then used to transform competent bacteria, which were then grown for midiprep purification of the plasmid. After this, the purified plasmid was digested with *Eco*RI, presenting a fragment of the expected size (*mbpa* promoter ~ 2000bp, vector ~ 2800bp) (**figure 5.1A**). The plasmid was also sequenced to confirm the sequence of the *mbpa* promoter. The 5'entry-multiple-cloning-site (5'E-MCS) vector – obtained from the Lawson lab (Kwan et al., 2007)- was linearised, and the *mbpa* promoter insert excised from the pGEM-*mbpa* promoter construct, both via sequential digestion with *Sac*II and *Sal*I (**figure 5.1B**). The linearised vector and promoter fragment were then gel purified, and the vector dephosphorylated using rSAP. The agarose gel showed bands of the correct sizes (**figure 5.2A**).

Competent bacteria were transformed with the ligated 5'E-[*mbpa* promoter] plasmid, and some of them released a DNA band of the correct insert size after linearizing them with *Sal*I (**figure 5.2B**). These plasmids were then sequenced to confirm that the insert was indeed the desired *mbpa* promoter.

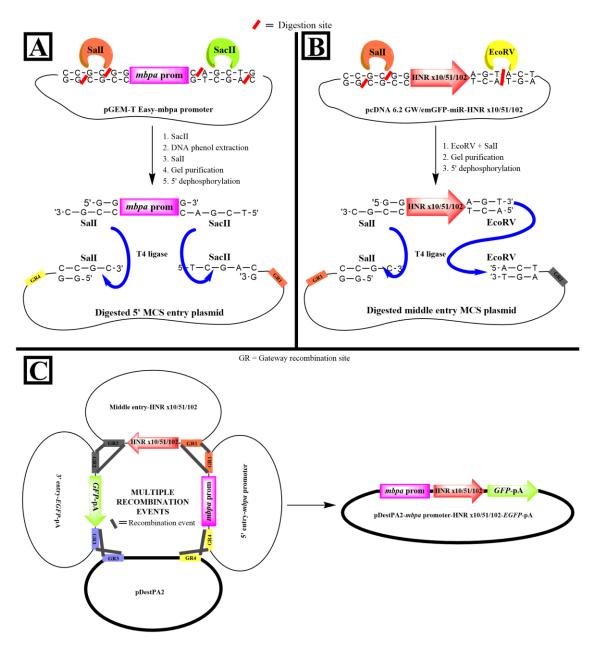
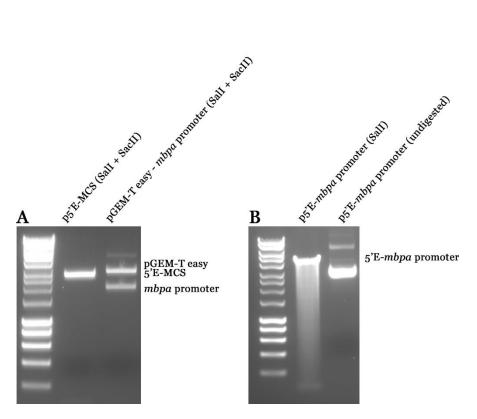


Figure 5.1: Gateway cloning strategy to construct the hexanucleotide repeat (HNR) expression vector for zebrafish. Putting interrupted C9HNRs and a fluorescent reporter under the influence of the *mbpa* promoter should direct their transcription specifically to oligodendrocytes in zebrafish. A: The mbpa promoter was excised from its original plasmid by using the restriction enzymes SacII and SalI. It was then cloned into a similarly digested 5' entry Gateway plasmid. B: The interrupted sequence of C9HNRs was purified from the original plasmid by digesting it with SalI and EcoRV, and then cloned into the middle entry Gateway plasmid. C: Mixing the two resulting vectors, along with the p3E-GFPpA and pDestPA2 vectors, and Gateway clonases, should trigger recombination events to provide the desired expression vector, with the different fragments in the correct orientation: The *mbpa* promoter would have allowed for the transcription of the C9HNRs and GFP mRNA to happen specifically in oligodendrocytes in the CNS; the C9HNR sequence did not include a Kozak consensus sequence so that it would not be translated by ribosomes, but the GFP gene was ATGdriven and included an upstream Kozak consensus. This should have allowed for the translation of GFP and the easy visualisation of cells which transcribed C9HNR sequences.



**Figure 5.2: Construction of the 5'-entry vector containing the** *mbpa* **promoter.** A: Digested pGEM-*mbpa* promoter (pGEM-T easy ~3000bp; *mbpa* promoter ~2000bp) and 5'-entry-MCS vectors (~2800bp), using *Sac*II and *Sal*I. B: Purified and linearised 5'-entry construct containing the *mbpa* promoter (~4800bp). Marker: Hyperladder 1kb.

# 5.2.2 Sub-cloning of hexanucleotide repeats

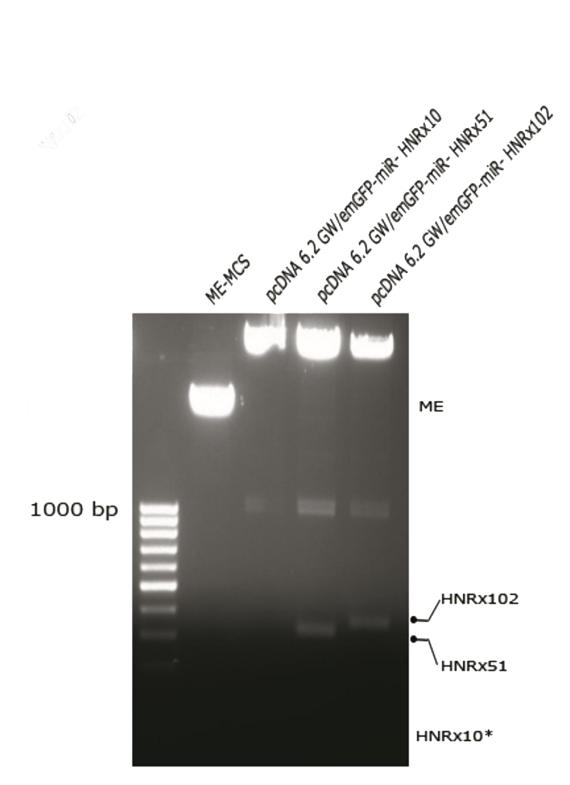
pcDNA 6.2 GW/emGFP-miR plasmids containing inserts with 10, 51 and 102 interrupted GGGGCC C9HNRs were previously generated by Dr Adrian Higginbottom in SITraN. The interruptions consisted of TCGA insertions every 10-12 GGGGCC repeats and were incorporated to improve the stability of the C9HNRs (Stopford et al., 2017). The plasmids were midi-prepped and sequenced to check the repeat lengths and linearised using EcoRV to visualise the size of the inserts in a gel (figure 5.3). The interrupted C9HNRs were excised from their respective plasmids, via double digestion with EcoRV and SalI in a single reaction. This led to visible DNA fragments of the correct size for 10x and 51xHNR. However, for the 102xHNR, just 1 or 2 colonies survived after plating the whole transformation content, and these contained contracted versions of the C9HNR (~30-50 repeats). This led us to conclude that the 102xHNR is toxic for bacteria and that the only bacteria that survive the transformation process are those which recombine the 102xHNR repeats to form shorter versions of it. Due to this problem, only the 10xHNR and 51xHNR fragments were purified from an agarose gel. In retrospect, a different strain of E. coli should have been used, as the one being used was not prepared to amplify unstable sequences.

The ME-MCS plasmid was obtained from the Lawson laboratory and showed a single band after *Sal*I digestion (**figure 5.3**) since the ME-MCS plasmid has only one *Sal*I digestion site (**figure 5.4**). The ME-MCS vector was linearised with *Sma*I and *Sal*I to perform a cloning reaction in conjunction with the C9HNR fragments.

The 10x/51xHNR sequences were then ligated into the *SmaI/Sal*I-linearised ME-MCS plasmid, transformed, grown and purified. The constructs were then digested to confirm the vector and insert sizes (**figure 5.5A**, **B**). They were then

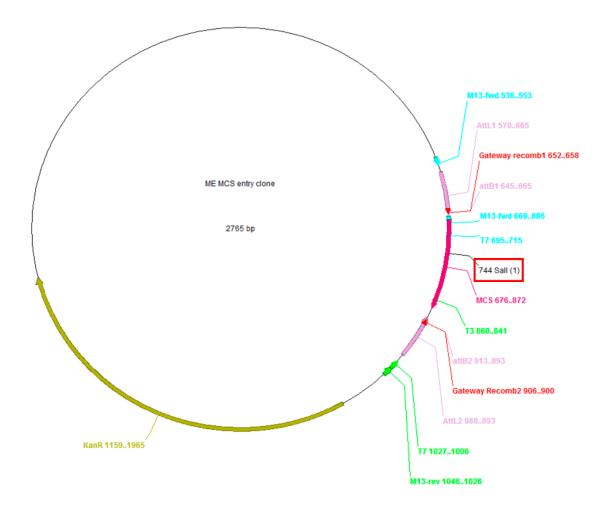
sequenced to confirm the insert size since the small size of the C9HNRx10 insert means that a considerable mass of DNA is needed for it to be observed using agarose gel electrophoresis. All colonies picked from the ME-10xHNR plate contained the correct insert (**figure 5.5B**). However, the colonies picked from the ME-51xHNR plate yielded chromatograms which were complicated to read: The beginning of the C9HNR sequence could be easily read in the sequence file, but the repeats became more difficult to read after about ~15 GGGGCC repetitions. Nonetheless, the chromatogram peaks can still be traced to get an approximation of the expansion length. This problem was due to the difficulties which arise when trying to sequence GC-rich strands of DNA. Sequencing the DNA with betaine (used for sequencing of CG-rich sequences) did improve the quality of the sequencing slightly (**figure 5.6**). **Figure 5.7** shows an example of the shortening of the 102xHNR : Only 51 repeats were identified tracing the peaks on the chromatogram.

Due to the low resolution of the sequencing of the ME-51xHNR clones, 1  $\mu$ g of each of 4 clones was digested with *Sal*I and *Not*I to check the C9HNR size. The gel in **figure 5.5B** shows that 3 out of 4 of the DNA preparations showed an insert of the correct size (~360bp). However, only one construct showed no additional bands, meaning that it contained only the vector and the correct insert, whereas the others showed smaller bands suggesting instability of the repeat. This construct was used to make the final Gateway construct.

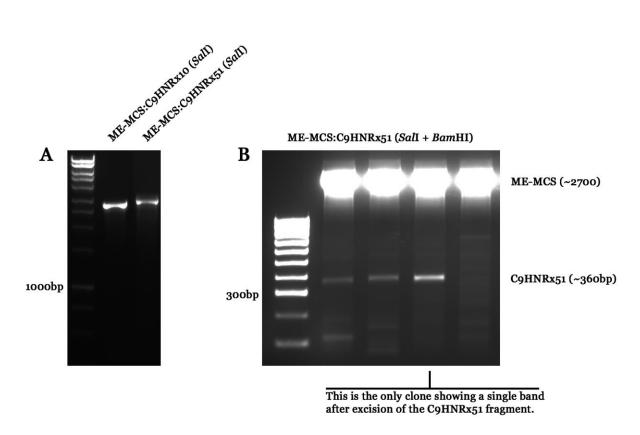


\*faintly visible in transilluminator

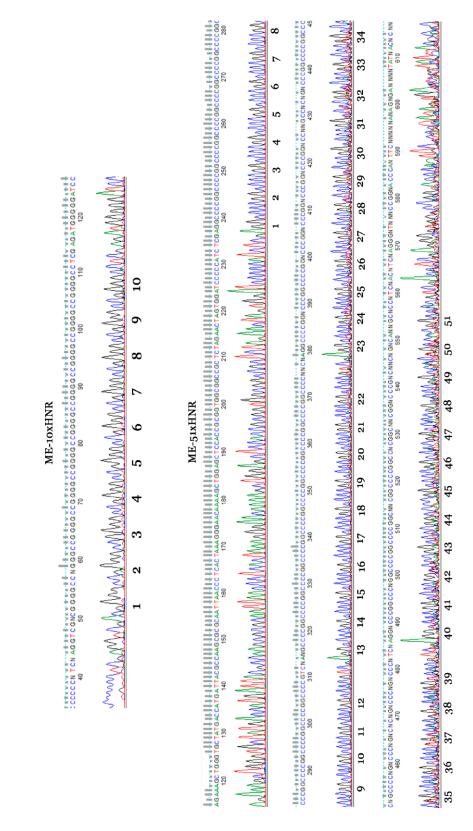
**Figure 5.3: Digestion of middle-entry vectors containing the three different C9HNR constructs.** Result of performing double digestion of the plasmids with *Sma*I + *Sal*I for ME-MCS, and *Sal*I + *Eco*RV for HNR-containing plasmids. The HNR bands were gel purified.



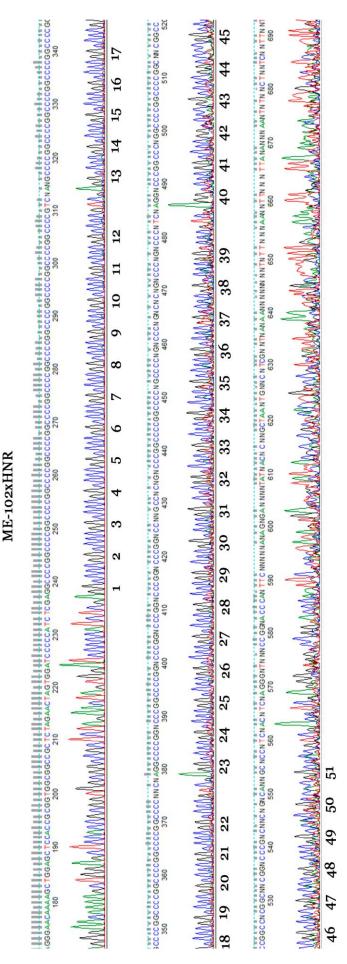
**Figure 5.4: Map of the ME-MCS plasmid.** Plasmid map highlighting the single digestion site for *Sal*I (in red square).



**Figure 5.5: Digestion of the ME-MCS:HNRx10/51 clone.** A: The plasmids showed the correct size (~2700bp and ~3000bp respectively) after digestion with *Sal*I. B: Digestion of different ME-MCS:HNRx51 clones. Only the third one from the left showed a single band of the correct size (~360bp) when the C9HNRx51 fragment was excised using *Sal*I and *Bam*HI.



**Figure 5.6: Sequencing of purified middle entry plasmids for recombination containing 10xHNR and interrupted 51xHNR sequences.** The GGGGCC repeats were normally sequenced for the ME-10xHNR plasmid and using betaine for the ME-51xHNR plasmid, for which the reverse primer was used and therefore shows GGCCCC repeats. Numbers have been placed under the traces at the beginning of each repeat.



**Figure 5.7: Sequencing of purified middle entry recombination plasmid containing the interrupted 102xHNR sequence.** Betaine and the reverse primer were used to sequence the interrupted GGCCCC repeats in this plasmid. Numbers have been placed at the beginning of each repeat. This sample presented the most repeats of any of the clones obtained attempting to generate the ME-102xHNR plasmid.

# 5.2.3 Obtaining the 3'E-GFP construct

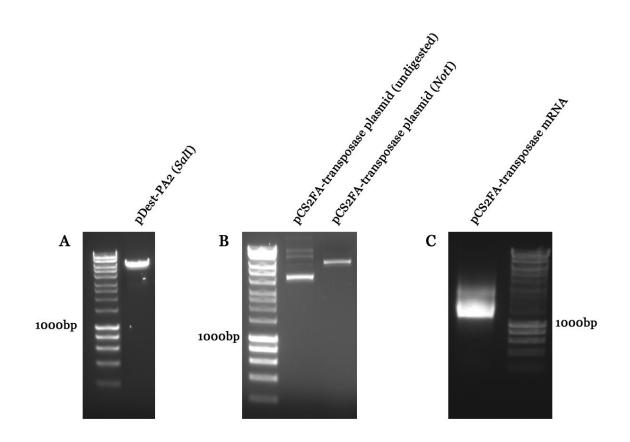
The third component wanted in our Gateway constructs was a GFP reporter downstream of the repeats. Expression of this gene should indicate which cells in the zebrafish transcribed the C9HNR sequences. The 3'E-GFP plasmid is contained in the tol2-kit and harbours both a Kozak consensus and an ATG codon at the beginning of the coding sequence which were cloned into the final expression vectors along with the *GFP* coding sequence. The size of this plasmid was confirmed by linearising it with *Not*I followed by agarose gel electrophoresis (not shown).

# 5.2.4 Obtaining the destination vector pDestPA2

The pDestPA2 vector is the backbone with which the other three plasmids will recombine to generate the final Gateway expression construct. It also came with the tol2-kit and contains the *ccdb* gene which is toxic to bacteria, necessitating the use of a *ccdb*-resistant strain of *E. coli* for propagation. The plasmid size was confirmed by linearising it with *Sal*I followed by agarose gel electrophoresis (**figure 5.8A**).

#### 5.2.5 Obtaining the capped transposase mRNA

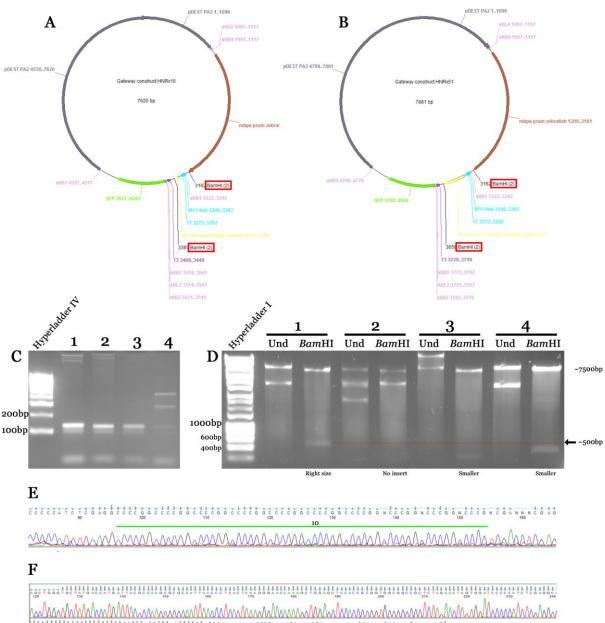
The pCS2FA-transposase plasmid includes the gene for a transposase which is necessary for the Gateway constructs to incorporate in to the zebrafish genomic DNA. The pCS2FA-transposase plasmid is also contained in the tol2-kit. This plasmid was grown and purified, then linearised using *Not*I. One microliter of the digest was used to confirm it was the right size (6034bp, **figure 5.8B**). The linearised DNA was used as a template to transcribe the transposase mRNA using SP6 RNA polymerase. It was then purified, alcohol-precipitated and resuspended in RNAse-free TE buffer. **Figure 5.8C** shows the synthesised mRNA on a non-denaturing agarose gel.



**Figure 5.8: Preparation of the pDest-PA2 destination vector and the pCS2FA-transposase mRNA.** A: *Sal*I digestion of the destination vector pDest-PA2. B: *Not*I digestion of the plasmid containing the gene for the transposase, needed for efficient integration into zebrafish genomic DNA. C: SP6 transcription product of the pCS2FA-transposase gene.

#### 5.2.6 Gateway recombination reaction

Once all the Gateway entry vectors and reagents had been prepared, the Gateway recombination reaction was performed using the purified 5'E-[*mbpa*-promoter], ME-10x/51xHNR, 3'E-GFP, and pDestPA2 plasmids. When mixed according to the protocol, the Gateway LR Clonase II enzyme should make them recombine as seen above in **figure 5.1C**. The resulting plasmids were expected to be 7620bp for the 10xHNR-containing construct (figure 5.9A) and 7881bp for the 51xHNR (figure 5.9B). After the Gateway reaction, the mix was transformed into bacteria, colonies grown, and the plasmids purified. Plasmids obtained from the 10xHNR reaction were used in a PCR reaction with primers flanking the HNR, producing a 120bp band. Figure 5.9C shows that there were some 10xHNR-containing constructs generating the right size product. Plasmids containing the 51xHNR were digested using *BamH*I, which cuts 159bp upstream and 10bp downstream of the HNR, to check the size of the plasmid and the size of the C9HNR. This process was expected to release a 490bp band and a vector band at 7385bp. As figure 5.9D shows, one clone contained the correct insert and vector size. These were sequenced and were confirmed to contain the desired inserts in the correct orientation (Figure 5.9E, F). These plasmids were injected in zebrafish 1-cell embryos along with the capped transposase mRNA, which integrates the insert of the Gateway construct in the zebrafish genomic DNA.



**Figure 5.9: Gateway recombination results. A, B**: Gateway recombination constructs including the pDest-PA2 backbone, the *mbpa* promoter (brown), the C9HNR sequence (yellow), and the GFP gene (green). The *Bam*HI digestion sites flanking the C9HNR sequences are indicated by red rectangles. **C**: PCR of the Gateway construct containing the C9HNRx10 sequence. The product size indicates the correct insert was found in the first three samples (single band at ~120bp), but sample 4 shows three bands, which was not what was expected. **D**: *Bam*HI digestion of the Gateway construct containing the C9HNRx51 sequence. Only sample 1 shows a DNA fragment of ~500 bp, which was the expected size. **E**, **F**: Sequencing results of the samples labelled 1 in **C** and **D**. Green lines indicate the location of the C9HNR sequences. For **F**, the sequence is less clear due to the lack of efficiency of the DNA polymerase with repeat sequences. However, 51 interrupted HNR (blocks of 12-10-17-12) could be counted on the chromatogram.

# 5.3 Injection of the Gateway constructs with the transposase mRNA into zebrafish embryos

The promoter]:10x/51xHNR:GFP:pDestPA2 (final [mbpa constructs concentration: 30  $pg/\mu l$ ) were mixed with diluted transposase mRNA (final concentration: 25  $pg/\mu l$ ) and phenol red solution (final concentration: 0.1%) in the appropriate amounts, and this mix was injected directly into either the yolk sac (NwT = 86,  $N_{10XHNR}$  = 32,  $N_{51XHNR}$  = 26) or the fertilised cell (NwT = 101,  $N_{10XHNR}$ = 28,  $N_{51xHNR}$  = 40) of 1-cell stage zebrafish embryos . Figures 10A and 10B show how the injections were detrimental to the survival of the embryos when compared to wild-type controls from the same cohorts. About 25% more embryos died when injected with the plasmid containing C9HNRx10, and 50% when injected with that containing C9HNRx51 into the yolk mass. Of the surviving embryos, only 5-10% did not present with deformities which would have killed the embryos at later stages of development. The rest of the embryos (~70% for the 10xHNR construct and ~45% for the 51xHNR construct) presented normal development. None of the surviving embryos showed any green fluorescence in the brain or posterior lateral line nerve apart from that observed in autofluorescent structures, which also fluoresced in uninjected wild-type embryos.

To increase the chance of early integration of the plasmids, injection into the fertilised cell of 1-cell embryos was performed multiple times (as recommended by the Tol2 kit provider). **Figure 5.10B** indicates that the injection resulted in the death of  $\geq$ 80% of injected embryos, and this was consistent every time the embryos were injected with the plasmids into the cell. Initially, this lethality was caused by poor injection technique. However, even with good injection technique, poor survival rates were observed and reducing the amount of DNA injected 10-fold to reduce the effects of DNA toxicity also had little effect. After each round of

injections, the surviving embryos that developed normally (~10-15% for both constructs) did not show any fluorescence different from the autofluorescence observed in the controls, which suggests that the DNA had not been integrated into the genome or was not expressing.

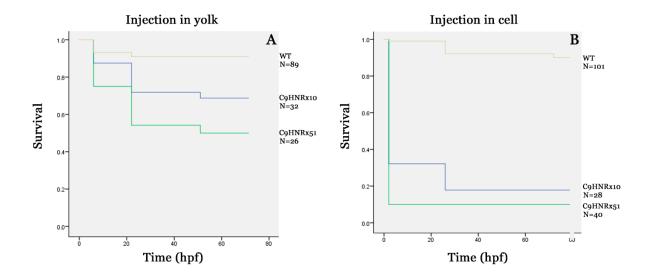


Figure 5.10: Kaplan-Meyer plots for zebrafish injected with the Gateway constructs. Injecting the plasmids into the yolk mass was less detrimental to the survival of the zebrafish embryos than injecting them into the cell. A: Kaplan-Meyer plot of the only time the embryos were injected in the yolk. B: Kaplan-Meyer plot from experiments where the embryos were injected into the cell.

#### **5.4 Discussion**

After correct synthesis of the [*mbpa* promoter]:10x/51xHNR:GFP:pDestPA2 DNA expression constructs, its injection into 1-cell zebrafish embryos did not produce any fluorescence, which was our marker to confirm integration of the construct in the host genome and expression. This was also our decision point as to whether to raise the injected embryos.

The main weakness of the different approaches assayed in our experiments was the lack of positive controls. Without positive controls, knowing where the experiments failed is difficult to assess. Additionally, it could be argued that the C9HNR repeats, which seem prone to self-recombine due to the high homology of the repeat sequences, could have been spliced from the sequence during the Gateway recombination reaction. However, this seems unlikely because the data above (**figure 5.9**) clearly shows that the repeats were present in the final construct. Below is an account of possible reasons as to why injected fish did not show any expression.

# 5.4.1 The mbpa promoter might not be active enough to provide a sufficient oligodendroglial expression of GFP in the constructs used.

This option is very unlikely because this promoter has been used previously in an efficient manner (Jung et al., 2010). In that research, an expression construct consisting of the *mbpa* promoter inserted directly into the Tol2-GFP vector, upstream of the *GFP* gene, was injected in zebrafish, obtaining selective fluorescence in myelinating oligodendrocytes. A way to confirm whether it was a problem with the promoter that caused the lack of fluorescence in this specific construct would have been to inject embryos with plasmids identical to the above HNR-containing plasmids, but regulated by the Gal4-activated upstream

activator sequence (UAS) instead. This is not an ideal solution because of the low and inefficient expression shown by the UAS in zebrafish expressing transgenic Gal4 under the *svtk* promoter and the thymidine kinase promoter from Herpes simplex (Scheer and Campos-Ortega, 1999). Some improvements have been made in this respect since then to enhance the transcription of UAS-promoted sequences (Halpern et al., 2008), but their complexity rendered them out of the scope of this project.

Other promoters that have been used to target oligodendroglial expression in zebrafish include the promoters for the zebrafish *olig2* (Shin et al., 2003, Park et al., 2007) and *oliq1* (Schebesta and Serluca, 2009) genes and the mouse *Plp* gene (Yoshida and Macklin, 2005). Expression driven by the *olig2* promoter labelled oligodendroglial precursor cells at early stages of growth, with the disadvantage (in our case) that these cells later follow different fates. For example, some neurones, such as the eurydendroid/Purkinje cells in the cerebellum, also utilise the *olig2* promoter at embryonic stages and show fluorescence when harbouring a Tg[olig2:egfp] bacterial artificial chromosome (BAC) (Shin et al., 2003). However, only oligodendrocyte-fated glia showed expression of olig2 expression at post-embryonic stages (Park et al., 2007). Using the Tq[olig1:eqfp] BAC, differentiating oligodendrocytes have been successfully labelled in zebrafish embryos (Schebesta and Serluca, 2009), but it remains to be ascertained whether the expression is maintained in adult zebrafish. The same can be said about zebrafish that express EGFP under the murine *Plp* (proteolipid protein) promoter (Yoshida and Macklin, 2005).

# 5.4.2 The C9HNR segments might have prevented genomic DNA integration or expression of the GFP gene.

Pure *C9orf72* expansions allow the mRNA to form complex secondary structures known as G-quadruplexes (Reddy et al., 2013). It would be interesting to elucidate whether G-quadruplexes still occur even in the interrupted sequences used in this experiment. However, another interrupted C9HNR sequence with a similar pattern of interruptions demonstrated that the tertiary structure is not affected by small interruptions in the sequence every 12 repeats (Mizielinska et al., 2014). However, as it was shown above, polymerases can struggle to copy these expansions with high efficiency. These factors could have contributed towards a poor recombination of the desired DNA fragment into the zebrafish genome, or to inefficient transcription or translation of the resultant 51xHNR-containing mRNA. The latter is less likely to apply to the 10xHNR-containing fragment since it can be easily amplified by Taq polymerase and may form fewer or less complex secondary structures.

Embryos injected with a plasmid containing the *mbpa* promoter followed by the GFP marker gene lacking C9HNR sequences would have helped to ascertain whether the lack of genomic integration was caused by an unviable expression vector due to the C9HNR fragments. There is also the possibility that low levels of expression would have produced sub-threshold levels of fluorescence which could have been tested using more sensitive detection methods. Grown injected embryos could have thus been subjected to *in situ* hybridisation or immunohistochemistry using probes for *GFP* or antibodies for GFP respectively.

However, the possibility of HNR expansions blocking GFP expression seems unlikely because other groups have successfully produced zebrafish models harbouring *C90rf72* expansions with fluorescent reporters downstream of the repeats. A zebrafish strain harbouring ubiquitously expressed 80×(GGGGCC) upstream of a fluorescent GFP reporter has very recently been utilised (Ohki et

al., 2017) to investigate the effects of dipeptide repeats and GFP fluorescence was observed. Additionally, strains harbouring different numbers of GGGGCC repeats (8, 38, and 72) after a GFP gene and a stop codon were used to investigate the effect of the expansion size in GFP synthesis (Lee et al., 2016). These constructs were also successfully injected into neuronal cell models. Drosophila melanogaster models harbouring C90rf72 expansions with expression markers have also become abundant since Xu and colleagues created the first of such models. These flies carried a 30×GGGGCC:GFP construct interrupted in the middle of the expansion with an in-frame restriction site and preceded by either CNS or motor neurone promoters (Xu et al., 2013, Zhang et al., 2015). Later, a ubiquitous 58×(GGGGCC):GFP construct was also successfully used to investigate the effects of DPR polypeptides in flies (Freibaum et al., 2015). However, this construct would only allow for the expression of GFP if one of the three possible reading frames was translated into DPR, while the other two would not yield fluorescence. Another Drosophila model was created using a 48×GGGGCC:(FLAG-HA-MyC) construct, having three different markers, each in frame with a different DPR product (Burguete et al., 2015). All these models show that it is possible to produce viable animals harbouring C90rf72 repeats with functional downstream reporters.

Fluorescent *in situ* hybridisation for the C9HNR would have been useful to ascertain whether the expansions used in our studies were being transcribed in the zebrafish genome. Finally, if this technique did not report any HNR transcription, Southern blotting could have been performed in injected embryo DNA extracts to determine whether the construct was being integrated into the genome. However, this would be limited by the effects of mosaicism and the efficiency of integration.

### **CHAPTER 6**

### Studying the influence of TDP-43 over MBP mRNA transport using zebrafish models

#### 6.1 Introduction

The aim of this part of the project was to determine whether *TARDBP* mutations interfere with MBP synthesis either by a toxic gain of function of the mutant protein –sequestering relevant proteins such as hnRNP-A2- or a loss of function –TDP-43 could be an important component of mRNA transport granules.

## 6.2 Analysis of oligodendrocyte development in *tardbp* knock-out zebrafish

In order to address the loss of function hypothesis, the possible developmental consequences driven by depletion of the *tardbp*'s gene's protein product, Tardbp, were studied in zebrafish. A knock-out (KO) zebrafish strain for *tardbp* that had been generated by TILLING (Targeting Induced Local Lesions in Genomes) was available to us (Hewamadduma et al., 2013). This strain contains an early stop codon in the *tardbp* gene, which truncates the resultant protein at its second RNA-recognition motif, which likely results in its rapid degradation by the cell since no traces of it can be found in homozygous fish. This feature makes the model useful to investigate oligodendrocyte development in the absence of Tardbp, although with the caveat that the *tardbpl* gene is up-regulated to compensate for this loss (Hewamadduma et al., 2013).

To study oligodendrocyte development in this model, OPCs positive for *sox10* mRNA were counted in 3 dpf (days post-fertilisation) embryos, and oligodendrocytes and commissures positive for *mbp* were counted in the same

area of the brain in 5 dpf larvae. Both types of counting were performed on wild type (*tardbp*<sup>+/+</sup>), homozygous mutant (*tardbp*<sup>-/-</sup>), and heterozygous (*tardbp*<sup>+/-</sup>) animals. Due to the variability that was seen with the employed staining method and chromogen (BCIP/NBT), a quantitative measurement of the colour density would not provide accurate results. This means that quantification of each type of mRNA was not possible using this methodology.

#### 6.2.1 Counting of sox10-positive OPCs in 3 dpf zebrafish

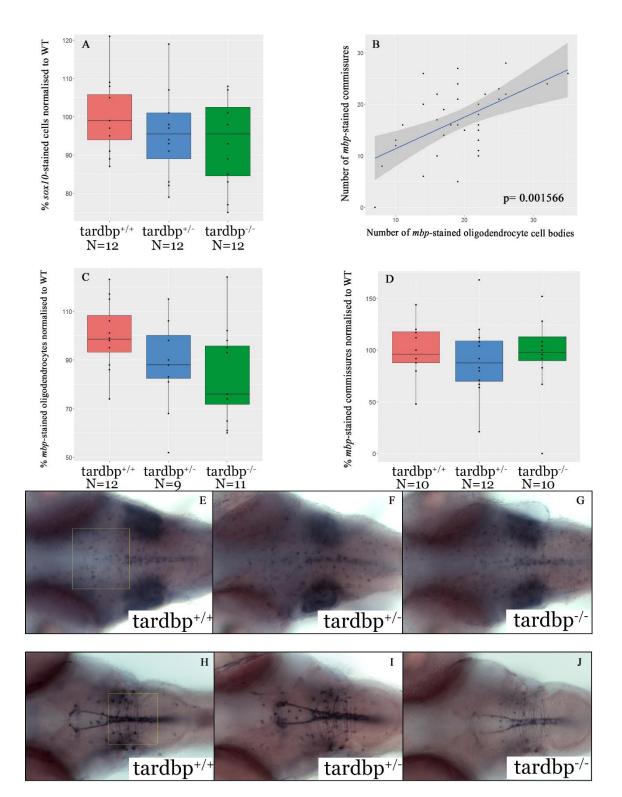
After counting *sox10*-positive cells in the area shown in **figure 6.1E** (**figure 6.1E-G**), a Kruskal-Wallis test showed that 3 dpf embryos do not differ in the amount of OPCs regardless of their *tardbp* genotype (KW p= 0.7109, **figure 6.1A**). This means that depletion of Tardbp does not lead to a loss of cells with an oligodendroglial fate at the 3 dpf stage.

#### 6.2.2 Counting of mbp-positive structures in 5 dpf zebrafish

The first myelin sheaths begin to appear between 4 and 5 dpf. We hypothesised that a lack of Tardbp may hinder myelination in zebrafish by impairing the transport of *mbp* mRNA to the oligodendrocyte's myelin compartment. Oligodendrocytes within the hindbrain's midline could not be counted due to poor contrast. For this analysis, only those oligodendrocytes that were lateral to the hindbrain's midline were counted.

*mbp*-positive oligodendrocyte cell bodies and axons were counted in the area shown by **figure 6.1H**. Due to the fact that multiple axons can be myelinated by a smaller number of oligodendrocytes, the number of oligodendrocyte cell bodies could potentially not have a direct effect on how many axons become myelinated. For this reason, we first analysed whether a higher number of oligodendrocyte cell bodies correlated with a greater number of myelinated axons in that area. A Kendall test encompassing all the study animals corroborated this (Kendall p= 0.001566. tau= 0.3846805), indicating that a smaller amount of Schwann cells is indeed correlated with less myelinated axons at this stage (**figure 6.1B**).

The next step was to ascertain whether Tardbp-deficient zebrafish presented a different overall number of oligodendrocyte cell bodies or axons. *tardbp*-/- and *tardbp*+/- zebrafish showed an average of 20% fewer Schwann cells than *tardbp*+/+. However, a Kruskal-Wallis test did not show statistical significance amongst the groups (K-W p= 0.06315, **figure 6.1C**). This result might be caused due to the low statistical power this analysis presents at  $\alpha$ = 0.05 (achieved power= 0.52). This indicates that a positive result will not be detected 48% of the time for this effect size at  $\alpha$ = 0.05, using this number of embryos. Regarding the number of myelinated axons, it was not any different from group to group (K-W p= 0.6117, **figure 6.1D**). It could be the case that the number of *mbp* mRNA-positive axons was the same, but that the actual amount of *mbp* mRNA present at the end of the oligodendrocytic processes varied from group to group. Qualitatively, we noticed that *tardbp*-/- fish presented a paler staining pattern than *tardbp*+/+ or *tardbp*+/- (**figure 6.1H-J**). To investigate this, a quantitative fluorescent technique could be used in a future experiment.



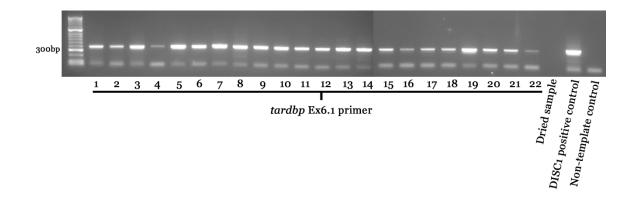
**Figure 6.1:** Whole-mount *in situ* hybridisation analysis of the expression of *sox10* and *mbp* mRNAs in zebrafish embryos. A: Relative number of *sox10*-positive cells compared to the average of the WT control group in 3 dpf embryos. B: Positive significant correlation between the numbers of oligodendrocytes and *mbp*-positive commissures. C, D: Intergroup comparison of the numbers of mature oligodendrocyte cell bodies and the number of commissures containing *mbp* mRNA. E-G: Representative images of *sox10* staining for the different *tardbp* fish genotypes at 3 dpf. H-J: Representative images of *mbp* staining for the different *tardbp* fish genotypes at 5 dpf. Yellow rectangles in E and H show the areas in which oligodendrocyte cell bodies and commissures were counted for each marker.

# 6.3 Producing zebrafish lines that express mutant Tardbp using CRISPR/Cas9

In the previous section, no reduction could be seen in the number of oligodendrocytes in *tardbp*-/- zebrafish. Therefore, we aimed to produce a line of zebrafish that included human TDP-43 exon 6 point mutations in well-conserved residues of the zebrafish *tardbp* gene using clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) technology (as detailed in the Materials and Methods section, **2.2.3**).

#### 6.3.1 Testing the target sequences for the presence of SNPs

Once the target sequences had been identified, we had to confirm that there were no SNPs in the G nucleotides of the PAM sequences, which are essential for Cas9 to cleave the DNA. To achieve this, 24 WT zebrafish embryos were raised for two days then genomic DNA extracted for PCR and sequencing of the target sequences. The PCR was conducted using the exon6 primers described in **section 2.2.3.2**. The PCR products were run on an agarose gel as shown in **figure 6.3**. Twenty-two of the samples showed a single product. These were purified using the ExoSAP protocol (**section 2.2.2.3**) and sequenced using the exon6 primers. The results indicated that there were no common SNPs in the target sequences or the p.A<sub>379</sub> $\rightarrow$ T PAM site. However, the p.G<sub>347</sub> $\rightarrow$ V PAM sequence contained the SNP (A/C)GG (**figure 6.4**). Due to the fact that the SNP does not affect the G nucleotides of the PAM site, it should not influence the efficiency of Cas9 cleavage.



**Figure 6.2:** *tardbp* exon 6 **SNP-screening PCR.** 1-22: PCR of the exon 6 of the zebrafish *tardbp* gene for 22 WT embryos. Sample 23 dried out during the PCR. Primers for the *DISC1* gene were used as a positive control, presenting a band at the right size. Non-template control shows no PCR product. All samples were run on the same gel with two running fronts, and for visualisation purposes, samples 15 onwards have been moved next to the rest.

B CAGAATCAGTCGGGTACTTC A **GGCAGCAGCTCAGCTGCTCT**CGG GGCAGCAGCTCAGCTGCTCTCGG CAGAATCAGTCGGGTACTT NGG CAGAATCAGTCGGGTACTTC **GGCAGCAGCTCAGCTGCTCT**CGG AGG CAGAATCAGTCGGGTACTTC **GGCAGCAGCTCAGCTGCTCT**CGG CAGAATCAGTCGGGTACTTC<mark>A</mark> **GGCAGCAGCTCAGCTGCTCT**CGG CAGAATCAGTCGGGTACTTCNGG GGCAGCAGCTCAGCTGCTCTCGG **GGCAGCAGCTCAGCTGCTCT**CGG CAGAATCAGTCGGGTACTTC GGCAGCAGCTCAGCTGCTCTCGG CAGAATCAGTCGGGTACTTC **GGCAGCAGCTCAGCTGCTCT**CGG CAGAATCAGTCGGGTACTTCAGG CAGAATCAGTCGGGTACTTC GGCAGCAGCTCAGCTGCTCTCGG GGCAGCAGCTCAGCTGCTCTCGG CAGAATCAGTCGGGTACTTCAGG **GGCAGCAGCTCAGCTGCTCT**CGG CAGAATCAGTCGGGTACTTC GG **GGCAGCAGCTCAGCTGCTCT**CGG CAGAATCAGTCGGGTACTTCNGG GGCAGCAGCTCAGCTGCTCTCGG CAGAATCAGTCGGGTACTTC GGCAGCAGCTCAGCTGCTCTCGG CAGAATCAGTCGGGTACTTC<mark>A</mark>GG GGCAGCAGCTCAGCTGCTCTCGG CAGAATCAGTCGGGTACTTC **GGCAGCAGCTCAGCTGCTCT**CGG CAGAATCAGTCGGGTACTTCNNG GGCAGCAGCTCAGCTGCTCT CAGAATCAGTCGGGTACTTCAGG CAGAATCAGTCGGGTACTTCNGG GGCAGCAGCTCAGCTGCTCTCGG CAGAATCAGTCGGGTACTTCAGG **GGCAGCAGCTCAGCTGCTCT**CGG **GGCAGCAGCTCAGCTGCTCT**CGG **CAGAATCAGTCGGGTACTTC**MGG AGAATCAGTCGGGTACTT GGCAGCAGCTCAGCTGCTCTCGG GG 

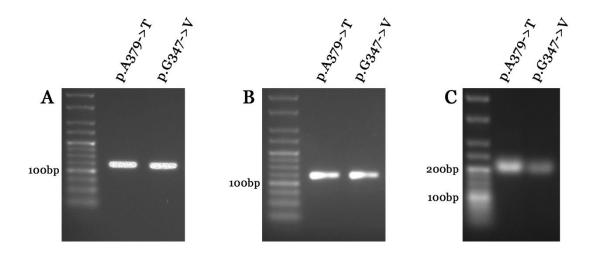
**Figure 6.3:** Alignment of the *tardbp* exon six target sequences. A: The target sequence containing the coding sequence for *tardbp*'s p.A379 (yellow) and its PAM site (grey) do not contain any SNPs, so the gRNA should easily bind to it, and Cas9 should nick the sequence before the PAM site. B: The target sequence containing the coding sequence for *tardbp*'s p.G347 (yellow) does not present any SNPs, and the PAM site (grey) contains an SNP in the first nucleotide. This nucleotide is allowed to be variable, so this should not present an impairment to the gRNA and Cas9 activities.

#### 6.3.2 Guide RNA synthesis

The guide RNAs need to be transcribed from a DNA template. These DNA templates need to include the protospacer sequence, the gRNA scaffold plus a T7 RNA transcription promoter for *in vitro* transcription. This promoter is not included in the gRNA because the T7 polymerase only transcribes what comes after the promoter.

The oligonucleotides (Integrated DNA Technologies) were subjected to PCR for rapid amplification using the T<sub>7</sub> primer and the gRNA primer. The product bands at 120bp can be seen in **figure 6.5A**. The bands were extracted from the gel and precipitated to purify the template DNA. **Figure 6.5B** shows the DNA bands after ethanol precipitation. The template DNA was next used for the production of the guide RNAs.

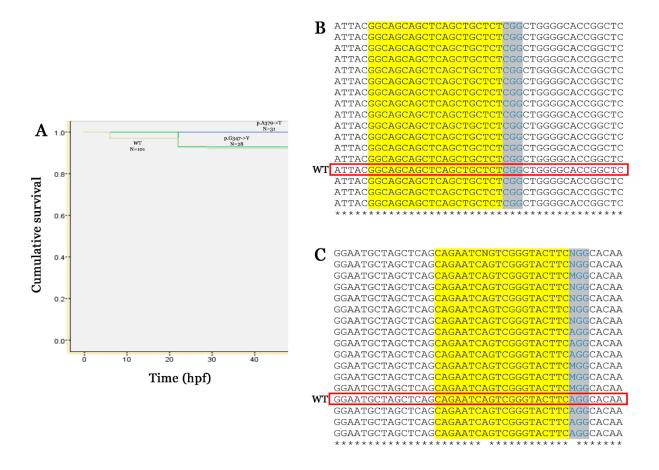
To obtain the guide RNAs, *in vitro* transcription with RNA polymerase was performed. The template DNA was then digested with DNAse, and the RNA molecules were then ethanol precipitated. **Figure 6.5C** shows the RNA products in a non-denaturing agarose gel. They ran slightly slower than similar-length double-stranded DNA due to the lower negative charge of single-stranded RNA of the same length, but the bands were clean and free of other nucleic acid residues. These guide RNAs were then ready for injection along with Cas9 protein.



**Figure 6.4: Synthesis of the gRNAs for Cas9 guidance.** A: PCR products resultant from using T7 and gRNA primers with the oligonucleotide. They both should be 120bp long, so the bands indicate a successful PCR. B: Gel-purified and ethanol precipitated PCR products. C: T7-transcription products using the DNA templates from B. The ladder is a DNA ladder and the RNA molecules, which should have a size of 100bp, run higher (slower) than their DNA counterparts.

### 6.4 Microinjection of zebrafish with gRNA/Cas9

We sought to test the efficiency of our gRNAs before designing donor sequences to introduce the desired mutations. WT zebrafish embryos at the 1-cell stage were microinjected in the yolk with 2 nl of solutions containing one of the gRNAs (1.6fold dilution), Cas9 protein (40 pg) and phenol red, while uninjected embryos served as controls. The embryos were grown for 51 hours and then culled in tricaine. Figure 6.6A shows the survival curve for each of the groups showing that the injected embryos survived normally compared to the controls. Genomic DNA was extracted from the gRNA-injected embryos to determine whether Cas9 had cut the tardbpa gene by amplifying the target sequence using PCR and sequencing the purified amplicon with custom primers directed at that region. The sequence should include indel mutations surrounding the target region if efficient mutagenesis has occurred because of error prone homology-directed repair. After PCR amplification using the Ex6.1 primers and sequencing the products using the forward Ex6.1 primer, no insertions or deletions could be observed beyond the target sequences (Figure 6.6B, C). The same results were found after multiple trials, indicating that our guide RNAs were not effective at all. The same Cas9 protein and phenol red solution were used by another person who was using a different gRNA on embryos from the same lay. The success of this person's injections indicated that there was not a problem with the Cas9 protein or the phenol red, and the lack of success of our injections was likely due to the inefficiency of our gRNA. gRNA inefficiency is very common, and it can be found in a number of studies (Xu et al., 2015, Doench et al., 2014, Moreno-Mateos et al., 2015).



**Figure 6.5: gRNA/Cas9 injection results.** A: The survival plot shows that none of the cohorts suffered a significant amount of death during the 51 hours the embryos were raised. B, C: Sequence alignment of segments of the PCR products obtained using Ex6.1 primers from embryos injected with the p.A379->T gRNA (B) and the p.G347->V gRNA (C). There is no evidence that Cas9 nicked the genomic DNA in these regions since the target sequence (yellow) and the PAM site (grey) remain intact, and no mutations or loss of sequence quality were observed anywhere near the target sequences. The reference sequence of these regions is marked WT and boxed in red for comparison.

#### 6.5 Discussion

To study the effects of *tardbp* dysfunction, we used *in situ* hybridisation of mRNA to characterise the development and maturation of oligodendrocytes in the Tardbp-lacking zebrafish model (Hewamadduma et al., 2013). Our results showed no overall differences in the numbers of OPCs or mature, myelinating oligodendrocytes. It is of importance to note that this Tardbp-lacking strain of zebrafish uses an alternatively spliced form of the *tardbpl* mRNA which provides a full-size Tardbp protein (Tardbpl) to counteract the loss of Tardbp. This could rescue any dysfunctions that could be caused by a lack of Tardbp in zebrafish. It will be of interest for future studies to assess myelination in double *Tardbp/Tardbpl* mutants and in embryos produced by breeding *Tardbpl* knock-outs and *Tardbp* knock-in mutants to ascertain whether a total loss of function or a toxic gain of function impair myelination.

Future work should address the amount of Mbp generated and compare it to that of Plp or other myelin proteins synthesised in the cell body. This can be achieved using both Western blotting and whole-mount fluorescent immunohistochemistry on embryos from the same lines discussed here. This could be done by staining embryos using fluorescently-tagged antibodies which would follow a 1:1 relationship between fluorescence intensity and protein concentration. As mentioned in **chapter 4**, C9ALS human post-mortem tissue does show a loss of MBP in the dorsolateral motor tracts and the dorsal sensory tracts of the spinal cord which is not accompanied by a loss of PLP in the same regions. Due to the wide variety of ALS-causing *TARDBP* mutations, a stable zebrafish line with specific human mutations in *Tardbp* is desirable to model the disease and understand the functions that TDP-43 plays in ALS

Therefore, the design and testing of guide RNAs for *tardbp* which was pursued as the first steps towards making *tardbp* mutant knock-in zebrafish, where translated protein would harbour human mutations in conserved residues. We investigated this

gene under the hypothesis that Tardbp is involved in the transport of Mbp, and that ALS-causing mutations in Tardbp impair MBP mRNA transport and correct axonal myelination by oligodendrocytes. The overall process to produce the guide RNA molecules to target Cas9 nuclease activity was successful, producing good purified RNA with no traces of RNA/DNA contaminants. Unfortunately, injection of neither of the guides resulted in mutations around the PAM site. This lack of effectiveness was likely due to one of two reasons: suboptimal methodology or the guide RNAs were not effective in targeting the genomic regions. The latter option is unlikely for one of the guides since very recently a group successfully created the p.A379->T model using the same gRNA sequence utilized in this study in the same zebrafish strain (Armstrong et al., 2016). However, they used a modified version of the Streptococcus pyogenes Cas9 mRNA containing a double nuclear localisation signal which has a 75-99% biallelic mutagenesis success rate (Jao et al., 2013). This mutant line will be of interest to investigate the effects of human TARDBP mutations in the zebrafish nervous system. In particular, it will display any toxic effects that may be caused by mutant Tdp-43 (gain of function), but any loss of function will be compensated by Tardbpl (Hewamadduma et al., 2013). Thus, this strain can help to address the loss of function/gain of function conundrum regarding mutations in TARDBP.

The zebrafish Tardbp protein shares significant homology with human TDP-43 including other amino acids which are also found mutated in ALS cases. The reason why we did not target other mutations is that there would have been no easy way to screen injected fish to detect the mutation. What made the selected sequences ideal for the later screening is the fact that they contained a restriction site that would be turned into a different restriction site after modifying the sequence. This would make it straightforward to genotype the fish using PCR amplification and restriction enzyme digests.

It is true that there are other available methods by which the desired directed mutagenesis could have been achieved. For example, Zinc-finger nucleases (ZFN) and Transcription activator-like effector nucleases (TALENS) are similar to the CRISPR/Cas9 system in that fact that they cause double-stranded breaks at specific points in the zebrafish genome. Both ZFN and TALENS rely on the engineering and screening of proteins, making them costly and very time-consuming and challenging. That added to the fact that engineered ZFN and TALENS would not guarantee mutagenesis even when they have demonstrated binding activity, made these systems unfeasible to use given the budget and the time scope for this project. However, the CRISPR/Cas9 system relies on the engineering and screening of gRNA sequences, reducing the costs and time scale considerably and making this approach much more desirable. Additionally, the efficiency of the CRISPR/Cas9 system has been shown to be superior to other nuclease-based systems, and obtaining new gRNA sequences in case the designed one do not work as desired can be done relatively quickly and inexpensively.

Another possible approach by which it would have been possible to obtain the desired mutations or even other ALS-causing mutations in *tardbp*, would have been the use of TILLING mutants. These are fish whose male progenitor has been subjected to a highly mutagenic agent before fertilisation. Those embryos constitute a living library and are posteriorly grown to breed and screened for single point mutations in the target gene (Wienholds et al., 2002). As an example, the *tardbp*<sup>-/-</sup> strain used in this study was generated and screened by TILLING (Hewamadduma et al., 2013). Two obvious disadvantages of this system are the possibility of introducing additional off-target mutations, and the fact that it needs to be done on a much larger scale than that needed for CRISPR/Cas9. High-throughput techniques have been generated to alleviate the latter (Draper et al., 2004), but the problem of the off-target effects still remains.

DISCUSSION

#### **CHAPTER 7**

### Discussion

#### 7.1 Neuronal research and glial research in ALS

The roles that different ALS-causing mutations have in the degeneration of neurones and glial cells still need to be fully understood. A vast number of studies have investigated neuronal pathology in ALS to date, finding commonalities in the pathology presented by different genetic mutations and in sporadic cases, such as the cytoplasmic deposition of phosphorylated TDP43 in the ventral horn motor neurones. Such studies have also differentiated distinct forms of neuronal pathology affecting specific areas of the brain of patients harbouring different ALS-causing genetic mutations. This has greatly helped with investigating the molecular mechanisms which underlie the different neuronal pathologies found in ALS caused by different mutations. Regarding glial cells, the mechanisms underlying glial involvement in ALS have been studied for about 20 years, when reactive astrogliosis was found in G85R SOD1 mice (Bruijn et al., 1997) and decreased kinetics of sodium-dependent glutamate transport was seen in ALS brains (Rothstein et al., 1992). Since then, the involvement of astrocytes and microglia in ALS has been studied thoroughly. However, oligodendrocytes were mostly ignored as mechanistic sources of neurodegeneration in ALS up until a few years ago (Lee et al., 2012, Kang et al., 2013, Philips et al., 2013), even though evidence for oligodendroglial involvement in ALS was presented almost 30 years ago (Yamada et al., 1990). That study showed the abundance of complementactivated oligodendrocytes in the motor cortex, brainstem and spinal cord of ALS patients compared to controls. These are oligodendrocytes which are labelled with antibodies for proteins of the complement system, suggesting that they are in the process of being opsonised in ALS tissue.

### 7.2 MBP shortage in the cervical spinal cord does not correlate with motor axon degeneration or with a general shortage of myelin proteins

In this doctoral investigation, neuropathological and mechanistic approaches were taken to ascertain to which extent oligodendrocytes contribute to the neuropathology seen in ALS. We determined that the levels of MBP are diminished in ALS patients, while PLP levels remain similar to controls in the lateral corticospinal tracts. It seems, therefore, that there might be a selective reduction of MBP in the motor areas of the spinal cord white matter in ALS. The fact that this reduction in MBP does not correspond to a reduction of the axonal density in the corticospinal motor tracts suggests that axonal degeneration is not the cause of oligodendroglial degeneration in the lateral corticospinal tracts of the spinal cord. Previous studies revealed not only oligodendroglial dysfunction in ALS using human tissue (Lee et al., 2012, Kang et al., 2013), but also some molecular mechanisms underlying the dysfunction using G93A SOD1 mice (Lee et al., 2012, Kang et al., 2013, Philips et al., 2013). Some of these studies show a reduction in monocarboxylate transporter 1 (MCT1) in ALS patient tissue and in SOD1 mice, which indicates that oligodendrocytes are not providing enough metabolic support to the neighbouring axons (Lee et al., 2012, Philips et al., 2013). Additionally, the oligodendroglial turnover rate was abnormally high in ALS patients' brains, which was accompanied by demyelination in the grey matter of the motor cortex and the ventral horns of the spinal cord of ALS patients (Kang et al., 2013). In the same study, a substantial reduction in MBP was demonstrated

in these two regions, which was not shown for 2',3'-Cyclic-nucleotide 3'phosphodiesterase (CNPase), a myelin protein present everywhere in the cells.

Some of the pathological studies in human *post mortem* tissue presented in this thesis could benefit, however, from the inclusion of a larger pool of tissue pertaining to neurologically healthy controls and sporadic cases of ALS. This is especially relevant for the Western blot and qPCR studies, where it would be desirable to obtain data for a larger number of cases from all the cohorts to increase confidence in the data and the statistical power. However, some of the large changes observed between the cohorts for the MBP/PLP ratio suggest an mRNA-transport-dependent myelin loss in the motor tracts of C9ALS cases is likely. Also, the generalised loss of both MBP and PLP in the dorsal column -an extramotor region- in either ALS group compared to controls, provides further evidence of general oligodendroglial dysfunction in ALS. If this was indeed the case for the rest of the brain, the fact that ALS affects mainly the body's motor function could be due to the fact that motor neurones are very energy-demanding compared to smaller neurones. It would have been of interest to study whether this reduction in the dorsal columns was accompanied by a loss of axonal fibres in the same area since secondary sensory neurones also extend over long distances.

### 7.3 The aggregation of C9ALS-related dipeptide repeats and TDP-43 cannot explain all the glial protein inclusion pathology in C9ALS brains

We also showed that glial cells have a distinct pathology in some relevant regions of the frontal lobe such as the precentral gyrus and the prefrontal cortex in C9ALS compared to sporadic cases of ALS. There is a large number of p62-positive protein aggregates which cannot be explained by just C-terminal pTDP43 or DPR

aggregation in our C9ALS cohort. RNA-Binding Motif Protein 45 (RBM45) (Collins et al., 2012), Rho guanine nucleotide exchange factor (RGNEF) (Erschbamer et al., 2005) and N-terminal TDP43 seem like good candidates for proteins which could potentially be part of those inclusions. It would be advisable to perform further immunohistochemical studies to elucidate whether they actually are part of the inclusion bodies, including co-localisation studies where aggregation of p62-labelled possible. Cvtoplasmic protein in some neurodegenerative diseases has also been related to a dysfunction of nucleocytoplasmic transport, especially that of TDP43 in most forms of ALS: Molecules larger than ~40 kDa-this includes TDP43 (43 kDa) - need active transport to shuttle into the nucleus through the nuclear pore complex (NPC) (Rout and Aitchison, 2000). The proteins which form the NPCs are extremely long-lived (Savas et al., 2012) and they assemble in an elastic network after mitosis, with a very low turnover rate in non-dividing cells (Daigle et al., 2001, D'Angelo et al., 2009), such as neurones and oligodendrocytes. With ageing, NPCs deteriorate, and this might lead to the cytoplasmic aggregation of proteins which normally shuttle between the nucleus and the cytoplasm (D'Angelo et al., 2009). Irregularities in the nuclear membrane shape and mislocalisation of nuclear transporters were found in sALS and SOD1 fALS cases (Kinoshita et al., 2009). However, this alone does not explain why aggregated proteins are mostly specifically located to the cytoplasm. To address this, it is important to consider that shuttling into the nucleus through the NPCs is only possible through the interaction of importin proteins with the nuclear localisation signal (NLS) found in karyophilic proteins. This interaction is tightly regulated by the local concentration of GTP-bound Ran-GTPase (Ran-GTP) in the cytoplasm, which disrupts the binding of importins to their cargo. For Ran-GTP to hydrolyse GTP to GDP and form Ran-GDP -which does not disrupt importin-cargo interactions-, it needs first to bind Ran-GTPase activating protein 1 (RanGAP1) (Freitas and Cunha, 2009). This means that a disruption of the Ran-GTP/RanGAP1 interaction in the cytoplasm would result in the impairment of the nuclear import of importin-dependent molecules. Interestingly, a recent study found that RanGAP1 accumulates with expanded C9orf72 mRNA in post mortem brain tissue, induced pluripotent stem cell (iPSC)-derived neurones from C9ALS patients, and expansion-expressing Drosophila cells (Zhang et al., 2015). These authors also claim to hold unpublished evidence of RanGAP1 mislocalisation in brain tissue of sALS patients (Zhang et al., 2016a). A decrease of 50% in protein import and the mislocalisation of Ran-GTP to the cytoplasm was also found in iPSC-derived neurones, along with the cytoplasmic aggregation of NPC scaffolding proteins (Zhang et al., 2015). This is highly relevant to the observed mislocalisation of TDP43 in ALS, which was observed to happen later than nucleocytoplasmic transport deficiency in iPSC-derived neurones (Zhang et al., 2015). This also explains why most ventral horn neurones present abnormalities in the nuclear envelope shape, even those without TDP43 aggregation (Kinoshita et al., 2009). Additionally, TDP43 mislocalisation to the cytoplasm coincided with Ran-GTP cytoplasmic mislocalisation in the hepatic tsBN2 cell line (Winton et al., 2008). All this evidence strongly suggests that the disruption of nucleocytoplasmic transport is one of the main causes of protein cytoplasmic mislocalisation and aggregation in ALS. Therefore, it would be useful for our study to use immunohistochemistry to determine whether the p62-positive and TDP43 inclusions found in the cortical and subcortical oligodendrocytes of C9ALS and sALS cases also contain RanGAP or other proteins related to nuclear transport. This could potentially affect other similar proteins such as FUS, which is also rapidly shuttled between the nucleus and the cytoplasm (Zinszner et al., 1997). FUS aggregation has been observed not only in ALS cases bearing FUS 242

mutations (Vance et al., 2009, Huang et al., 2010), but also in sALS and non-SOD1 fALS (Keller et al., 2012, Deng et al., 2010). Some of the reports showed no trace of FUS aggregation in sALS cases (Neumann et al., 2009, Vance et al., 2009) but this may have been due to a very small number of sALS cases in their study (N = 2 and N not mentioned respectively). TDP43 and FUS are both part of transport granules (Fujii et al., 2005, Freibaum et al., 2010). Therefore, disruption of their normal function due to their cytoplasmic aggregation may have an impact on mRNA processing and transport, which is crucial for the synthesis of MBP and the subsequent formation of myelin. Our results are consistent with this hypothesis, but in vivo experiments should be carried out to study the development of MBP loss in sALS and C9ALS and establish whether there is a cause-effect relationship between the observed protein aggregation and defects in mRNA transport. A recent study found that iPSC-derived neurones from C9ALS patients harbouring 600-1600 GGGGCC repeats present impaired mRNA transport in the dendrites along with dendrite branching defects (Burguete et al., 2015). Furthermore, they also show evidence of the GGGGCCcontaining mRNA being itself transported and localised to the dendrites. It would also be relevant to optimise the *in situ* hybridisation for *MBP* mRNA attempted here, to clarify whether the glial pathology is accompanied by the impaired transport of it.

One promising reported model that could help understand how the glial and neuronal aggregates found in C9ALS develop is the mouse line expressing (GGGGCC)<sub>66</sub> by adeno associated virus. This strain replicates the human TDP43, DPR and RNA foci pathology of C9ALS, which other models focusing on specific pathogenic mechanisms of mutant *C9orf72* have failed to show (Chew et al., 2015). Other interesting models are the bacterial artificial chromosome (BAC)- transgenic mice that also express GGGGCC expansions, but at a relatively lower level (O'Rourke et al., 2015, Peters et al., 2015, Liu et al., 2016). These models contain an artificial gene with 100 to 1000 GGGGCC repeats, and they all replicate the pathology found in C9ALS, including *C9orf72* mRNA foci and DPR inclusions. However, two of these models do not present neurodegeneration or p62 and TDP-43 pathology as seen in the patients' brains (O'Rourke et al., 2015, Peters et al., 2015), while the other does (Liu et al., 2016). It would be interesting to use these models in future studies in order to study oligodendroglial dysfunction and demyelination in C9ALS and to test new drugs which may alleviate the pathology shown by cell models of C9ALS. An example of this can be found in the Huntington's disease zQ175 mouse model whose neurones show disruption of the nucleocytoplasmic transport, caused by mutant Huntingtin expression of RAN-translated proteins (Grima et al., 2017).

# 7.4 iOligodendrocytes will be useful to understand oligodendroglial dysfunction in different types of ALS

Unfortunately, sporadic ALS does not have a generalised model. However, in the last decade, the induction of patient-derived fibroblasts directly into motor neurones (iMotor Neurones) and glia (iAstrocytes, iOligodendrocytes) has been developed and used to investigate neurodegenerative diseases (reviewed in Myszczynska and Ferraiuolo, 2016). This has allowed for the production of neuronal and glial models with the exact genome ALS patients have without artificial genetic alterations which could affect the expression of endogenous genes. Also, an advantage of these directly induced neurones and glia over those derived from human induced pluripotent stem cells is the fact that they do not lose the age-dependent signatures which are lost in the latter (Mertens et al., 2015). Directly-induced neurones and glia from ALS patients will likely provide scientists with the possibility of investigating the pathology and mechanisms of degeneration unique to individual patients while they are still alive, resulting in personalised drug screening and therapies. Being able to use the patients' own cells as models of their own disease, researchers may find in these *in vitro* models a way to understand the mechanisms underlying sALS. Related to the study presented in this thesis, iOligodendrocytes from different types of familial ALS and from sporadic ALS have shown a reduced secretion of lactate compared to those derived from controls (Ferraiuolo et al., 2016). Interestingly, this was not observed for iOligodendrocytes from patients harbouring the *C90rf72* mutation, which secreted as much lactate as those from controls. These authors' results also suggest that oligodendrocytes from different ALS origins are toxic to motor neurones using a common mechanism that involves SOD1 and a lower amount of lactate production. However, this was not seen for the C9ALS group, suggesting that the toxicity oligodendrocytes exert has a different underlying mechanism in these cases.

## 7.5 The limitations of human *post mortem* studies can be overcome by the use of *in vivo* models

Future studies will need to perform *in vivo* studies in ALS models to overcome the limitations that *post mortem* studies offer. Human *post mortem* tissue provides a great window into the disease pathology at the time of death but is weak at providing details about the development of that pathology. However, applying different techniques on *post mortem* brains can determine genomic and proteomic targets to investigate further using *in vivo* models such as mice, zebrafish, or drosophila. In the case presented here, it would be ideal to ascertain the molecular pathways which lead to the observed oligodendroglial pathology and the motor tract loss of MBP. Here, we intended to accomplish this by producing transgenic and mutation-carrying zebrafish lines.

One of those strains would have expressed *C9orf72* expansion mRNA specifically in oligodendrocytes, using a GFP reporter to indicate transgenesis. Unfortunately, this was unsuccessful, and it would be desirable that it is retried in the future using positive control fluorescence-inducing Gateway constructs to troubleshoot potential problems and mistakes. The other two strains were intended to harbour point mutations that cause ALS in humans, in conserved amino acids of the Tardbp protein. CRISPR/Cas9 technology was used to achieve this, but the choice of gRNA did not seem to trigger the nuclease activity of Cas9 upon the desired sequence.

If these zebrafish lines were made in the future, there is the possibility that *tardbp* mutants will be rescued by their non-mutant *tardbp-like* (*tardbpl*) gene. *tardbpl's* mRNA undergoes alternative splicing in response in *tardbp-/* fish (Hewamadduma et al., 2013, Schmid et al., 2013), producing a larger Tardbpl protein which mimics Tardbp. Double knock-outs (*tardbp-/*;*tardbpl-/*), however, show motor neurone and axonal defects and vascular dysfunction, unlike single knock-outs of either gene (Hewamadduma et al., 2013, Schmid et al., 2013, Schmid et al., 2013). These two studies are based on nonsense mutations which introduce a stop codon prematurely in the mRNA reading frame, rendering dysfunctional truncated proteins. It would be of interest to see how missense mutations in Tardbp, which cause ALS in humans when present in TDP-43, affect the splicing pattern of *tardbpl* in zebrafish. A promising strain to study one of the Tardbp mutations was recently produced using CRISPR/Cas9 (Armstrong et al., 2016), although their study did not look into the matter any further. This is due to the fact that the new line needs to be out-crossed for a few generations to eliminate any off-target

mutations, usually caused by the high homology of the gRNA's target sequence with other regions of the genome.

The use of the CRISPR/Cas9 system for the editing of genomic DNA has increased exponentially. This is due to the fact that it presents clear advantages over other similar methods such as Transcription activator-like effector nucleases (TALENS) and Zinc finger nucleases (ZFN), which rely on protein-DNA interactions rather than RNA-DNA interactions. The production of specific proteins is much more costly than producing specific gRNAs to target the desired sequence, and it makes it easier to introduce multiple mutations at once. A few years ago, this was demonstrated by mutating five different genes with single injections in embryonic stem cells using five specific gRNAs and mRNA encoding Cas9 (Wang et al., 2013). In 10% of cells, all alleles for the five genes were mutated together, which shows just how efficient the system is.

#### 7.6 Final remarks

The pathology data presented here certainly provides evidence that mRNA transport disruption should be studied thoroughly in oligodendrocytes as a possible consequence of protein sequestration and aggregation, and a cause of ALS. Future studies should address whether TDP-43 is part of the *MBP* mRNA transport granules and whether the interaction of hnRNP-A2 and TDP-43 and/or expanded *C90rf72* mRNA is detrimental to the transport of hnRNP-A2-dependent granules and to MBP synthesis.

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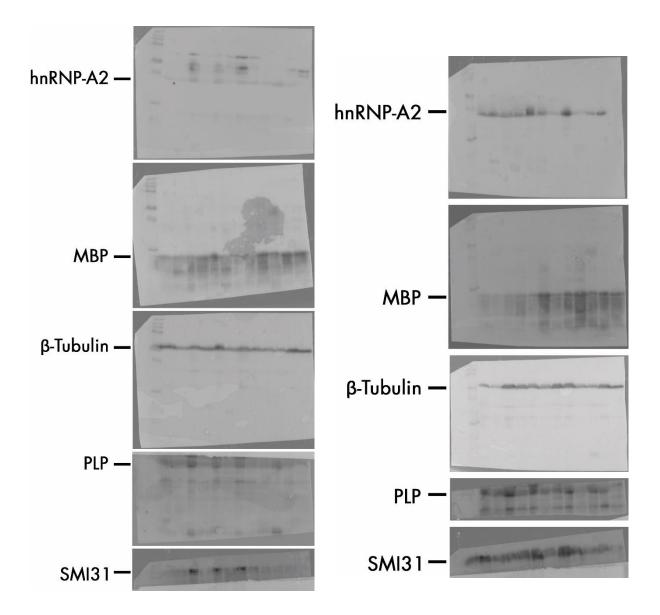
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## **APPENDIX 1**



## Full membrane images of the Western blot membranes

These are the overlay images of the membranes used for the densitometries described in section 2.1.3.5. The black lines indicate the height at which the protein levels were measured. Any artifacts caused by the contact of the containing plastic bag were not visible in the analysed images. The images on the **left** and the **right** correspond to the **dorsal column** and **dorsolateral motor tract** samples respectively. As explained in section 2.1.3.4, the three first proteins were probed in ascendant order of abundance (hnRNP-A2 $\rightarrow$ MBP $\rightarrow$  $\beta$ -Tubulin) to avoid photographic saturation of the images for less-abundant proteins. For PLP and SMI31, the membrane was cut because their antibody had been raised in the same species animal as one of the other three, which would cause cross-reactivity of the secondary antibodies. The lanes, from left to right, belong to the following cohorts in both membranes: 1: Size marker; 2-5: C9ALS; 6-9: sALS; 10-13: controls.