

The protective function of the oxidation resistance 1 gene in ALS

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Abstract

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that is characterized by the selective degeneration of motor neurons in the brain and spinal cord, resulting in muscle wasting, paralysis and eventually death usually within 2-5 years of symptom onset. Despite decades of intense research to ultimately find a cure, there are still only a few treatments available to patients, none of which are effective in slowing disease progression. Therefore, novel therapeutic strategies will be increasingly important to alleviate this health burden. While the underlying pathogenic mechanisms remain unclear, increasing evidence suggests that oxidative stress (OS) is a key contributory factor early in ALS pathology. Therefore, understanding the role of proteins involved in antioxidant defence networks will facilitate the development of new therapeutic approaches. Oxidation resistance 1 (OXR1) has emerged as an essential antioxidant protein that regulates neuronal survival in response to OS, however its precise molecular functions remain unclear.

Thus, the overall aim of this thesis was to investigate the neuroprotective function of Oxr1 in motor neurons, particularly focusing on its role in modulating disease-relevant phenotypes observed in ALS. To ascertain the importance of Oxr1 to motor neuron function and survival, mice in which *Oxr1* was conditionally deleted in motor neurons were behaviourally and pathologically characterised. My data indicates that loss of Oxr1 specifically in motor neurons does not increase their susceptibility to degeneration or cause motor dysfunction in aged mice, but can induce a local neuroinflammatory response in the spinal cord. Next, to investigate the neuroprotective function of Oxr1 in ALS caused by mutations in TAR DNA-binding protein 43 (TDP-43), longitudinal behavioural and pathological analysis was carried out on a novel mutant TDP-43 mouse model of ALS in the presence or absence of increased Oxr1 expression. I demonstrate that neuronal overexpression of Oxr1 protects against motor dysfunction and neuromuscular defects in TDP-43 mutant mice. Furthermore, using primary motor neurons from TDP-43 mutant mice, I show that overexpression of Oxr1 can reduce the mislocalisation of TDP-43 protein, which is a characteristic feature in more than 95% of ALS cases. Finally, in order to begin to determine whether endogenous Oxr1 expression can be manipulated as a therapeutic strategy, I investigated its transcriptional regulation by characterizing Oxr1 promoter regions. I identified a highly conserved functional promoter region of Oxr1 that was subsequently used in establishing a new cell-based luciferase reporter assay that can be employed to identify compounds that upregulate Oxr1. Taken together, these studies deepen our understanding of Oxr1 as a neuroprotective antioxidant protein and offers new insights into its potential use as a future therapeutic target for the treatment of ALS.

Declaration

The work presented in this thesis was performed in the Department of Physiology, Anatomy and Genetics, University of Oxford, from 2014-2018. This work was funded by the Motor neuron disease association (MND), Medical Research Council (MRC) and the European Research Council (ERC). All work presented was conducted by the author (Matthew Grantley Williamson) unless otherwise stated, and no part of this thesis has been submitted for any other degree in this or any other university or institute of learning.



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*I dedicate this thesis to my parents
Daniel and Lee Williamson,
Without whom none of my success would be possible*

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Abbreviations

2H3: Neurofilament (NF-M)	CD68: Cluster of differentiation 68
4-HNE: 4-hydroxynonenal	CDKN2a: Cyclin-dependent kinase inhibitor 2A
8-OHdG: 8-hydroxy-2'-deoxyguanosine	CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
α-BTX: α -Bungarotoxin	ChAT: Choline acetyltransferase
Abra: Muscle Activator of Rho Signalling	CHCHD10: Coiled-coil-helix-coiled-coil-helix domain-containing protein 10
AChRs: Acetylcholine receptors	ChIP: Chromatin immunoprecipitation
AD: Alzheimer's disease	ChIP-seq: Chromatin immunoprecipitation sequencing
ALS: Amyotrophic lateral sclerosis	CNS: Central nervous system
ALS-FTD: ALS-FTD spectrum disorder.	CNTF: Ciliary neurotrophic factor
ANG: Angiogenin	CRAL: Crotonaldehyde-lysine
ARE: Antioxidant-response element	CRISPR: Clustered regularly interspaced short palindromic repeats
ATP: Adenosine triphosphate	CSF: Cerebrospinal fluid
ATXN2: Ataxin 2	DAT: Dopamine transporter
BAC: Bacterial artificial chromosome	DCTN1: Dynactin 1
BBB: Blood brain barrier	DIV: Day in vitro
BDNF: Brain derived neurotrophic factor	DJ-1: Parkinson protein 7
BSA: Bovine serum albumin	DMD: Duchenne muscular dystrophy
C/EBPB: CCAAT/Enhancer Binding Protein Beta	DMSO: Dimethyl sulfoxide
C9ORF72: Chromosome 9 open reading frame 72	DNAJC19: DnaJ homolog, subfamily C, member 19 (also known as HSP40)
C20ORF118: Chromosome 20 open reading frame 118	Dox: Doxycycline
CA: Cornu ammonis	DPR: Dipeptide repeat
CCT2: Chaperonin containing TCP1 subunit 2	DRP1: Dynamin-related protein 1
CCT6A: Chaperonin containing TCP1 subunit 6A	dRTA: Distal renal tubular acidosis
CCT7: Chaperonin containing TCP1 subunit 7	DTT: Dithiothreitol

E: Embryonic day	GATA1/2/3: GATA binding protein 1/2/3,
EAAT2: Excitatory amino acid transporter 2	GC: Granule cell
EDL: Extensor digitorum longus	GDNF: Glial derived Neurotrophic Factor
EIF2: Eukaryotic initiation factor 2	GFAP: Glial fibrillary acidic protein
ELF-1: Ets-related transcription factor	GFP: Green fluorescent protein
ENU: N-ethyl-N-nitrosourea	CNF: Centrally nucleated fibre
EphA4: Ephrin receptor tyrosine kinase	GPI: Glucose-6-phosphate isomerase
ER: Endoplasmic reticulum	GPX: Glutathione peroxidase
ERAD: ER-associated degradation pathway	GPX2: Glutathione peroxidase 2
ETC: Electron transport chain	GPX4: Glutathione peroxidase 4
ETS-1: Ets Proto-Oncogene 1	GRAM: Glucosyltransferases, Rab-like GTPase activators and Myotubularins
FACS: Fluorescent activated cell sorting	GRIK4: Glutamate receptor ionotropic kainate 4
fALS: Familial amyotrophic lateral sclerosis	GSH: Glutathione
FDA: Food and Drug Administration	GSHe: GSH monoethyl ester
FIG4: Polyphosphoinositide phosphatase	H&E: Haematoxylin and eosin
Flp: Flippase	H₂O₂: Hydrogen peroxide
FLuc: Firefly luciferase	HD: Huntington's disease
FMN: Facial motor nucleus	HDAC6: Histone Deacetylase 6
FOXL1: Forkhead box L1	HeLa: Negroid cervix epitheloid carcinoma
FOXM1: Forkhead Box M1,	HNEH: 4-hydroxy-2-nonenal-histidine
FOXO3: Forkhead Box O3,	hnRNPA1: Heterogeneous nuclear ribonucleoprotein A1
FRDA: Friedreich's ataxia	hnRNPs: Heterogeneous ribonucleoproteins
FRT: Flippase recombinase target	HO-1: Heme-oxygenase 1
FTD: Frontotemporal dementia	HRP: Horseradish peroxidase
FUS: Fused in sarcoma	HSF-1: Heat shock factor protein 1
FXN: Frataxin gene	HSP: Heat shock protein
G3BP1: GTPase Activating Protein (SH3 Domain) Binding Protein 1	
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase	

IBA1: Ionized calcium-binding adapter molecule 1

IHC: Immunohistochemistry

IL-6: Interleukin-6

IP: Immunoprecipitation

iPSCs: Induced pluripotent stem cells

ISGF-3: Interferon-Stimulated Gene Factor 3 Gamma,

JNK: c-Jun N-terminal kinase

kDa: Kilodaltons

KIAA1609: TBC/LysM-Associated Domain-Containing Protein 1

L: Lumbar region

LCM: Laser capture microdissection

LHX3: LIM Homeobox 3,

LMD-3: LysM Domain (Peptidoglycan binding) protein 3 (*C. elegans* OXR1 homologue)

LysM: Lysin motif

MATR3: Matrin 3

MIO-M1: Human retinal Müller glial cell line

miR: MicroRNA

MLS: Mitochondrial localisation signal

MNGM: Motor neuron growth medium

mPrnp: Mouse prion protein promoter

MSCs: Mesenchymal stem cells

mtd: Mustard (ortholog of OXR1 in *Drosophila*)

mTOR: Mechanistic target of rapamycin

N2a: Neuroblastoma 2a cell line

NADPH: Reduced nicotinamide adenine dinucleotide phosphate

NCOA7: Nuclear receptor coactivator 7

NCOA7-B: Short isoform of NCOA7

NCOA7-FL: Full length isoform of NCOA7

NEF: Neurofilament heavy chain

NFATc/p/x: Nuclear Factor of Activated T-cells cytoplasmic 1/2/3,

NFκB: Nuclear factor-κ B

Nkx2.5: NK2 homeobox 5

NLS: Nuclear localisation signal

NMJ: Neuromuscular junction

NO: Nitric oxide

NQO1: NAD(P)H dehydrogenase, quinone 1

NRF2: Nuclear factor E2-related factor 2

NT-3: Neurotrophin-3

NTg: Non-transgenic

O₂^{·-}: Superoxide anion

[·]OH: Hydroxyl radical

OM: Mitochondrial outer membrane

ONOO⁻: Peroxynitrite

OPTN: Optineurin

OS: Oxidative stress

OTX2: Orthodenticle homeobox 2

OXR1: Oxidation resistance 1

OXR1-C: Short isoform of Oxr1

OXR1-FL: Full length isoform of Oxr1

P: Postnatal day

p21: Cyclin dependent kinase inhibitor 1A

PBS: Phosphate buffered saline

PBX1: Pre-B-cell leukemia homeobox 1

PD: Parkinson's disease

p-DRP1: Phospho-dynamain-related protein 1	STAT3: Signal transducer and activator of transcription 3
PET: Positron emission tomography	SV2: Synaptic vesicle protein 2
PFA: Paraformaldehyde	TA: Tibialis anterior
PFN1: Profilin 1	TBC: Tre-2/Bub2/Cdc16
PLD5: Phospholipase D family member 5	TBC1D24: TBC1 domain family, member 24
POU1F1: Pou Class 1 Homeobox 1	TBK1: Serine/threonine protein kinase TBK1
PPP: Pentose phosphate pathway	TDP-43: Transactive response DNA binding protein 43 kDa
PRDX2: Peroxiredoxin 2	Tet: Tetracycline
PRDX4: Peroxiredoxin 4	TFBS: Transcription factor binding sites
PTC-124: {3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl] benzoic acid}	Thy1.2: Thy-1.2 cell surface antigen
qRT-PCR: Quantitative real-time polymerase chain reaction	TLDC: TBC LysM domain catalytic domain
R26RlacZ: Rosa26 locus lacZ reporter	Tm1a: Knockout-first conditional allele
RAN: Repeat-associated, non-ATG initiated	Tm1c: Conditional allele
Rluc: Renilla luciferase	Tm1d: Constitutive knockout allele
ROS: Reactive oxygen species	TNF-α: Tumor necrosis factor alpha
RRM: RNA-recognition motif	TRE: tTA-activated promoter
RT: Room temperature	tTA: Tet-controlled transactivator
RXRA: Retinoid X Receptor Alpha	TUBA4A: Tubulin- α 4A
sALS: Sporadic amyotrophic lateral sclerosis	UBQLN2: Ubiquilin 2
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis	UPR: Unfolded protein response
SMI32: Neurofilament H non-phosphorylated	UPS: Ubiquitin proteasome system
SOD1: Superoxide dismutase 1	UV: Ultra violet
SOD2: Superoxide dismutase 2	VAPB: Vesicle-Associated Membrane Protein-Associated Protein B
SP1: Specificity Protein 1	V-ATPase: Vacuolar proton pumping ATPase
SQSTM1: Sequestosome 1	VCP: Valosin-containing protein

Chapter 1

Introduction

1.1 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common form of adult-onset motor neuron disease and the third most common neurodegenerative disease (Renton, Chiò and Traynor 2014). It is characterised by the progressive loss of upper motor neurons that project from the cortex to the brainstem and spinal cord, and lower motor neurons that project from the brainstem and spinal cord to muscle (Figure 1.1A; Kiernan et al. 2011). As lower motor neurons degenerate, skeletal muscles show features of denervation and re-innervation, with fibre type grouping and clusters of angular atrophic fibres (“*amyotrophic*”). Additionally, degeneration of the spinal processes of upper motor neurons results in scarring in the lateral tracts of the spinal cord (“*lateral sclerosis*”; Hardiman et al. 2017). Microscopic examination of motor neurons usually reveals the cytoplasmic accumulation of rounded or thread-like deposits of aggregated proteins, which are often ubiquitinated (Blokhuys *et al.* 2013). In addition to these findings in motor neurons, there is also abundant evidence of relevant pathology in non-neural cell types, such as astrocytes and microglia (Liu and Wang 2017).

The initial presentation of the disease partly depends on which motor neurons are primarily affected and in what order. Some patients initially present with symptoms such as spasticity and muscle weakness, which is predominantly caused by upper motor neuron degeneration with the involvement of lower motor neurons only becoming evident at later stages of the disease. Conversely, other patients present symptoms such as fasciculation, cramp and muscle wasting, which is predominantly caused by lower motor neuron degeneration (Kiernan *et al.* 2011; Hardiman *et al.* 2017). The focality of symptom onset has also been used to further stratify the subtype of ALS. Spinal-onset ALS, characterised by

muscle weakness of the limbs, accounts for ~60% of cases. However, ~30% of cases initially present with bulbar-onset, which is characterised by dysarthria (difficulty with speech) and dysphagia (difficulty swallowing). Cognitive-onset and respiratory-onset is also observed and each account for ~5% of ALS cases (Bäumer, Talbot and Turner 2014). Ultimately, all types of disease onset in ALS lead to one final outcome - progressive muscle wasting due to denervation and degeneration of the neuromuscular junction (NMJ), paralysis and eventually death usually within 2-5 years (Andersen and Al-Chalabi 2011). Preceding death, there is a near total loss of respiratory function, as well as a loss of the ability to chew, swallow and speak (Turner and Swash, 2015).

Although the primary symptoms of ALS are associated with motor dysfunction, up to 50% of patients develop cognitive and/or behavioural impairment (Phukan *et al.* 2012; Elamin *et al.* 2013). Furthermore, ~15% of patients with ALS are concomitantly diagnosed with frontotemporal dementia (FTD), a condition of impaired judgment and executive skills, which often leads to behavioural disturbances and dementia (Phukan *et al.* 2012; Elamin *et al.* 2013). Indeed, some patients with ALS show signs of extra-motor cerebral pathology in the prefrontal, frontal and temporal cortices, parts of the brain responsible for the expression of thought, planning, personality and speech (Figure 1.1B; Neumann *et al.* 2006; Arai *et al.* 2006; Forman *et al.* 2006). Therefore, it is no longer justifiable to consider ALS as a disease restricted to the motor system. Instead, ALS and FTD are located on opposite ends of a disease spectrum.

In Europe, the incidence of ALS is 3.0 per 100,000 and 2.4 per 100,000 for men and women, respectively (Beghi *et al.* 2006; Logroscino *et al.* 2010). Like all neurodegenerative diseases, the biggest risk factor for ALS is age. The risk of ALS significantly increases after the age of 40 years and peaks at 65 years, before a decline in incidence occurs (Logroscino *et al.* 2010). As we are considered an aging population, it is argued that the number of ALS

cases will increase in the future. This theory is supported by the increasing incidence of ALS in countries such as Sweden, France and USA (Stallones *et al.* 1989; Neilson *et al.* 1994; Fang *et al.* 2009; Neilson, Gunnarsson and Robinson 2009). However, more research is needed to exclude alternative explanations such as increasing awareness of ALS in the general public or better diagnostic criteria. Nonetheless, it will be important in the future for international consortia to collect data from a wider demographic, across multiple continents to fully understand the causative role of ancestry, genetics and environmental factors in ALS.

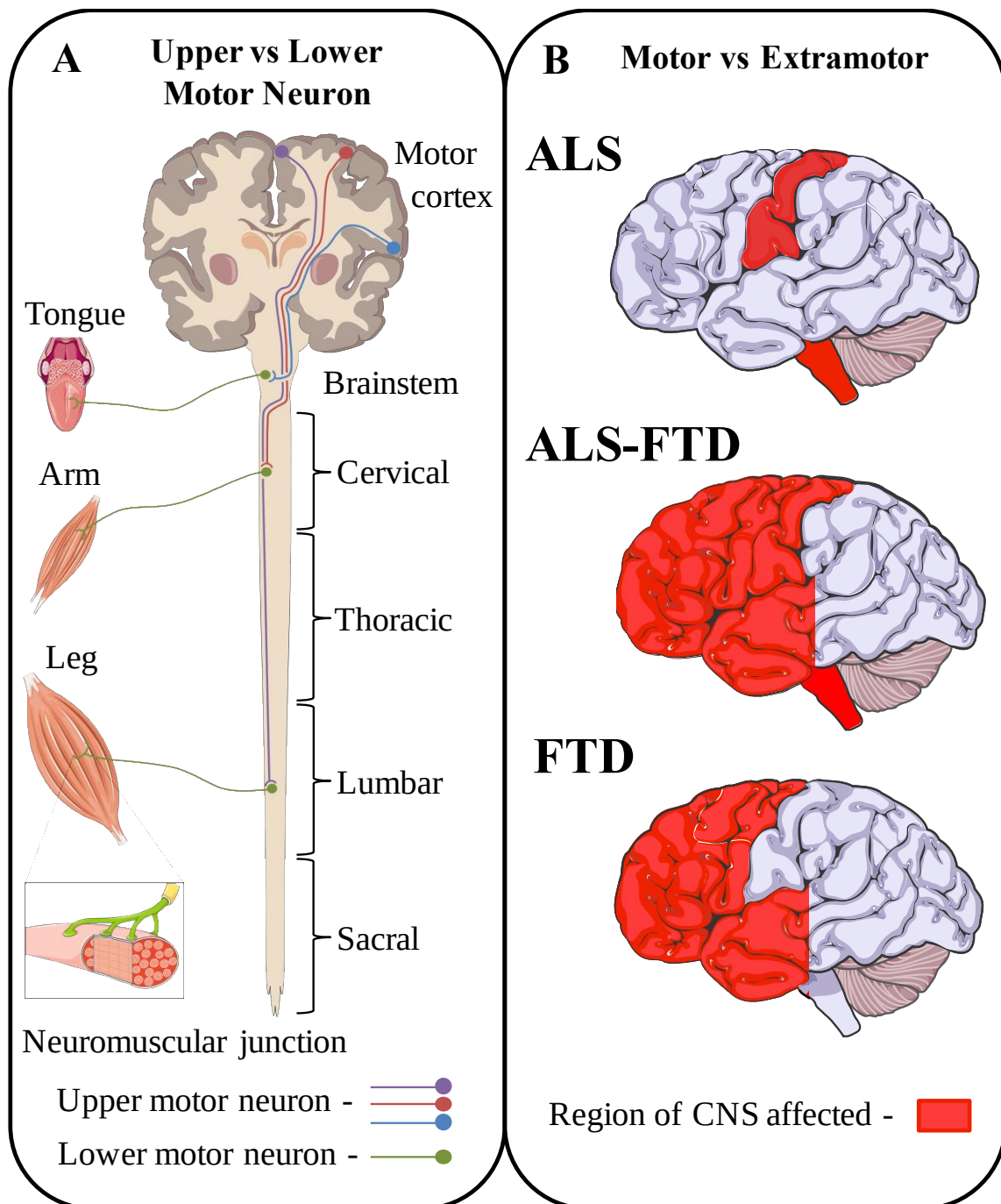


Figure 1.1. Components of the neuromuscular system affected by ALS. (A) ALS is caused by the degeneration of upper motor neurons in motor cortex that project into synapses in the brainstem and spinal cord, and lower motor neurons in the brainstem and spinal cord that project into specialised synapses called neuromuscular junctions (NMJ) found on muscles. (B) In ALS, the motor cortex and spinal cord are affected. However, in some cases, patients with ALS are also diagnosed with FTD (ALS-FTD), which is caused by additional pathology in extra-motor regions of the brain such as the prefrontal, frontal and temporal cortices. Drawn in Microsoft PowerPoint, adapting images from Servier Medical Art® (<http://www.servier.com>)

1.2 Genetics of ALS

Although the majority of ALS cases seem sporadic (sALS), with no discernible family history, there are a number of cases (~10%) that are familial (fALS), with a Mendelian pattern of inheritance (Ewis *et al.* 2001). It is believed that the proportion of fALS cases have been underestimated in the past, probably due to inadequate recording of family history, small family sizes, misdiagnosis and incomplete penetrance (Bäumer, Talbot and Turner 2014). As a result, many cases thought to be sporadic may well be familial, a misdiagnosis likely due to the fact that they are both clinically indistinguishable and genes found mutated in fALS have also been found mutated in sALS. For example, it is now evident that ~2% of sALS cases are caused by missense mutations in superoxide dismutase 1 (SOD1) and ~20% of sALS cases are caused by intronic hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (C9orf72) (Gamez *et al.* 2006; Cooper-Knock *et al.* 2012). Taken together, while the traditional dichotomy of ALS into familial and sporadic subtypes would have been convenient for genetic counselling, it is now considered to be a gross oversimplification and is not particularly meaningful in terms of disease biology (Talbot 2011).

It is now believed that the genetic etiology of ALS is also attributable to genetic pleiotropy (a single gene has multiple phenotypic manifestations) and oligogenic inheritance (a phenotypic trait is determined by more than one gene), which may explain why pedigrees of ALS show incomplete penetrance (Van Blitterswijk *et al.* 2012). This model supports the notion that ALS patients inherit primary ‘at risk’ genetic variants that increase disease susceptibility and secondary genetic loci that influence clinical manifestation and disease progression (Van Blitterswijk *et al.* 2012; Renton, Chiò and Traynor 2014). From the growing list of potential ALS genes (>50), there are a number that are unequivocally implicated in disease pathogenesis (Table 1). These genes can be grouped in to several loose

categories that affect RNA metabolism, protein homeostasis, autophagy, endosomal trafficking, cytoskeletal dynamics and oxidative stress (OS). Up to 70% of fALS cases have now been accounted for by missense mutations in SOD1, TAR DNA-binding protein 43 (TARDBP, which encodes TDP-43), fused in sarcoma (FUS) and intronic hexanucleotide repeat expansions in C9orf72 (Chiò *et al.* 2012b; Millecamps *et al.* 2012). Interestingly, this repeat expansion was present in up to 62% of the cases with ALS-FTD, 46% of the cases with fALS and ~20% of the cases with sALS (DeJesus-Hernandez *et al.* 2011; Renton *et al.* 2011; Gijselinck *et al.* 2012). Therefore, it is currently the most important genetic risk factor for ALS.

Other genetic ‘at risk’ variants that increase susceptibility to ALS or that influence the clinical manifestation of ALS are still of interest as they could, in part, explain the general complexity and heterogeneity of the disease and have implications on therapeutic strategies. For example, intermediate expansions of CAG repeats in the Ataxin 2 gene (ATXN2) increase the risk of developing ALS and ATXN2 protein is a potent modifier of TDP-43 toxicity in animal and cellular models (Elden *et al.* 2010). By contrast, variants that reduced expression of an ephrin receptor tyrosine kinase (EphA4), which regulates axonal outgrowth, correlated with delayed onset and improved survival of ALS patients from a Dutch cohort, supporting the notion that other genetic loci can influence or modulate the manifestation of ALS. (Van Hoecke *et al.* 2012). The discovery of ALS-associated genes has accelerated over the last 25 years, largely due to advances in sequencing and enhanced association studies that identify rare genetic variants. While this has helped unravel the genetic etiology of fALS, it is clear this will be helpful in understanding the cellular and molecular mechanisms involved in the pathology of sporadic cases as well.

Table 1.1 Major genes implicated in ALS in order of discovery.

Gene (Protein)	Locus	Mutations	Frequency	Implicated disease mechanism	Date of discovery	Refs
SOD1 (Superoxide dismutase 1)	21q22.1	>180	20% fALS 2% sALS	Oxidative stress Protein aggregation	1993	(Rosen <i>et al.</i> 1993)
DCTN1 (Dynactin 1)	2p13	10	1% fALS <1% sALS	Vesicular transport Cytoskeletal dynamics	2003	(Puls <i>et al.</i> 2003)
VAPB (vesicle associated membrane protein-associated protein B)	20q13.33	2	<1% fALS	ER stress Protein aggregation	2004	(Nishimura <i>et al.</i> 2004)
ANG (Angiogenin)	14q11.2	29	<1% fALS <1% sALS	RNA metabolism Stress response	2006	(Greenway <i>et al.</i> 2006)
TARDBP (TDP-43)	1p36.2	>50	5% fALS <1% sALS	RNA metabolism Protein aggregation Mitochondrial dysfunction Oxidative stress Cytoskeletal dynamics	2008	(Kabashi <i>et al.</i> 2008; Sreedharan <i>et al.</i> 2008)
FUS (Fused in sarcoma)	16p11.2	>50	5% fALS <1% sALS	RNA metabolism Protein aggregation	2009	(Kwiatkowski <i>et al.</i> 2009; Vance <i>et al.</i> 2009)
FIG4 (polyphosphoinosi tide phosphatase)	6q21	10	<1% fALS <1% sALS	Endosomal trafficking	2009	(Chow <i>et al.</i> 2009)
VCP (Valosin- containing protein)	9p13	5	1-2% fALS <1% sALS	Protein degradation Autophagy Oxidative stress	2010	(Johnson <i>et al.</i> 2010)
OPTN (Optineurin)	10p13	1	4% fALS <1% sALS	Autophagy Vesicular transport	2010	(Maruyama <i>et al.</i> 2010)
ATXN2 (Ataxin 2)	12q24	Intermediate- length CAG repeats (polyQ) expansions	<1% sALS	RNA metabolism Stress response Modifier of TDP-43 pathology	2010	(Elden <i>et al.</i> 2010)
C9orf72 (C9orf72)	9p21.3	Intronic GGGGCC repeat	>40% fALS ~20% sALS	RNA toxicity Abnormal protein translation Autophagy	2011	(DeJesus- Hernandez <i>et al.</i> 2011; Renton <i>et al.</i> 2011)

UBQLN2 (Ubiquilin 2)	Xp11	26	<1% fALS <1% sALS	Protein degradation Autophagy and UPS	2011	(Deng <i>et al.</i> 2011)
SQSTM1 (Sequestosome 1)	5q35	17	<1% fALS <1% sALS	Protein degradation Autophagy	2011	(Fecto <i>et al.</i> 2011; Teyssou <i>et al.</i> 2013)
PFN1 (Profilin-1)	17p13	12	<1% fALS <1% sALS	Cytoskeletal dynamics	2012	(Wu <i>et al.</i> 2012)
hnRNPA1 (heterogeneous nuclear ribonucleoprotein A1)	12q13	3	<1% fALS <1% sALS	RNA metabolism	2013	(Kim <i>et al.</i> 2013)
MATR3 (Matrin-3)	5q31.2	4	<1% fALS <1% sALS	RNA metabolism	2014	(Johnson <i>et al.</i> 2014)
TUBA4A (Tubulin- α 4A)	2q35	7	<1% fALS <1% sALS	Cytoskeletal dynamics	2014	(Smith <i>et al.</i> 2014)
CHCHD10 (Coiled-coil- helix-coiled-coil- helix domain- containing protein 10)	22q11.23	5	<1% fALS <1% sALS	Mitochondrial maintenance	2014	(Bannwarth <i>et al.</i> 2014)
TBK1 (serine/threonine protein kinase TBK1)	12q14.1	10	Unknown	Protein degradation Autophagy	2015	(Freischmidt <i>et al.</i> 2015; Matsumoto <i>et al.</i> 2015)

ER – Endoplasmic reticulum; UPS – ubiquitin proteasome system. A list of ALS genes and mutations can be found at <http://alsod.iop.kcl.ac.uk>

1.3 Pathogenic mechanisms of ALS

The recent advance in understanding pathogenic mechanisms underlying ALS is mostly due to the discoveries of >50 ALS-associated genes and the generation of animal models that recapitulate certain disease phenotypes. ALS is highly complex, involving defects not only in motor neurons but also in non-neuronal cells such as glial, muscle and immune cells. Furthermore, as previously mentioned (Table 1; section 1.2), ALS pathology involves a combination of mechanisms including oxidative stress (OS), mitochondrial dysfunction, impaired protein homeostasis, altered RNA metabolism, cytoskeletal defects and impaired axonal transport. As ALS is highly heterogeneous and known to be associated with mutations in a wide variety of genes, it is wrong to assume that all these processes contribute to the pathology of all ALS cases. However, the separation of different mechanisms is somewhat artificial as many of these processes are interlinked. In fact, disruption in one mechanism often leads to disruption in another. One example of this would be OS, which has a detrimental effect on other cellular processes and is key and early feature in ALS and other neurodegenerative diseases (Lin and Beal 2006).

1.3.1 Oxidative stress

OS arises from the imbalance between the production of reactive oxygen species (ROS) and the ability of a system to remove or repair the damage caused by ROS (Lin and Beal, 2006). To protect against ROS-induced damage, eukaryotic organisms have developed an elaborate network of antioxidants, from enzymatic scavengers such as SOD1 and glutathione peroxidase (GPX), to non-enzymatic molecules such as glutathione and vitamins (Ott *et al.* 2007). Cellular ROS are generated by both exogenous and endogenous sources. Exogenous sources of ROS include ultra violet (UV), ionizing radiation and certain drugs (Pham-Huy, He and Pham-Huy 2008). Endogenous ROS is mostly generated as a natural

by-product of aerobic metabolism, usually due to leakage of electrons from the mitochondrial electron transport chain (ETC). This results in incomplete, premature reduction of oxygen during oxidative phosphorylation, to produce the superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2 ; Turrens 2003; Murphy 2009). Neither superoxide nor hydrogen peroxide is highly reactive, but each undergoes further reactions to produce more potent oxidants. For example, superoxide can react with nitric oxide, a weak oxidant produced by nitric oxide synthase, to produce peroxynitrite ($ONOO^-$; Pryor & Squadrito 1995). In addition, dismutation of superoxide, a reaction catalysed by SOD1 and SOD2, gives rise to hydrogen peroxide, which slowly decomposes to the highly reactive hydroxyl radical ($\cdot OH$) in a process called the Fenton reaction (Fridovich 1995; Beckman and Koppenol 1996).

Cellular ROS can act as signalling molecules, but when excessively produced or not detoxified can cause damage to DNA, proteins and lipids. Such damage includes; altered DNA and RNA species, protein modifications and misfolding, and altered cellular membrane dynamics by oxidation of unsaturated fatty acids (Stadtman and Berlett 1997; Ott *et al.* 2007; Phaniendra, Jestadi and Periyasamy 2015). The central nervous system (CNS) as a whole is particularly susceptible to OS for a few reasons: (1) its high metabolic demand (neurons exhibit ~10-fold increase in oxygen consumption compared to other tissues); (2) it has high concentrations of redox-active transition metals but a relatively low concentration of antioxidants (compared to the relative level of OS); (3) the neuronal membrane contains a high abundance of polyunsaturated fatty acids, which are susceptible to oxidation (Cosgrove, Church and Pryor 1987; Antonio Contestabile 2001).

Evidence of OS has been repeatedly found in multiple pathological studies from ALS patients. First, increased markers of ROS damage have been detected in post-mortem tissue from ALS patients. For example, 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of

oxidized DNA, and 4-hydroxy-2-nonenal-histidine (HNEH) and crotonaldehyde-lysine (CRAL), markers for lipid oxidation were found elevated in spinal cords from ALS patients (Fitzmaurice *et al.* 1996; Shibata *et al.* 2001). In addition, elevated protein carbonyl levels have been detected in the motor cortex and spinal cord of ALS patients and increased 3-nitrotyrosine levels, a marker for peroxynitrite-mediated damage were observed in spinal motor neurons of ALS patients (Abe *et al.* 1995; Shaw *et al.* 1995; Beal *et al.* 1997; Ferrante *et al.* 1997a). Second, studies analysing cerebrospinal fluid (CSF) from ALS patients, which enables the measurement of OS markers at earlier stages of the disease reveals similar results. For example, 8-OHdG, 3-nitrotyrosine, 4-hydroxynonenal (4-HNE) and ascorbate free radical have been detected in CSF from ALS patients (Smith *et al.* 1998; Tohgi *et al.* 1999; Bogdanov *et al.* 2000; Simpson *et al.* 2004; Ihara *et al.* 2005). Taken together, these studies suggest a clear role of OS in ALS, even in the earlier stages of the disease.

Further supporting the hypothesis that OS plays a major role in ALS pathology is provided by the observation that mutations in the antioxidant enzyme SOD1 were the first reported in ALS patients (Deng *et al.* 1993; Rosen *et al.* 1993). Mutations in SOD1 results in aberrant redox chemistry with the majority of evidence suggesting a toxic gain of function (Gurney *et al.* 1994; Allen *et al.* 2003). Indeed, transgenic mouse and cellular models of SOD1-related ALS recapitulate the oxidative damage to DNA, protein and lipids observed in the human disease (Ferrante *et al.* 1997b; Liu *et al.* 1999; Cookson *et al.* 2002). This emphasizes that inability to detoxify ROS and repair oxidative damage efficiently can lead to pathogenic outcomes in ALS.

It is now known that OS also plays a major role in non-SOD1 related ALS as well. For example, in a yeast model, expression of mutant TAR DNA-binding protein 43 (TDP-43; encoded by TARDP; Table 1) results in increased markers of OS, apoptosis and necrosis (Braun *et al.* 2011). In *Drosophila*, motor neuron-specific overexpression of TDP-43 display

increased levels of protein carbonylation and glutathione S transferase D1, both known markers of OS (Zhan, Xie and Tibbetts 2015). These observations have also been recapitulated in mammalian systems as well. For example, expression of mutant TDP-43 in a motor neuron-like cell line induces OS, mitochondrial dysfunction and nuclear accumulation of nuclear factor E2-related factor 2 (Nrf2), which is an indicator and modulator of OS (Duan *et al.* 2010). Activation of Nrf2 leads to its nuclear translocation from the cytoplasm, where it interacts with antioxidant-response element (ARE) enhancer sequences to increase expression of proteins involved in antioxidant defence systems, such as heme-oxygenase 1 (HO-1; Nguyen *et al.* 2009). Interestingly, despite nuclear accumulation of Nrf2 in mutant TDP-43 expressing cells, HO-1 mRNA and protein was unexpectedly downregulated (Duan *et al.* 2010). This suggests mutant TDP-43 inhibits expression of HO-1 independent of Nrf2-antioxidant response element pathway and could explain the increased oxidative damage observed in these cells.

Similarly, two more recent studies also support the role of TDP-43 in inhibiting antioxidant signalling pathways and promoting OS. The first study, using another motor neuron-like cell line expressing human TDP-43 (hTDP-43) with the ALS-associated M337V mutation, observed that Nrf2 translocated to the nucleus, but the expression of Nrf2 and downstream phase II detoxifying enzyme NAD(P)H Quinone Dehydrogenase 1 (NQO1) was decreased in cells expressing TDP-43^{M337V} (Tian *et al.* 2017). In addition, expression of TDP-43^{M337V} resulted in more rounded cells with reduced neurites, shortened axons, increased levels of lipid peroxidation and increased cell death (Tian *et al.* 2017). The second study, using ALS patient fibroblasts harbouring a TDP-43^{M337V} mutation, observed that Nrf2 and downstream antioxidant transcripts were increased, yet there was no increase in corresponding protein expression (Moujalled *et al.* 2017). Furthermore, levels of glutathione (GSH; downstream Nrf2 signalling) were decreased in TDP-43^{M337V} patient fibroblasts,

suggesting elevated OS and failure of some upregulated antioxidant genes to be translated into protein (Moujalled *et al.* 2017). Taken together, these studies further support that mutant TDP-43 inhibits antioxidant response pathways and increases cell susceptibility to OS-induced damage.

Despite the increasing evidence OS occurs during ALS pathogenesis, it is still debated with this is a primary cause of disease or a secondary consequence. Indeed, presence of ROS and OS markers alone does not necessarily mean that it is actively driving pathogenesis. However, multiple studies from ALS patients and animal models suggest that OS is one of the earliest pathological changes, occurring at pre-symptomatic stages of the disease (Smith *et al.* 1998; Bogdanov *et al.* 2000; Higgins *et al.* 2002; Ihara *et al.* 2005). Furthermore, other studies have shown that inhibiting ROS production or decreasing levels of OS delay pathogenesis in ALS. For example, treatment of motor neuron-like cells expressing mutant TDP-43 with GSH monoethyl ester (GSHe), which directly increases intracellular GSH levels, protected against TDP-43 protein aggregation, decreased ROS production and improved cell survival (Chen *et al.* 2018b). Taken together, this argues that OS is more likely a early cause of disease and not just a late consequence. The notion that OS is central to ALS pathology is further exemplified by its intimate links to many of the other pathogenic mechanisms involved, including mitochondrial dysfunction, protein aggregation, altered RNA metabolism, ER stress, impaired axonal transport and inflammation (Figure 1.2; Lin & Beal 2006; Ott *et al.* 2007; Kim *et al.* 2015). In the following sections, each of these pathogenic mechanisms will be introduced, evidence for their involvement in ALS pathology will be provided and the effect of OS on these mechanisms will be discussed.

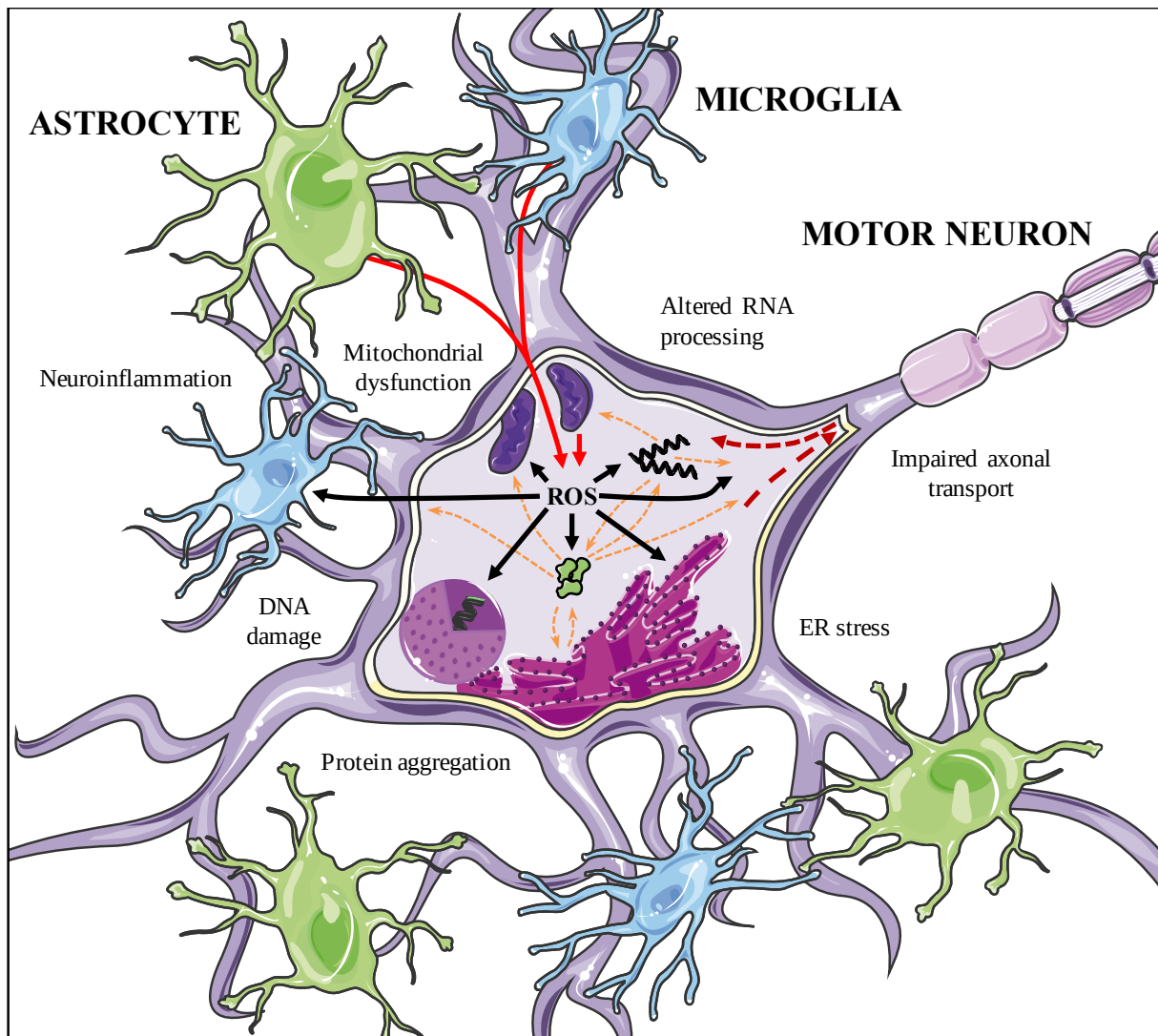


Figure 1.2. Crosstalk between OS and other pathogenic mechanisms in ALS. OS and mitochondrial dysfunction are tightly dependent on each other and form the basis of redox dysregulation in ALS. Increased production of ROS by mitochondria can directly contribute to motor neuron death by deregulating other cellular processes such as transcription, RNA processing, protein homeostasis, axonal transport and mitochondrial function. In addition, ROS and ROS-induced damage in motor neurons activates a neuroinflammatory response and activated microglia and astrocytes can cause further oxidative damage by the production and release of ROS. Subsequently, as these cellular processes are so tightly dependent and connected, these pathogenic events can cause secondary detrimental effects, exacerbating disease. Links between pathogenic mechanisms are discussed in more detail in sections 1.3.2 – 1.3.6. Red arrows signify sources of ROS, black arrows denote direct effects of ROS on cellular process and orange arrows indicate secondary effects of dysfunction in one mechanism on another. *Drawn in Microsoft PowerPoint, adapting images from Servier Medical Art® (<http://www.servier.com>)*

1.3.2 Mitochondrial dysfunction

Mitochondria are key to cell survival and metabolism with their major function being the production of adenosine triphosphate (ATP) through the oxidative phosphorylation system (Saraste 1999). Other functions include calcium homeostasis and apoptosis (Smith, Shaw and De Vos 2017). Mitochondria are particularly important in neurons due to their high metabolic requirements (Nicholls and Budd 2000; Engl and Attwell 2015). Furthermore, as neurons are long-lived cells that persist throughout the lifespan of an organism it can be argued that they are more susceptible to accumulating damage due to mitochondrial dysfunction (Payne and Chinnery 2015). In line with the OS hypothesis, the death of motor neurons has been largely attributed to mitochondrial dysfunction (Strong 2003). Furthermore, this dysfunction can be loosely subcategorized into the following; structural damage to mitochondria, defective mitochondrial respiration, impaired mitochondrial dynamics, overproduction of ROS and induction of apoptosis.

Structurally damaged mitochondria with a swollen and vacuolated appearance was one of the first alterations observed in motor neurons from ALS patients (Atsumi 1981; Sasaki and Iwata 2007). More recently, mutations in the mitochondrial protein coiled-coil-helix-coiled-coil-helix domain-containing protein 10 (CHCHD10; see table 1) have been found associated with ALS (Bannwarth *et al.* 2014). CHCHD10 is localized to contact sites between the inner and outer mitochondrial membrane and ALS-linked mutations disrupt the mitochondrial cristae, providing direct evidence that disruption of mitochondrial structure can cause ALS (Genin *et al.* 2016). Animal and cell models with mutations in other ALS-linked genes have also reported similar observations. For example, expression of mutant TDP-43^{M337V} resulted in aggregated, fragmented and vacuolated mitochondria (Hong *et al.* 2012; Wang *et al.* 2013). Mitochondrial structure and the mitochondrial network appears to be disrupted early in most if not all models of ALS and in ALS patients, suggesting this

occurs as an upstream cause rather than a secondary consequence (Vande Velde *et al.* 2011; Magrané *et al.* 2014).

Defective mitochondrial respiration and reduced ATP production is also well documented in ALS. *Post mortem* samples from ALS patients reveal a decreased activity of all (I, II, III and IV) ETC complexes (Borthwick *et al.* 1999; Wiedemann *et al.* 2002). In support of this, primary motor neurons from TDP-43^{M337V} mice demonstrate a depolarization of mitochondria (Wang *et al.* 2013), and in ‘motor neuron-like’ NSC-34 cells expressing TDP-43^{M337V}, reductions in mitochondrial membrane potential and complex 1 activity has been observed (Duan *et al.* 2010; Lu *et al.* 2012). Taken together, these studies support the involvement of ALS-associated genes in mitochondrial dysfunction by impairing respiration and reducing ATP production.

Several studies have suggested that impaired mitochondrial dynamics including aberrant fission and fusion, disrupted quality control and impaired axonal transport also contribute to overall mitochondrial dysfunction in ALS. Mitochondrial morphology is tightly regulated by the balance between fission and fusion events. Mitochondria can fuse to or fission from the mitochondrial network in response to various stimuli (Westrate *et al.* 2014). Fusion permits mitochondria to share mitochondrial metabolites such as DNA and enzymes improving efficiency of energy production, whereas fission allows the isolation of damaged parts of the mitochondrial network prior to disposal by mitochondrial-specific autophagy, also known as mitophagy (Chan 2012). Evidence points towards an imbalance in these two opposing pathways in ALS. For example, expression of TDP-43^{M337V} in rat primary motor neurons resulted in reduced mitochondrial length and a misbalance of fusion-fission events, tipped more towards mitochondrial fission (Wang *et al.* 2013). The relevance of increased mitochondrial fission in ALS pathology still remains unclear, however it is hypothesized that smaller mitochondria, which tend to be less energetically favourable and

more prone to accumulating ROS induced damage if they cannot fuse back to the mitochondrial network (Hoitzing, Johnston and Jones 2015). This could potentially explain the accumulation of aggregated, fragmented mitochondria seen in ALS.

By proximity, mitochondria are particularly susceptible to ROS-induced damage to mitochondrial DNA, RNA, proteins and lipids, which have been shown to affect mitochondrial function in ALS (Fujita *et al.* 1996; Swerdlow *et al.* 1998; Smith, Shaw and De Vos 2017). Such damage can lead to activation of apoptosis, which involves upstream signalling cascades converging on mitochondria to initiate cytochrome c release and activation of downstream executioner caspases (Ashkenazi and Salvesen 2014). The importance of ROS in cell death is further exemplified by the fact that inhibition of ROS production can protect against apoptosis (Marchetti *et al.* 1997; Sidoti-de Fraisse *et al.* 1998; Fleury, Mignotte and Vayssi re 2002). In summary, like OS, mitochondrial dysfunction is a common and early event in ALS that occurs by defects in mitochondrial structure, ATP production, mitochondrial dynamics and OS. However, OS and mitochondrial damage cannot represent the only mechanisms operating in the pathogenesis of ALS. Thanks to recent advances in genetics, we now know that ALS is associated to mutations in >50 genes, coding for proteins involved in diverse cellular functions, such as protein homeostasis.

1.3.3 Impaired protein homeostasis

Impaired protein homeostasis is also undoubtedly a critical factor in ALS pathology and is involved in the dysfunction of other cellular processes (Figure 1.2). These changes occur as a primary consequence of mutations in the affected protein and/or as secondary phenomena induced by other underlying mechanisms. For example, although SOD1 itself does not play a role in the maintenance of other proteins, pathogenic mutations cause SOD1 protein to form cytoplasmic aggregates in motor neurons (Bruijn *et al.* 1997; Pasinelli *et al.*

2004). By contrast, mutations in optineurin causes a decrease in its ability to perform its autophagic function, which subsequently increases the abundance of protein aggregates in cells (Maruyama *et al.* 2010; Wong and Holzbaur 2014).

Many genes implicated in ALS encode proteins that play a role in the various stages of protein homeostasis, including protein synthesis, folding and clearance. ALS-associated mutations in TDP-43 and FUS disrupt the normal range of interactions with DNA and RNA, which in turn can lead to marked changes in transcription, alternative splicing, and abnormal protein translation on a broad scale (Lagier-Tourenne *et al.* 2012; Arnold *et al.* 2013; Zhou *et al.* 2014; Amlie-Wolf *et al.* 2015). In addition, TDP-43 and FUS possess low-complexity, aggregation-prone prion-like domains, which facilitate oligomer self-assembly and increase susceptibility to TDP-43 and FUS aggregation (Gitler and Shorter 2011; Molliex *et al.* 2015; Patel *et al.* 2015). Unsurprising then, that TDP-43 and FUS are major components of protein aggregates found in motor neurons of both familial and sporadic forms of ALS, even in the absence of TDP-43 and FUS mutations (Arai *et al.* 2006; Neumann *et al.* 2006; Deng *et al.* 2010).

The aggregation of misfolded proteins within a cell tend to accumulate in the ER, leading to ER stress (Schröder 2008). This can induce a protective response from the ER, called the unfolded protein response (UPR). The UPR initially aims to restore normal function of the cell by halting protein translation, degrading misfolded proteins, and activating the signalling pathways that lead to increasing the production of molecular chaperones involved in protein folding (Rozas *et al.* 2017). However, if this is not achieved and ER stress persists, ROS are produced as by-products, leading to OS (Tu and Weissman 2004). As the protein folding process is dependent on redox homeostasis, OS can disrupt the UPR and enhance the production of misfolded proteins, causing further ER stress and even activation of apoptotic pathways (Figure 1.2; Kikuchi *et al.* 2006; Atkin *et al.* 2008; V. Ayala

et al. 2011). Interestingly, it has been shown that vulnerable fast motor neurons are most prone to activating the UPR, which could potentially explain, in part, why these types of motor neurons are more susceptible to degeneration (Saxena, Cabuy and Caroni 2009; Rozas *et al.* 2017). In addition, ALS-linked mutations in vesicle associated membrane protein-associated protein B (VAPB) triggers VAPB aggregation (including wild type VAPB) and disrupts its function in activating the UPR. This leads to ER stress, further protein aggregation and neuronal death (Nishimura *et al.* 2004; Kanekura *et al.* 2006; Chen *et al.* 2010). Taken together, this highlights the importance of the ER and UPR in maintaining protein homeostasis and how OS can contribute to their disruption and reduce motor neuron survival.

Protein aggregation is not only the result of disruption in protein synthesis and folding, but is also the result of a failure in either/both of the two major degradation pathways, the ubiquitin proteasome system (UPS) and autophagy. The fact that the majority of aggregates in ALS contain ubiquitin, suggests that cells have recognised and attempted to clear the misfolded proteins through either the UPS or autophagy, but failed (Kabashi *et al.* 2004; Li, Zhang and Le 2008; Cheroni *et al.* 2009; Chen *et al.* 2012). For example, mutations in C9orf72, a key regulator of autophagy initiation, may contribute to the presence of ubiquitin positive, p62-positive and TDP-43 negative inclusions in extra motor regions of the CNS in ALS-FTD (Al-Sarraj *et al.* 2011; Webster *et al.* 2016). UBQLN2 and SQSTM1 function as adapters that bring polyubiquitinated proteins to the proteasome or autophagosome for degradation, which is disrupted by the presence of ALS-linked mutations (Gal *et al.* 2009; Osaka, Ito and Suzuki 2016). Interestingly, both SOD1 and TDP43 are known substrates of autophagy, suggesting that defective autophagy could contribute to the toxic accumulation of these proteins in some cases of ALS (Kabuta, Suzuki and Wada 2006; Bose, Huang and Shen 2011). Finally, ALS-linked mutations in VCP, a ubiquitin-dependent

segregase, impairs its role in the proteasomal sorting of ubiquitinated proteins as well as the clearance of misfolded proteins through the ER-associated degradation pathway (ERAD; Meyer & Wehl 2014; Moustaqim-Barrette et al. 2014). Taken together, this highlights the importance of efficient degradation and clearance of misfolded proteins in maintaining protein homeostasis, disruption of which can lead to the development of ALS.

It is well established that OS affects protein conformation thereby promoting protein aggregation in ALS. For example, OS-induced damage to both mutant and wild-type SOD1 causes dissociation of SOD1 dimers to monomers and subsequent aggregation (Rakhit *et al.* 2004). Interestingly, since misfolded and aggregated SOD1 has been shown to disrupt mitochondrial function and increase ROS production, a vicious cyclical mechanism emerges (Pickles *et al.* 2013, 2016). OS is also known to promote the mislocalisation of the predominantly nuclear TDP-43 protein to the cytoplasm, where it is prone to cross-linking and aggregation via cysteine oxidation, incorrect disulphide bond formation and also acetylation of key residues (Ayala *et al.* 2011a; Cohen *et al.* 2012, 2015; Chang *et al.* 2013). In agreement, treatment of fibroblast-like cells with HNE was shown to cause insolubilisation, phosphorylation, and cytoplasmic mislocalisation of TDP-43 (Kabuta *et al.* 2015). As previously mentioned, CSF HNE levels are increased in ALS patients and therefore could potentially contribute to TDP-43 aggregation (Smith *et al.* 1998).

Clearly the widespread appearance of protein aggregation in ALS exemplifies a generalised protein misfolding phenomenon that can affect both mutated and wild-type proteins. We know this disruption occurs at multiple levels of protein homeostasis in ALS and is exacerbated by OS. Furthermore, discovering that RNA binding protein TDP-43 is found aggregated in ubiquitin-positive neuronal inclusions in >98% ALS patients and ~50% of FTD patients was a landmark event in our understanding of the pathogenic mechanisms

underlying ALS (Arai *et al.* 2006; Neumann *et al.* 2006). In addition, this discovery strongly implicated altered RNA metabolism as another central mechanism of disease.

1.3.4 Altered RNA metabolism

The discovery of TDP-43 mutations in ALS and FTD families lead to subsequent identification of ALS-associated mutations in related proteins that bind RNA, including FUS and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1; (Kabashi *et al.* 2008; Sreedharan *et al.* 2008; Kwiatkowski *et al.* 2009; Vance *et al.* 2009; Kim *et al.* 2013). TDP-43, FUS and hnRNPA1 are members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins that function in almost every aspect of RNA metabolism (Colombrita *et al.* 2011). They bind to thousands of RNA targets, which means that an altered function in one of these proteins can greatly dysregulate RNA metabolism (Polymenidou *et al.* 2011; Tollervey *et al.* 2011; Lagier-Tourenne *et al.* 2012). For example, in a mutant TDP-43 mouse model of ALS, it was observed that motor neuron degeneration, NMJ degeneration and muscle wasting occurred due to the loss and gain of splicing function of specific RNA targets without the formation of toxic insoluble TDP-43 aggregates, suggesting altered RNA metabolism can drive ALS pathology independently of impaired protein homeostasis (Arnold *et al.* 2013).

Nevertheless, altered RNA metabolism and impaired protein homeostasis are not mutually exclusive mechanisms as some RNA-binding proteins such as TDP-43 and FUS are especially prone to aggregation due to their low complexity prion-like domains (Gitler and Shorter 2011; Patel *et al.* 2015). However, these domains are functionally important for mediating phase separation, which is the process by which complex RNA-protein assemblies separate from the surrounding nucleoplasm or cytoplasm and coalesce into membraneless organelles such as nucleoli or stress granules (Molliex *et al.* 2015). Stress granules are

formed in neurons under OS (or other cellular stress such as heat shock, glucose deprivation, viral infection) as a mechanism to rapidly modulate gene expression. These protective structures allow the prioritized translation of stress response genes by storing house-keeping mRNAs until the stress conditions persist (Li *et al.* 2013). However, this process presents a risk as RNA-binding proteins with low-complexity prion-like domains are placed in close proximity, increasing the chance of aggregation. Mutations in ALS are frequently found to cluster in the low-complexity domains of FUS, hnRNPA1 and especially TDP-43 (Kim *et al.* 2013; Peters, Ghasemi and Brown 2015). As a result, the dynamics of these membraneless organelles are altered and can entrap endogenous wild-type protein acting as seeds in accelerating protein aggregation (Parker *et al.* 2012; Vance *et al.* 2013).

As well as its effect on mislocalisation of RNA-binding proteins and stress granule formation, OS also increases the oxidation of mRNA species in ALS patients and in a SOD1 mouse model of ALS at pre-symptomatic stages of the disease (Chang *et al.* 2008). Furthermore, mRNA transcripts encoding proteins involved in the mitochondrial ETC and protein synthesis, folding and degradation were more vulnerable to oxidation than others (Chang *et al.* 2008). This suggests that OS can cause mitochondrial dysfunction and impair protein homeostasis indirectly by altering RNA metabolism. OS has also been implicated in altering RNA splicing. For example, human SH-SY5Y neuroblastoma cells treated with paraquat, an inducer of oxidative stress and mitochondrial damage, caused changes in the splicing pattern of some pre-mRNA that are crucial for motor neuron viability (Maracchioni *et al.* 2007). This suggests OS alters RNA splicing, further supporting its contribution to deregulating RNA metabolism in ALS.

1.3.5 Cytoskeletal defects and impaired axonal transport

The identification of mutations in Dynactin (DCTN), Profilin 1 (PFN1) and Tubulin α 4A (TUBA4A) in patients with ALS suggest that abnormalities in cytoskeletal proteins and axonal transport also play major roles in ALS pathology (Puls *et al.* 2003; Wu *et al.* 2012; Smith *et al.* 2014). In addition, RNA binding proteins such as TDP-43, FUS and hnRNPA1 have also been implicated in deregulating cytoskeletal function and impairing axonal transport. It is well established that TDP-43 forms cytoplasmic messenger ribonucleoprotein (mRNP) granules that undergo bidirectional, microtubule-dependent transport to deliver target mRNA to distal neuronal compartments (Alami *et al.* 2014). Interestingly, expression of mutant TDP-43 impairs axonal transport of mRNA in *Drosophila*, cultured mouse cortical neurons and motor neurons derived from ALS patients with TDP-43 mutations (M337V, G298S and A315T; Alami *et al.* 2014), suggesting mutations in TDP-43 could lead to axonal transport dysfunction. Additionally, TDP-43 is also known to regulate the expression and transport of mRNAs and proteins important for axonal outgrowth or the stabilization of microtubules at the synapse (Hummel *et al.* 2000; Roos *et al.* 2000; Coyne *et al.* 2014).

There is growing evidence that OS is also involved in cytoskeletal defects and axonal transport in the pathogenesis of ALS (Parakh *et al.* 2013). Indeed, the main components of the neuronal cytoskeleton, such as actin microfilaments, microtubules and neurofilaments are susceptible to oxidation, which can lead to impaired cytoskeletal dynamics (Kim *et al.* 2004; Sparaco *et al.* 2006; Munnamalai and Suter 2009; Hung *et al.* 2010; Morinaka *et al.* 2011). In addition, using live cell imaging in rat primary neurons, it was observed that treatment with hydrogen peroxide or sodium azide (ATP synthesis blocker) inhibited axonal transport of mitochondria and Golgi-derived vesicles. Furthermore, this occurred before any detectable changes in mitochondrial morphology or signs of axonal degeneration (Fang,

Bourdette and Banker 2012). This suggests that OS can lead axonal transport dysfunction, which may contribute to axonal degeneration and motor neuron death in ALS.

1.3.6 Neuroinflammation

Motor neuron degeneration in ALS is considered a combination of cell-autonomous and non-cell autonomous events, as surrounding non-motor neuronal cells such as immune cells and glia are actively involved. Mechanisms by which these cells contribute to neuronal pathology is often termed neuroinflammation, which is mostly characterised by astrocytic and microglial activation, T lymphocyte infiltration and the production of pro-inflammatory cytokines (Engelhardt, Tajti and Appel 1993; Hu *et al.* 2015). In fact, whichever mechanism initially involved in motor neuron pathology, it appears to be accompanied by neuroinflammation at some stage (Nagai *et al.* 2007; Swarup *et al.* 2011b; Schludi *et al.* 2017).

Microglia are a component of the innate immune system and considered the resident macrophages of the CNS, acting as the first line of defence against injury and infection (Cherry, Olschowka and O'Banion 2014). Microgliosis has been observed in the motor cortex, along the corticospinal tract and in the ventral horns of the spinal cord of ALS patients (Brettschneider *et al.* 2012). Using positron emission tomography (PET), microgliosis has also been detected in motor and extra-motor regions in the brains of living symptomatic ALS patients (Turner *et al.* 2004). Furthermore, the extent of microgliosis correlated with disease severity, suggesting an active involvement of microglia in the early stages of disease progression (Turner *et al.* 2004; Brettschneider *et al.* 2012). Further evidence of the active and early role of microglia in ALS has been found in transgenic models of the disease (Beers *et al.* 2006; Boillee *et al.* 2006; Xiao *et al.* 2007; Wang *et al.* 2009a). The fact microglial activation has been observed well before symptom onset, prior

to any motor deficit, motor neuron loss but concomitantly with NMJ degeneration (Alexianu, Kozovska and Appel 2001; Gerber *et al.* 2012), further suggests neuroinflammation occurs in early pre-symptomatic stages of ALS.

Astrocytes, another type of glial cell, regulate extracellular neurotransmitter concentrations, maintaining metabolic/ionic homeostasis, providing structural and trophic support, and contributing to the immune response (Farina, Aloisi and Meinl 2007; Sofroniew and Vinters 2010). Like microglia, activated astrocytes secrete pro-inflammatory cytokines and neurotoxic factors, such as ROS, interleukin-6 (IL-6), tumour necrosis factor alpha (TNF- α) and nitric oxide (NO), which can induce the apoptosis of neurons through Fas Ligand and TNF- α signalling (Dric Raoul *et al.* 2002; Barbeito *et al.* 2004; Farina, Aloisi and Meinl 2007; Haidet-Phillips *et al.* 2011). Astrocytes have also been shown to cause motor neuron death by activating a caspase-independent form of programmed cell death called necroptosis, which involves the loss of plasma membrane integrity (Re *et al.* 2014).

In patients with ALS, astrogliosis (astrocyte activation) has been shown to increase with disease progression (Nagy, Kato and Kushner 1994). The active role of astrocytes driving ALS pathology, has been further demonstrated by *in vitro* and *in vivo* models of disease (Hall, Oostveen and Gurney 1998; Levine *et al.* 1999; Nagai *et al.* 2007; Lepore *et al.* 2008; Papadeas *et al.* 2011; Wang, Gutmann and Roos 2011). In particular, mutations in TDP-43 have been associated with increased astrogliosis. For example, characterization of a transgenic rat line showing doxycycline-inducible expression of mutant TDP-43^{M337V} selectively in astrocytes resulted in progressive motor dysfunction and eventually complete paralysis (Tong *et al.* 2013). In addition, denervation and atrophy of skeletal muscles, progressive loss of spinal cord motor neurons, microglial activation and ubiquitin-positive inclusions within astrocytes were observed (Tong *et al.* 2013). This suggests that mutant

TDP-43 has a toxic effect on astrocytes and causes non-cell autonomous motor neuron degeneration in ALS.

As mentioned before, microglia and astrocytes contribute to OS via the overproduction of ROS in their activated pro-inflammatory state. However, OS is also known to play a role in glial cell activation, resulting in further release of ROS, thereby completing a vicious cycle (Koutsilieri *et al.* 2002; Zhao *et al.* 2004; Mittal *et al.* 2014). Glial involvement may therefore be one way in which motor neuron degeneration, which often starts focally, spreads to contiguous motor neuron populations (Barber and Shaw 2010). While it is likely that neuroinflammation does not initiate neuronal injury, a clear link between neuroinflammation and disease progression has been established. Furthermore, this can be divided into two phases: the early slow progression phase suggests a neuroprotective role of immune and glial cells, whereas the second rapidly progressing phase suggests a more toxic role of immune and glial cells with the failing of extrinsic, anti-inflammatory, repair processes.

1.3.7 Concluding pathogenic mechanisms of ALS

Despite the complexity of ALS, our understanding of its biology is improving, especially contextualizing the role of TDP43, which seems to be implicated in almost every pathogenic mechanism. Previously, pathways of OS and mitochondrial damage were considered distinct from those of protein homeostasis, RNA metabolism, axonal transport and neuroinflammation. However, ever-growing evidence points towards OS as a central disease mechanism that can deregulate all of the aforementioned processes. With that in mind, it is still difficult to completely distinguish which pathogenic mechanisms are the primary cause of disease and which are a reflection of downstream damage, especially as many of these processes go awry in synchrony. Future success in understanding the

pathology underlying ALS will depend on the development of new cellular and animal models of ALS.

1.4 Modelling ALS

Over the years researchers have attempted to recapitulate certain aspects of ALS in numerous model systems ranging from *in vitro* biochemical and cell culture systems, to *in vivo* invertebrates, non-mammalian vertebrates and rodent models (Van Damme, Robberecht and Van Den Bosch 2017). More recently, the generation of induced pluripotent stem cells (iPSCs) has opened up new avenues in which researchers can study disease mechanisms in patient-derived neurons *in vitro* (with or without known disease-associated gene mutations; Takahashi et al. 2007; Matus et al. 2014; Lee & Eric J Huang 2017). In addition, iPSCs can be differentiated into spinal motor neurons in order to study the specific cell type of interest in ALS (Maury *et al.* 2015). Indeed, many of the first iPSC-derived motor neuron models have recapitulated several aspects of ALS neuropathology, such as the cytoplasmic mislocalisation and/or aggregation of TDP-43, SOD1, FUS, and in the case of C9orf72 repeat expansions, the formation of RNA foci and dipeptide repeat proteins (Matus, Medinas and Hetz 2014; Lee and Huang 2017).

Although the translational potential of stem cell models remains to be proven, they provide a good balance between throughput and relevance for human disease (Van Damme, Robberecht and Van Den Bosch 2017). Significant advantages of human iPSCs is that they remove the need to overexpress transgenes containing pathogenic ALS gene mutations, they carry endogenous gene mutations in the context of an individual patient's genetic background and can also be generated from patients with sporadic disease, which is not possible in the other model systems (Lee and Huang 2017; Tan *et al.* 2017). However, the major disadvantage of iPSC-derived neurons is the biological heterogeneity within and

across cell lines and some have been shown to resemble foetal rather than adult neurons (Ho *et al.* 2016; Lee and Huang 2017). Furthermore, it is considered that iPSC models could also be of greater value if robust systems were available to generate mature motor neurons that innervate muscle cells (Sances *et al.* 2016).

A key limitation of cellular models is that they lack the complex interplay that occurs between motor neurons and their surrounding environment in a living organism. Therefore, ALS has been modelled in several species, such as fish (*Danio rerio*), nematodes (*Caenorhabditis elegans*) and arthropods (*Drosophila melanogaster*) (Babin, Goizet and Raldúa 2014; Therrien and Parker 2014; Casci and Pandey 2015). However, rodent models remain the gold standard of preclinical ALS modelling as they are a mammalian model system with a high degree of genetic homology and physiological relevance to humans (Tan *et al.* 2017).

1.4.1 Rodent models of ALS

Mutant SOD1 mice were the first genetic mouse models of ALS reported in 1994 and have since dominated preclinical studies (Gurney *et al.* 1994; Tan *et al.* 2017). This is likely due to the similarity this model shares with ALS in humans, with regard to onset and progression compared to other transgenic rodent models (Philips and Rothstein 2015; Picher-Martel *et al.* 2016). Briefly, SOD1^{G93A} (1Gur) mice grossly overexpress the mutant SOD1^{G93A} protein (~17-fold) and presents with adult onset (~3-4 months of age), rapidly progressive motor symptoms, denervation and muscle wasting, motor neuron loss, gliosis, axonal degeneration and reduced survival of 4-5 months (Table 1.2; Gurney *et al.* 1994; Vinsant *et al.* 2013).

Other SOD1^{G93A} mouse lines, such as G1dl, have been generated with a different transgene copy number leading to different disease onset and progression rates (Table 1.2;

Gurney et al. 1994; Jonsson et al. 2006; A. Acevedo-Arozena et al. 2011). This suggests that the aggressiveness of the mutant SOD1-mediated phenotype is highly dependent on the level of transgene expression. Crucially, overexpression of human wildtype SOD1, at a level similar to that seen in the SOD1^{G93A} (1Gur) mouse model, can cause progressive motor neuron degeneration and mice develop an axonopathy at a later age (Dal Canto and Gurney 1994; Graffmo *et al.* 2013). Therefore, one has to be careful when interpreting results from the transgenic mutant SOD1 mice, and mice overexpressing an equal amount of wildtype human SOD1 should always be used as a control (Dal Canto and Gurney 1994; Benatar 2007). It is also important to consider that mutations in SOD1 only account for a small proportion of total ALS cases (~2%), therefore therapies which show promise in SOD1 models may not be appropriate for the majority of fALS or sALS cases (Benatar 2007; Chiò *et al.* 2012b).

Since TDP-43 pathology is observed in >98% of ALS cases, there has been a huge effort to generate transgenic rodents with human wild-type or mutant TDP-43 in the hope to model associated pathologies that have a wider applicability to sALS cases (Arai et al. 2006; Neumann et al. 2006). The first mutant TDP-43^{A315T} transgenic model presented with reduced survival, motor impairment, degeneration of motor neurons, axons and NMJs, muscle atrophy, TDP-43 protein aggregation and gliosis (Table 1.2; Wegorzewska et al. 2009). However, additional studies demonstrated that early mortality was due to transgenic expression in the myenteric plexus leading to gastrointestinal problems and gut paralysis, which is not observed in humans with ALS and is therefore not an ideal model (Guo *et al.* 2012; Esmaeili *et al.* 2013). Another caveat associated with the use of this model is that no human wild type TDP-43 expressing control mouse was generated, so it is difficult to distinguish effects of overexpression from effects inherent to the TDP-43 mutation (Wegorzewska *et al.* 2009).

To circumvent this, one study generated both human wild type TDP-43 and human mutant TDP-43^{A315T} expressing mice (Table 1.2; Stallings et al. 2010). In these lines, TDP-43^{A315T} mice presented with motor dysfunction, shortened survival, and histological examination of motor neurons revealed the presence of ubiquitinated protein aggregates containing TDP-43 protein (Stallings *et al.* 2010). However, a major caveat associated with the use of this model is that the phenotypic differences between mutant and wild type TDP-43 mice could potentially be explained by the slightly reduced expression level of the wild type TDP-43, compared to that reported in the mutant TDP-43 overexpression line (Table 1.2; Stallings et al. 2010). In fact, in another study in which mutant TDP-43^{M337V} was overexpressed at the same level as wild type TDP-43 (2.7 and 2.5-fold of the endogenous Tdp-43, respectively), there was no difference in disease severity, suggesting that transgene expression levels rather than the nature of the mutation were partly responsible for the phenotype observed (Xu *et al.* 2010, 2011).

In order to bypass potential effects from huge overexpression of TDP-43 protein, one study generated mice which overexpress either human wild type or human mutant TDP-43^{Q331K} or ^{M337V} only up to 1.5-fold of the endogenous mouse Tdp-43 levels (Arnold et al., 2013). TDP-43^{Q331K} mice develop adult-onset progressive motor neuron degeneration and neuromuscular denervation at the age of 10 months. Importantly, disease phenotypes were specific for the human mutant overexpressing lines and the human wild type TDP-43 line did not develop any phenotypic abnormalities (Arnold *et al.* 2013). Taking this further, a more recent study used clustered regularly interspaced short palindromic repeats (CRISPR) technology to create a knock-in mouse model with a human-equivalent mutation (Q331K) in the endogenous mouse *Tardbp* gene (White *et al.* 2018). However, while Tdp-43^{Q331K} mice developed motor dysfunction by 4 months of age, they did not present with protein aggregation, motor neuron degeneration or NMJ denervation (White *et al.* 2018).

Furthermore, it was revealed that the Q331K mutation perturbs Tdp-43 autoregulation leading to an increase in Tdp-43 expression in the spinal cord, but not in laser capture micro-dissected lumbar motor neurons (White *et al.* 2018). In addition, motor neurons of homozygous Tdp-43^{Q331K} mice demonstrated upregulation of genes that may confer neuroprotection, which also may explain why mutant mice did not demonstrate significant neuromuscular phenotypes (White *et al.* 2018).

In all of the TDP-43 transgenic models discussed so far, the mouse prion protein (mPrnp) promoter was used to drive transgene expression throughout the CNS during embryonic development (Borchelt *et al.* 1996). However, other models have used different promoters to control the temporal and spatial expression of the transgene. For example, the Thy1.2 promoter drives expression of the transgene in neuronal and immune cells from one week of age, and therefore avoids the deleterious effects of TDP-43 overexpression during development (Morris 1985; Vidal *et al.* 1990). In addition, lines of mice expressing human wildtype and mutant TDP-43 (A315T and G348C) have also been developed using a bacterial artificial chromosome (BAC), which is large enough to contain the entire TDP-43 gene including its own promoter and regulatory elements that drive transgene expression (Swarup *et al.* 2011a). In these BAC transgenic lines, the expression of either wild type or mutant TDP-43 was approximately 3-fold greater than the endogenous mouse Tdp-43. Interestingly, this resulted in a significant downregulation of endogenous TDP-43 levels, supporting the observation that TDP-43 regulates its own expression (Ayala *et al.* 2011b; Swarup *et al.* 2011a).

In addition to mouse models, several rat models of TDP-43-related ALS have also been developed (Zhou *et al.* 2010; Huang *et al.* 2012). Constitutive expression of mutant TDP-43^{M337V}, but not wildtype TDP-43, in founder lines caused early mortality and prevented the generation of further lines (Zhou *et al.* 2010). Therefore, in order to produce

mutant TDP-43 lines, the tetracycline (Tet)-off system was used to induce transgene expression of mutant TDP-43^{M337V} by postnatal day 10 (Zhou *et al.* 2010). The Tet-off system is commonly used in transgenic studies and is comprised of two elements, a Tet-controlled transactivator (tTA) and a tTA-activated promoter (TRE) (Yamamoto, Hen and Dauer 2001). In the absence of Doxycycline (Dox) administration, tTA constantly activates the TRE-TDP-43^{M337V} transgene, producing an expression pattern that is indistinguishable from constitutive transgene expression. This resulted in impaired rotarod performance by 1 month of age, significant motor neuron loss and paralysis by approximately 1.5 to 2 months of age (Zhou *et al.* 2010).

While results have shown large phenotype variation, nearly all TDP-43 models show impaired motor function (Table 1.2; references therein). Pathologically, gliosis is observed in all TDP-43 models, however motor neuron degeneration is inconsistent and usually mild in severity (Table 1.2; Wegorzewska *et al.* 2009; Wils *et al.* 2010; Arnold *et al.* 2013; Zhou *et al.* 2010; White *et al.* 2018). Axonal loss, NMJ degeneration, denervation and muscle atrophy is more frequent in TDP-43 rodent models, however it is unclear if these phenotypes are the cause of early mortality (Table 1.2; McGoldrick *et al.* 2013). Furthermore, the presence of TDP-43 inclusions does not seem to be necessary for the development of other ALS-like phenotypes, suggesting this is not an essential process in the development of ALS (Table 1.2; Shan *et al.* 2010; Swarup, Phaneuf, Bareil, *et al.* 2011; Arnold *et al.* 2013; Huang *et al.* 2012; White *et al.* 2018).

This variation in development and severity of phenotypes is likely due to differences in the choice of promoter, mutation, gender and level and timing of transgene expression, with higher levels of transgene expression being associated with earlier onset of disease phenotypes (Table 1.2; McGoldrick *et al.* 2013). However, transgene expression levels in TDP-43 models are typically low compared to SOD1 transgenic mice, as founder lines which

have the highest levels of overexpression do not survive long enough to generate transgenic lines with high levels of overexpression (Stallings *et al.* 2010). It could be argued that TDP-43 models are therefore more relevant to human ALS than SOD1 models as mutant protein expression is significantly closer to physiological levels. However, all TDP-43 models to date have failed to recapitulate all of the features of human ALS.

Studies examining rodent models of ALS has not only aided our understanding of the pathogenic mechanisms implicated in ALS, but also allowed the testing of new therapeutics. Unfortunately, previous therapeutic approaches have had limited translational success (Benatar 2007). This is likely because many studies test therapies in pre-symptomatic mouse models, which is of limited translational value as sALS patients are only treated after the onset of symptoms (McGoldrick *et al.* 2013). Moreover, it is believed that additional rodent models of ALS that more accurately model the human disease will be important for future therapeutic success.

Table 1.2 Summary of rodent models of ALS

Mutation	Promoter	Protein expression (fold change)	Phenotypes								Survival (months)	References
			Motor Defect (months)	Degeneration				Pathology				
				UMN (%loss)	LMN (%loss)	Axonal (%loss)	NMJ (%loss)	Protein aggregation	Gliososis	Muscle		
Mouse with SOD1												
G93A (1Gur)	Human SOD1	17	Yes (3-4)	Yes (>50% at 4m)	Yes (>50% at 5m)	Yes	Yes (55-60% at 3-4m)	Yes (SOD1+)	Yes	Yes	4-5	(Gurney <i>et al.</i> 1994; Johnston <i>et al.</i> 2000; Ozdinler <i>et al.</i> 2011; Vinsant <i>et al.</i> 2013)
G93A (G1dl)	Human SOD1	8	Yes (6)	-	Yes (>50% at 8m)	-	-	Yes (SOD1+)	Yes (Subtle)	Yes	8-9	(Gurney <i>et al.</i> 1994; Jonsson <i>et al.</i> 2006; Acevedo-Arozena <i>et al.</i> 2011)
Mouse with TDP-43												
A315T	mPrnp	3	Yes (3)	Yes	Yes (20% at 4-5m)	Yes	Yes	Yes (TDP-/Ub+)	Yes	Yes	5	(Wegorzewska <i>et al.</i> 2009)
WT	mPrnp	3	No	-	Nd	Nd	Nd	No	Yes	No	>11	(Stallings <i>et al.</i> 2010)
A315T	mPrnp	4	Yes (1)	-	Nd	Nd	Nd	Yes (TDP+/Ub+)	Yes	Yes	2-3	(Stallings <i>et al.</i> 2010)

Mutation	Promoter	Protein expression (fold change)	Phenotypes								Survival (months)	References
			Motor Defect (months)	Degeneration				Pathology				
				UMN (% loss)	LMN (%loss)	Axonal (%loss)	NMJ (%loss)	Protein aggregation	Gliososis	Muscle		
WT (line6/6)	Thy1.2	3.8	Yes (2)	Yes 15%	Yes 10%	-	-	Yes (TDP+/Ub+)	Yes	-	6-7	(Wils <i>et al.</i> 2010)
WT (line 4/4)	Thy1.2	5.1	Yes (0.5)	Yes 30%	Yes 25%	-	-	Yes (TDP+/Ub+)	Yes	-	<1	(Wils <i>et al.</i> 2010)
WT	Thy1.2	M=3.6 F=1.3	Yes (M=2-3 F=13)	-	No	M=Yes	Yes	No	Yes (Subtle)	Yes	-	(Shan <i>et al.</i> 2010)
WT	mPrnp	2.5	Yes (0.5)	-	No	Yes	-	Yes (TDP+/Ub+)	Yes	No	1-2	(Xu <i>et al.</i> 2010)
M337V	mPrnp	2.7	Yes (0.5-1)	-	No	-	-	Yes (TDP+/Ub+)	Yes	-	1-2	(Xu <i>et al.</i> 2011)
WT	Human TDP-43 (BAC)	2.5-3	Yes (9-10)	-	-	No	Yes (5%)	No	Yes	-	>12	(Swarup <i>et al.</i> 2011a)
A315T	Human TDP-43 (BAC)	2.5-3	Yes (9-10)	-	-	No	-	Yes (TDP+)	Yes	-	>12	(Swarup <i>et al.</i> 2011a)
G348C	Human TDP-43 (BAC)	3	Yes (9-10)	-	-	No	Yes (15%)	Yes (TDP+/Ub+)	Yes	-	>12	(Swarup <i>et al.</i> 2011a)
WT	mPrnp	1.5	No	No	No	No	No	No	Yes (Subtle)	No	>17	(Arnold <i>et al.</i> 2013)

Mutation	Promoter	Protein expression (fold change)	Phenotypes								Survival (months)	References
			Motor Defect (months)	Degeneration				Pathology				
				UMN (% loss)	LMN (%loss)	Axonal (%loss)	NMJ (%loss)	Protein aggregation	Gliososis	Muscle		
M337V	mPrnp	1.5	Yes (10)	No	ns (20% 10m)	No	No	No	Yes (Subtle)	No	>17	(Arnold <i>et al.</i> 2013)
Q331K	mPrnp	1.5	Yes (3)	No	Yes (35% at 10m)	Yes 32% at 10m)	Yes (30% at 10m)	No	Yes	Yes	>17	(Arnold <i>et al.</i> 2013)
A315T	Thy1.2 (TRE)	1.7	Yes (3)	Yes	No	-	-	Yes (TDP+/Ub+)	Yes	Yes	>15	(Ke <i>et al.</i> 2015)
WT	N/A	N/A	No	No	No	-	No	No	-	-	>19.5	(White <i>et al.</i> 2018)
Q331K	Endogenous Tardbp (Knock-in)	1.5	Yes (4)	No	No	-	No	No	-	-	>19.5	(White <i>et al.</i> 2018)

Rat with TDP-43

WT	Human TDP-43	-	No	-	No	No	-	-	-	-	>6-7	(Zhou <i>et al.</i> 2010)
M337V	Human TDP-43 (TRE)	- (induced by P10)	1	-	Yes (17% at 1.5-2m	Yes (40% at 1.5-2m)	Yes (40% at 1.5-2)	Yes (TDP+/Ub-)	Yes	Yes	1.5-2	(Zhou <i>et al.</i> 2010)

Mutation	Promoter	Protein expression (fold change)	Phenotypes								Survival (months)	References
			Motor Defect (months)	Degeneration				Pathology				
				UMN (% loss)	LMN (%loss)	Axonal (%loss)	NMJ (%loss)	Protein aggregation	Gliososis	Muscle		
M337V	NEF (TRE)	2 (induced 2m)	<2.5	-	Yes (20% at 3m)	Yes	Yes	No	-	Yes	3	(Huang <i>et al.</i> 2012)
M337V	ChAT (TRE)	3.5 (induced 2m)	<2.5	-	Yes (60% by <3m)	Yes	Yes	Yes (TDP-/Ub+)	Yes	Yes	<3	(Huang <i>et al.</i> 2012)

Where possible the age of phenotype onset and % loss of degeneration has been given. WT: wild-type, m: months. P: postnatal day, M: male, F: female, mPrnp: mouse prion protein, Thy1.2: Thy-1.2 cell surface antigen, NEF: neurofilament heavy chain, ChAT: choline acetyltransferase, TRE: tetracycline response element, BAC: bacterial artificial chromosome, SOD1+: inclusions positive for SOD1, TDP+/-: inclusions positive/negative for TDP-43, Ub+/-: inclusions positive/negative for ubiquitin, -: not done, ns: not significant, N/A: not applicable.

1.5 Treatments of ALS

Currently, the best treatment of ALS is a multidisciplinary approach led by teams of neurologists, physical and speech therapists, psychologists, nutritionists and specialized nurses to alleviate symptoms and improve quality of life (Miller *et al.* 2009; Andersen *et al.* 2012). Despite years of intense investigation in to chemical compounds and cell-based therapies, there is still no effective treatment for ALS. Riluzole was the first compound to be approved by the Food and Drug Administration (FDA) in 1996, but only extends survival by 1-3 months and has no significant effect on muscle strength or function (Bensimon, Lacomblez and Meininger 1994; Lacomblez *et al.* 1996). Moreover, while riluzole is a relatively safe drug, there are some common adverse effects including an increase in liver enzymes, asthenia (lack of energy) and in some cases fatal hepatic failure and pancreatitis (Bensimon, Lacomblez and Meininger 1994; Lacomblez *et al.* 1996). In ALS, the excitatory amino acid transporter 2 (EAAT2), an astroglial protein that is the main synaptic glutamate reuptake transporter is impaired (Howland *et al.* 2002). This results in synaptic glutamate abundance and motor neuron toxicity, often referred to as ‘excitotoxicity’ (Shaw and Bains 2002; Guo *et al.* 2003). The mechanism of action of riluzole is still not fully understood, however it is believed to reduce glutamate release by blocking voltage-gated sodium channels on presynaptic neurons (Kretschmer, Kratzer and Schmidt 1998; Wang, Wang and Wang 2004). While excitotoxicity is believed to be a common feature amongst all ALS cases, there are other important mechanisms that will need to be targeted for effective therapeutic intervention.

1.5.1 Targeting protein aggregation as a therapeutic strategy for ALS

The evidence discussed in section 1.3.3 strongly suggests protein aggregation due to impaired protein homeostasis is a major contributor to the pathology of ALS. Therefore,

restoring protein homeostasis has emerged as an attractive therapeutic target. To date, treatments have focused on either increasing protein folding to prevent protein aggregation or removal of aggregated proteins (Webster *et al.* 2017). For example, arimoclomol stabilizes the transcription factor heat shock factor protein 1 (HSF-1) in its active state leading to the upregulation of a number of heat shock protein (HSP) family chaperones, including HSP60, Hsp70, and Hsp90 (Vígh *et al.* 1997). Arimoclomol has been shown to prevent aggregation of mutant SOD1, increase muscle function, delay disease progression and extend the lifespan of SOD1^{G93A} mice (Kieran *et al.* 2004; Kalmar *et al.* 2008; Kalmar, Edet-Amana and Greensmith 2012). Alternatively, fluphenazine and methotrimeprazine are potent inducers of autophagy and efficiently enhance TDP-43 aggregate clearance and improve survival of murine primary cortical neurons overexpressing TDP-43^{A315T} and iPSC-derived motor neurons and astrocytes expressing TDP-43^{M337V} (Barmada *et al.* 2014). Taken together, these studies suggest that specific compounds that reduce the burden of protein aggregation hold great potential in finding new and improved treatments for ALS.

1.5.2 Targeting OS as a therapeutic strategy for ALS

As discussed in section 1.3.1, OS is also capable of causing considerable damage to motor neuron populations, and can also influence other mechanisms implicated in ALS. Therefore, it seems sensible to attempt to target the causes and consequences of OS for therapeutic intervention as well. Several pharmacotherapeutic agents with antioxidant properties such as vitamin E, acetylcysteine, selegiline hydrochloride, creatine, coenzyme Q10 and melatonin have been tested as an attempt to slow ALS progression; however, most of them failed to do so (Louwerse *et al.* 1995; Lange *et al.* 1998; Desnuelle *et al.* 2001; Groeneveld *et al.* 2003; Weishaupt *et al.* 2006; Kaufmann *et al.* 2009). Edaravone (MCI-186) is a neuroprotective drug with free radical scavenging properties (Watanabe *et al.*

1994). It eliminates lipid peroxide and hydroxyl radicals by transferring an electron to the radical, thereby exerting a protective effect on neurons (Watanabe *et al.* 1994). In a phase II clinical trial in Japan, edaravone diminished 3-nitrotyrosine levels, a marker of OS, in CSF of ALS patients (Yoshino and Kimura 2006). Following this, in a recent phase III clinical trial, edaravone was shown to slow disease progression in a particular group of patients with early onset and rapidly progressing disease (Abe *et al.* 2017). Importantly, there were a number of limitations to this trial that argue whether edaravone would be effective and safe to use for all ALS patients. In addition, while edaravone is approved by the FDA, it has still not yet been approved by the European Medicines Agency and further trials as a treatment for ALS are felt to be needed (Hardiman and van den Berg 2017).

Nearly all antioxidant medications used in ALS clinical trials have so far been unable to dramatically slow disease progression. By contrast, pre-clinical studies using antioxidants to treat animal models of ALS has been largely successful in diminishing OS, slowing disease progression and extending lifespan (Barber and Shaw 2010; Niedzielska *et al.* 2016). One explanation for this discrepancy is that there has been a lack of well-designed, randomised, placebo-controlled antioxidant clinical trials. Instead, trials have mostly been of poor methodological quality and lacked statistical power (Orrell, Lane and Ross 2007; Beghi *et al.* 2011). In addition, many pre-clinical studies test compounds in mice at pre-symptomatic stages of disease, unlike the treatment of ALS patients, which begins after the onset of symptoms (McGoldrick *et al.* 2013). Another explanation is that the contribution of various pathogenic mechanisms within individual patients has not been well characterized, and disease heterogeneity is likely to account in part for the failure of over 50 major phase II and phase III clinical trials in patients with ALS, despite positive studies in animal models (Beghi *et al.* 2011). Furthermore, a limited number of clinical studies have fully investigated

oxidative damage or changes in the antioxidant defence status after antioxidant therapy, which makes interpretation of compound effectiveness difficult (Niedzielska *et al.* 2016).

While a huge number of exogenous small molecule antioxidants with widely varying chemical structures have been investigated as potential therapeutic agents in ALS, the use of most of these compounds has been limited. This is likely because a great deal of compounds tested do not cross the blood brain barrier (BBB) and access the CNS (Gilgun-Sherki, Melamed and Offen 2001). In addition, most antioxidant compounds reduce only one subtype of ROS (Orrell, Lane and Ross 2007). For example, despite coenzyme Q10's ability to cross the BBB, it specifically targets superoxide radicals and has no effect on the other ROS such as hydroxyl radicals or peroxides (Gilgun-Sherki, Melamed and Offen 2001; Phaniendra, Jestadi and Periyasamy 2015). Furthermore, free radical scavenging alone is unlikely to reverse damage to DNA, proteins and lipids that would have accumulated by the time of treatment (Uttara *et al.* 2009). To overcome these limitations, an alternative logical approach would be to activate endogenous antioxidants that target a range of pathways present in the appropriate cellular locale of the CNS (Joshi and Johnson 2012; Chan and Chan 2015). Therefore, identifying and understanding the protective roles of novel antioxidant proteins that regulate OS in motor neurons could be extremely important for the development of more effective treatments of ALS.

1.6 Oxidation resistance 1 is a critical regulator of OS-induced cell death

Oxidation resistance 1 (OXR1) was first identified in a screen for human genes that that were able to rescue the DNA oxidation repair-defective phenotype of a spontaneous *E. coli* mutant (Volkert, Elliott and Housman 2000). Furthermore, it was also demonstrated that deletion of the Oxr1 homologue in *S. cerevisiae* resulted in increased sensitivity to hydrogen peroxide treatment compared to controls (Volkert, Elliott and Housman 2000). Interestingly,

this phenotype was rescued by expression of mitochondria-targeted human OXR1 (Elliott and Volkert 2004). Taken together these studies were the first to suggest that OXR1 has an OS-related function that protects cells against oxidative damage. Consistent with the original findings in bacteria and yeast, the anti-oxidative role of OXR1 orthologues has also been demonstrated in several other organisms. For example, in *C. elegans*, mutations in the OXR1 homologue (LMD-3) resulted in increased sensitivity to oxidative and heat stress and reduced lifespan compared to wild-type strains. This suggests LMD-3 protects cells against oxidative stress and improves *C. elegans* survival (Sanada *et al.* 2014). In *Drosophila*, overexpression of Oxr1 from silkworm (*B. mori*) significantly extended the lifespan of *Drosophila* fed on a diet with and without hydrogen peroxide solution (Kobayashi *et al.* 2014). This suggests that Oxr1 is beneficial for survival, both under induced OS and physiological conditions.

It has also been demonstrated that Oxr1 is essential for cell survival and CNS function in mammals. The *bella* mouse mutant, which contains a homozygous spontaneous deletion of the entire *Oxr1* loci, display increased levels of oxidative damage, degeneration of cerebellar granule cells (GCs), rapidly progressive ataxia followed by death by P24 (Oliver *et al.* 2011). This severe phenotype was completely rescued by ubiquitous expression of an Oxr1 transgene, confirming that the phenotype presented by the *bella* mutant was due to loss of *Oxr1* (Oliver *et al.* 2011). Furthermore, the evidence of oxidative damage occurring in the absence *Oxr1* was supported by additional *in vitro* experiments whereby cultured GCs from *bella* mutants were found to be more susceptible to cell death when exposed to OS (Oliver *et al.* 2011). These findings were also recapitulated when all Oxr1 isoforms were knocked-down by an shRNA to <10% of endogenous levels in wild-type GCs, supporting that Oxr1 levels are important for neuronal survival under OS conditions (Oliver *et al.*, 2011).

The anti-oxidative and protective role of OXR1 has also been demonstrated in human cell lines as well. For example, depletion of OXR1 was shown to sensitize several human cell lines to hydrogen peroxide-induced OS, reduce mitochondrial DNA integrity and increase apoptosis (Yang *et al.* 2014). Furthermore, expression of cyclin dependent kinase inhibitor 1A (p21) and the antioxidant genes GPX2 and HO-1 were reduced in OXR1-depleted cells (Yang *et al.* 2014). This suggests OXR1 regulates the expression of antioxidant genes via the p21 signalling pathway to suppress hydrogen peroxide-induced OS and maintain mitochondrial DNA integrity. A second example, using the human retinal Müller glial cell line (MIO-M1), it was shown that overexpression of Oxr1 attenuated an increase in 3-nitrotyrosine levels and reduced apoptosis when treated with 4-HNE, to induce OS conditions (Murray *et al.* 2013). Taken together, these studies further exemplify the importance of OXR1 in maintaining redox homeostasis and regulating cell survival in response to OS.

Increasing evidence suggests that Oxr1 may play an indirect regulatory role in OS response pathways by modulating the activity of effector proteins. First, *in silico* pathway analysis performed on Oxr1 binding partners suggested that Oxr1 interacts with proteins involved in mechanistic target of rapamycin (mTOR) and eukaryotic initiation factor 2 (EIF2) pathways, both of which play an important role in the OS response (Ron and Harding 2007; Teske *et al.* 2011; Maiese *et al.* 2012; Finelli *et al.* 2015). Second, a study using mosquitoes (*A. cambiae*) revealed that injection of hydrogen peroxide into female mosquitoes induced expression of several antioxidant enzymes including catalase and Gpx, c-Jun N-terminal kinase (JNK) and Oxr1 (Jaramillo-Gutierrez *et al.* 2010). Interestingly, silencing the mosquito homologue of OXR1 significantly decreased the expression of both catalase and Gpx by 92% and 82%, respectively (Jaramillo-Gutierrez *et al.* 2010). Furthermore, silencing JNK, a gene normally activated by OS, resulted in decreased Oxr1

expression (Jaramillo-Gutierrez *et al.* 2010). Taken together, these studies suggest OXR1 directly influences the activity of known cellular antioxidants and may also act downstream of the JNK pathway.

1.6.1 Oxr1 contains an evolutionary conserved TLDc domain

Oxr1 is found in all eukaryotic genomes and is expressed as many different isoforms, all of which contain the highly conserved C-terminal Tre2/Bub2/Cdc16 (TBC), Lysin motif (LysM), domain catalytic (TLDc) domain (Volkert *et al.* 2000; Durand *et al.* 2007). The full-length isoform (Oxr1-FL) has 3 known domains: LysM, a Glucosyltransferases, Rab-like GTPase activators and Myotubularins (GRAM) and the TLDc domain; whereas the shortest isoform (Oxr1-C) contains almost exclusively the TLDc domain (Figure 1.3A; Volkert *et al.* 2000; Fischer *et al.* 2001; Shkolnik *et al.* 2008). The TLDc domain consists of ~200 amino acids and is believed to show a combination of anti-parallel β -sheets and α -helices in a conformation distinct from any other known structures (Blaise *et al.* 2012). Originally hypothesized to react directly with ROS and have enzymatic activity, it was later demonstrated that while Oxr1 can directly react with hydrogen peroxide through oxidation of a conserved C-terminal cysteine residue (cys753), the rate of this reaction was not in the catalytic range (Doerks *et al.* 2002; Oliver *et al.* 2011). Furthermore, OXR1 protein does not possess any catalase or superoxide dismutase activity (Sanada *et al.* 2014). Together, this suggests that oxidation of Oxr1 has a more important functional and/or regulatory role than enzymatic activity.

The importance of the TLDc domain for the function of Oxr1 has been demonstrated by a number of studies. Firstly, *in vitro* peroxide sensitivity assays have demonstrated that Oxr1-C is as potent an antioxidant and conferred the same levels of neuroprotection against OS-induced cell death as Oxr1-FL (Oliver *et al.* 2011). Secondly, *in vivo* studies using a

gene-trap mouse that only express shorter isoforms of the Oxr1 gene, it was demonstrated that mice homozygous for the gene-trap insertion had almost completely ablated expression of Oxr1-FL, but importantly displayed no gait or pathological abnormalities in the CNS up to 12 months of age (Oliver *et al.* 2011). By contrast, an insertional mouse mutant that removes the last 101 amino acids of the TLDc domain of Oxr1 presented a phenotype that is identical to the recessive Oxr1 deletion *bella* mouse (Finelli *et al.* 2016). Taken together, these studies strongly support that the TLDc domain is critical for the function of Oxr1 and its protective role against OS-induced cell death.

Recently, a structural-functional study identified highly conserved critical residues in the TLDc domain important for its neuroprotective function and the OS response. For example, Glu773 in Oxr1-FL and Glu-293 in Oxr1-C are one of the most evolutionarily conserved residues in the TLDc domain and likely influence the position of the N-terminal region of the TLDc domain. Interestingly, oxidative stress assays using neuronal cells expressing Oxr1-C (Glu293Ala) mutants revealed that the proportion of cell death was significantly increased (~2-fold) compared to those expressing wild type Oxr1-C, suggesting that altering this amino acid was functionally disruptive. Furthermore, the same mutation in Oxr1-FL (Glu773Ala) almost completely abolished the protective activity of the protein, highlighting how the context of the TLDc domain within a larger protein structure may impart different functional properties.

1.6.2 Oxr1 belongs to a family of TLDc domain-containing proteins

Oxr1 is one member of a TLDc domain containing protein family, which also includes nuclear receptor coactivator 7 (NCOA7), TBC1 domain family, member 24 (TBC1D24), TBC/LysM-Associated Domain-Containing Protein 1 (TLDC1; also referred to as KIAA1609) and chromosome 20 open reading frame 118 (C20ORF118; Figure 1.3A;

Durand et al. 2007; Shkolnik et al. 2008). According to the alignment of TLDC domain containing proteins, NCOA7 is the closest family member to OXR1 (Shkolnik *et al.* 2008). Furthermore, similar to OXR1, NCOA7 exists as a full-length isoform (NCOA7-FL) and a shorter isoform (NCOA7-B; Figure 1.3A; Shkolnik et al. 2008). Interestingly, *in vitro* studies quantifying viability of neuroblastoma-2a (N2a) cells transfected with either full-length TLDC constructs or Ncoa7-B or Oxr1-C under arsenite treatment demonstrated that all TLDC domain-containing proteins are able to confer protection against OS-induced cell death (Finelli *et al.* 2016)

Systematic expression data of TLDC domain-containing genes in embryonic and adult tissues has demonstrated that each gene displays a distinct expression profile with some overlap between certain tissues and cell types (Finelli *et al.* 2016). For example, Oxr1-FL, Oxr1-C and Ncoa7-FL are the most highly expressed TLDC genes in the CNS, suggesting a tissue specific function of these isoforms (Figure 1.3B; Finelli et al. 2016). Furthermore, it was also demonstrated in several tissues that the relative expression of Oxr1-FL and Ncoa7-FL was significantly higher than their corresponding short isoforms (Oxr1-C and Ncoa7-B), suggesting that these specific isoforms are independently regulated via distinct promoters and is likely to be a reflection of their potentially different functions (Figure 1.3B; Finelli et al. 2016).

Using *in situ* hybridization to determine the anatomical localization of the TLDC domain-containing genes during embryonic development, it was demonstrated that Oxr1 and Ncoa7 are expressed throughout the developing embryo (E14.5), whereas Tbc1d24 expression is more localized to the brain, spinal cord, and liver (Figure 1.3C; Finelli et al. 2016). In addition, Kiaa1609 and C20orf118 were virtually undetectable at this developmental time point (Figure 1.3C; Finelli et al. 2016). Finally, it was also demonstrated that Oxr1, Ncoa7 and Tbc1d24 are expressed in all major structural regions in the adult brain,

particularly in the cortex, hippocampus, and cerebellum. However, Ncoa7 expression was found to be enriched in Purkinje cells as opposed to Oxr1 and Tbc1d24 expression, which was enriched in the cerebellar granule cell layer (Figure 1.3C), suggesting a specific role for Ncoa7 in this cell type. While expression data suggests that each gene in the TLDC domain-containing gene family has distinct expression profiles, the specific function of each of these genes remains largely unknown.

The importance of the TLDC domain to normal human brain development and function has been recently exemplified by the identification of mutations in the TLDC domain of TBC1D24 in patients with myoclonic, focal or generalised epilepsy, lethal early-onset epileptic encephalopathy and deafness, onychodystrophy, osteodystrophy, mental retardation syndrome (DOORS; Philippe M Campeau et al. 2014; Philippe M. Campeau et al. 2014; Mucha et al. 2015; Balestrini et al. 2016). Therefore, understanding the molecular mechanisms that govern the function of the TLDC domain will be important to understand its role in the OS response and a wide range of neurological diseases.

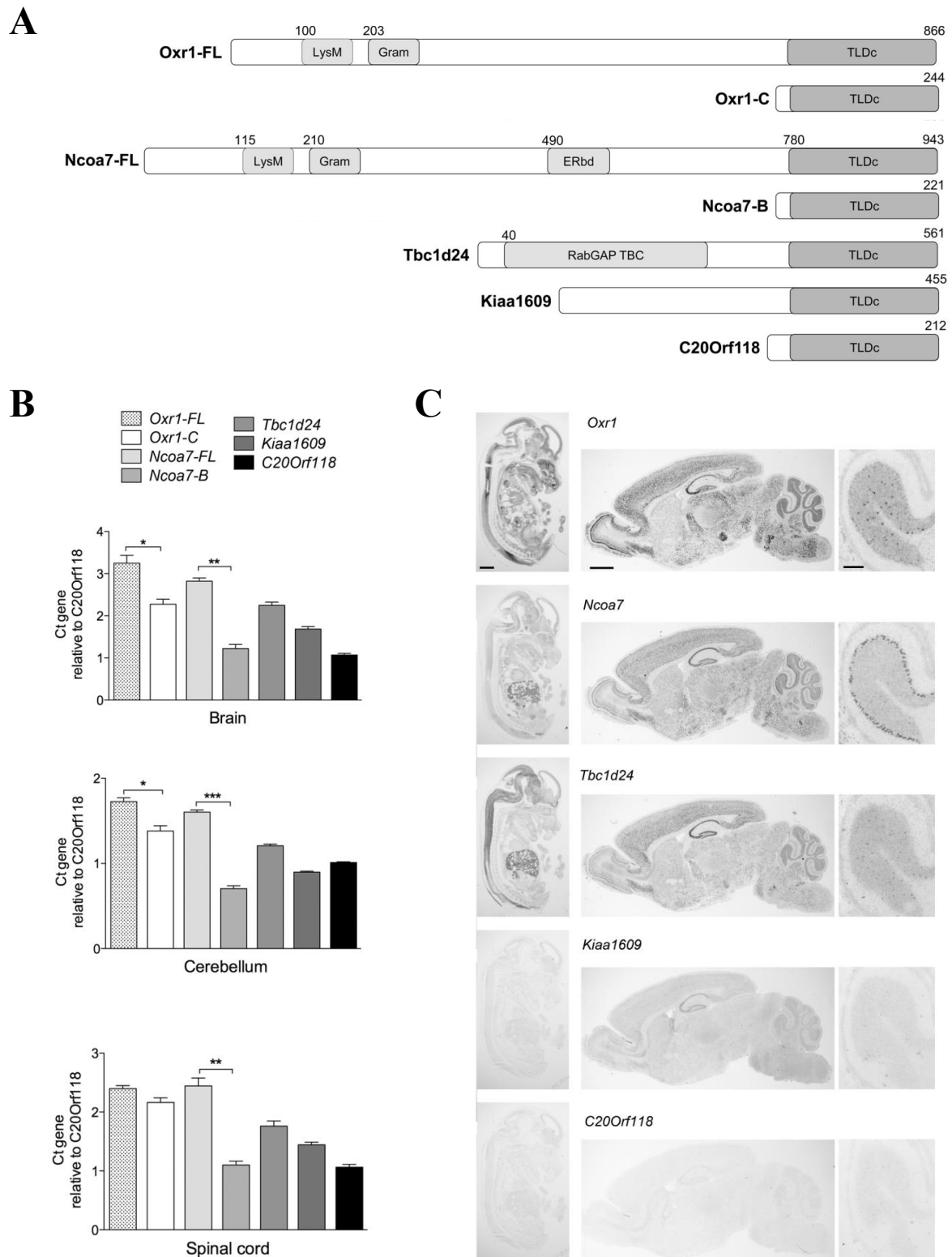


Figure 1.3. Schematics and expression of TLDC domain-containing proteins in embryonic and adult mouse. (A) A schematic illustration of the TLDC domain containing proteins: Oxr1-FL, Oxr1-C, Ncoa7-FL, Ncoa7-B, Tbc1d24, Kiaa1609 and C20Orf118. Both Oxr1-FL and Ncoa7-FL contain a LysM and GRAM domain; Ncoa7-FL also has an estrogen receptor binding domain (ERbd) and Tbc1d24 has a TBC domain similar to other RAB GTPase-activating protein (RabGAP) domains. **(B)** Expression of TLDC domain-containing

proteins in the CNS of adult male mice quantified by qRT-PCR. Data are shown relative to the expression of C20Orf118 and are represented as the means $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Student's *t* test. **(C)** *In situ* hybridization of TLDC domain-containing genes in the embryo (E14.5, left panel), adult brain (middle panel), or adult cerebellum (right panel) from wild-type male mice. Riboprobes were designed to span all isoforms of each gene. Scale bars: 1mm (left and middle panel) and 200 μ M (right panel). Figure adapted with permission from: M. J. Finelli, L. Sanchez-Pulido, K. X. Liu, K. E. Davies, P. L. Oliver (2016) "The evolutionary conserved Tre2/Bub2/Cdc16 (TBC), Lysin motif (LysM), Domain catalytic (TLDC) domain is neuroprotective against oxidative stress." *Journal of Biological Chemistry*, 291: 2751-2763.

1.6.3 The role of Oxr1 in mitochondria

Given that mitochondria are the major source of ROS within the cell, numerous antioxidant proteins have evolved to prevent or repair oxidative damage in this organelle (Takao *et al.* 1998; Arnér and Holmgren 2000). As mentioned earlier, it has been previously demonstrated that mitochondria-targeted OXR1 protects *S. cerevisiae* against oxidative damage (Elliott and Volkert 2004). However, a more recent study has demonstrated that specific isoforms of Oxr1 have distinct subcellular localisations. More specifically, out of the four main isoforms of Oxr1 detected in mouse brain homogenate, Oxr1-C (~24kDa) was almost exclusively localised to mitochondria; whereas Oxr1-FL (~84kDa) was almost exclusively localised in the cytosol (Wu, Davies and Oliver 2016). In addition, two intermediate isoforms (~55 and ~40kDa) were enriched in the mitochondrial fraction (Wu, Davies and Oliver 2016). Further to this, using a combination of ultracentrifugation and trypsin digestion assays, it was also demonstrated that the shorter isoforms of Oxr1 were found at the mitochondrial outer membrane (OM), facing the cytosol (Wu, Davies and Oliver 2016). Given that the human and mouse isoforms do not contain any known mitochondrial localisation signal (MLS), it is currently unclear how Oxr1 is translocated to this position (Wu, Davies and Oliver 2016). Nonetheless, taken together, these data suggest that only the shorter isoforms of Oxr1 are localised to and therefore likely function in the mitochondria.

Recent studies have also demonstrated that Oxr1 is involved in maintaining mitochondrial morphology in the stress response as part of its antioxidant function. For example, overexpression of Oxr1 in the cytoplasm of negroid cervix epitheloid carcinoma (HeLa) cells was capable of preventing morphological changes to mitochondria induced by rotenone (Wu, Davies and Oliver 2016). In agreement, using primary GCs from *bella* mice, it was also shown that while loss of Oxr1 did not affect mitochondrial DNA integrity, ETC protein activity or mitochondrial metabolism, it did cause a reduction in mitochondrial

length, a feature commonly observed in models of ALS as well (Tradewell *et al.* 2012; Wu, Davies and Oliver 2016). Furthermore, screening a panel of proteins that regulate mitochondrial morphology in *bella* GCs revealed increased expression of phospho-dynamin-related protein 1 (p-Drp1), a key mitochondrial fission regulatory factor (Wu, Davies and Oliver 2016). As mentioned in section 1.3.2, mitochondrial morphology is tightly regulated by fusion and fission processes and multiple lines of evidence have shown that under OS, the fission protein Drp1 is activated to facilitate mitochondrial fragmentation (Taguchi *et al.* 2007; Qi *et al.* 2011). Taken together, these studies highlight potential novel functions of Oxr1, revealing that regulating mitochondrial morphology may be an important feature of its protective function.

1.6.4 The role of Oxr1 in ALS

There is increasing evidence that suggests OXR1 could play an important role in ALS-associated pathogenesis. Firstly, a study investigating OXR1 expression in post-mortem spinal cord samples revealed that intermediate isoforms of OXR1 are upregulated in ALS patients (Figure 1.4A; Oliver *et al.* 2011). This observation was somewhat supported by additional *in vivo* studies demonstrating a threefold increase of Oxr1-FL expression in pre-symptomatic SOD1^{G93A} mice (Figure 1.4A; Oliver *et al.* 2011). Together, this suggests that Oxr1 up-regulation may be a consequence of neurodegeneration in the spinal cord; but more importantly, Oxr1 may serve as an early marker of neurodegeneration before any overt phenotypic abnormalities occur.

Following on from these findings, more recent studies have begun to focus on the potential neuroprotective role of Oxr1 in SOD1-related ALS. By crossing a transgenic mouse line overexpressing the full length isoform of Oxr1 in the CNS with the SOD1^{G93A} mouse model of ALS, it was shown that overexpression of Oxr1 extended the lifespan of SOD1^{G93A}

mice by 25-29 days (Liu *et al.* 2015). Longitudinal behavioural analysis demonstrated that overexpression of Oxr1 improved motor deficits and muscle strength of SOD1^{G93A} mice (Figure 1.4B; Liu *et al.* 2015). Furthermore, subsequent pathological analysis revealed that overexpression of Oxr1 delayed the expression of OS-related marker HO-1, improved motor neuron survival (Figure 1.4C & D), reduced NMJ abnormalities and delayed muscle pathology (Liu *et al.* 2015). Taken together, these findings identified OXR1 as the first neuron-specific antioxidant modulator of pathogenesis and disease progression in ALS.

Interestingly, using transcriptomic analysis of spinal cords from SOD1^{G93A} mice, Liu *et al.* further demonstrated that overexpression of Oxr1 rescued the expression of 63 deregulated genes in the spinal cord of SOD1^{G93A} mice (Liu *et al.* 2015). Pathway analysis of these genes revealed that overexpression of Oxr1 influenced a range of pathogenic mechanisms that have been previously found to be deregulated in ALS, such as immune cell trafficking (Olsen *et al.* 2001; Ferraiuolo *et al.* 2007; Liu *et al.* 2015). In addition, quantitative immunohistochemical staining revealed overexpression of Oxr1 decreased astrogliosis and microgliosis in the spinal cord of SOD1^{G93A} mice, suggesting that Oxr1 decreases pathological neuroinflammation (Liu *et al.* 2015). This was supported by additional gene expression analysis demonstrating that overexpression of Oxr1 delayed activation of the classic complement system and signal transducer and activator of transcription 3 (STAT3), a key regulator of the inflammatory response (Liu *et al.* 2015). Interestingly, STAT3 is activated in ALS patients (Shibata *et al.* 2009). Together, this suggests that overexpression of Oxr1 is sufficient to modulate the neuroinflammatory response by regulating the activation of multiple pathways in the spinal cord of SOD1^{G93A} mice.

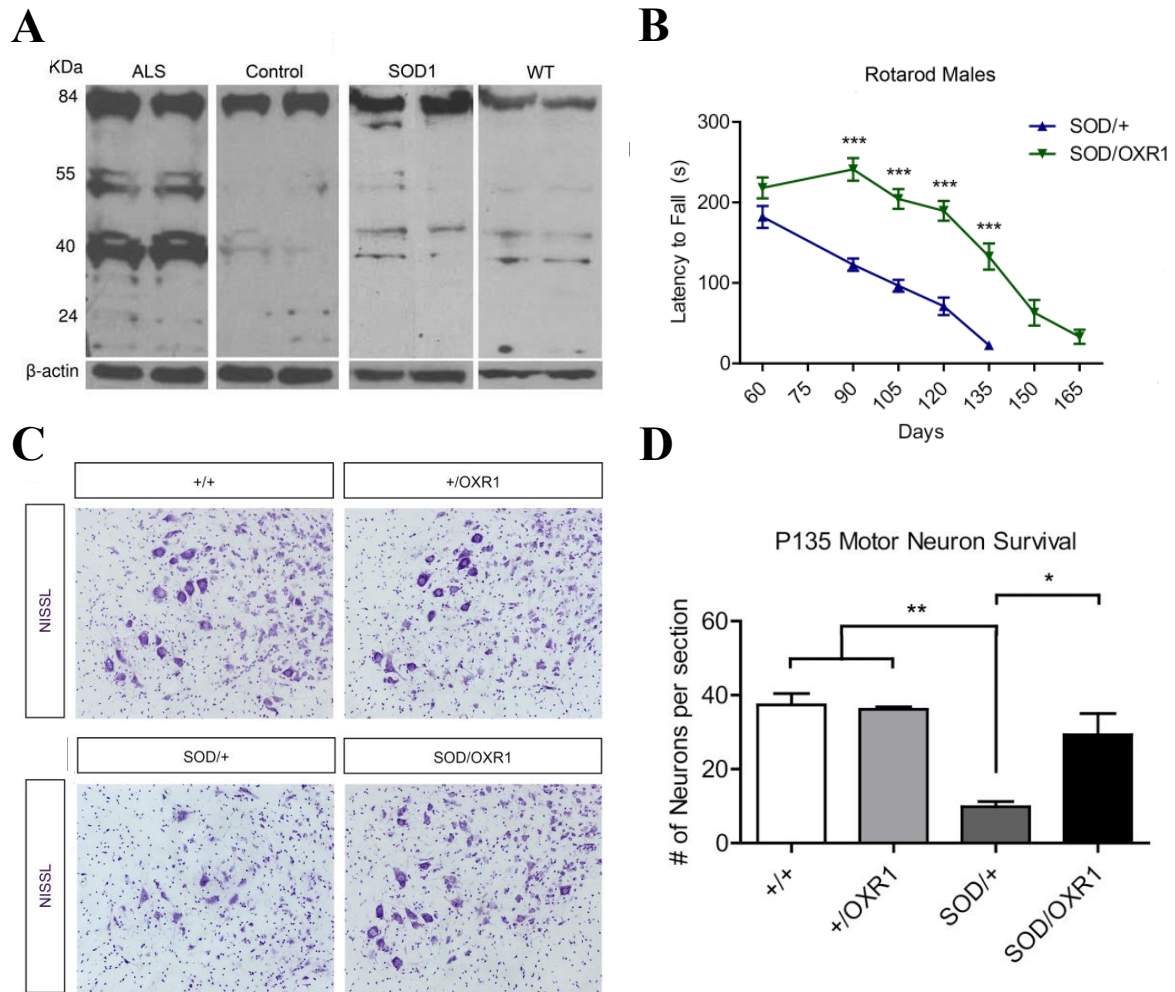


Figure 1.4. Oxr1 is upregulated in human ALS and has a protective role against ALS-associated pathogenesis. (A) Western blots showing up-regulation of intermediate isoforms of OXRF1 in spinal cord *post mortem* samples from ALS patients and upregulation of Oxr1-FL in spinal cord tissue from pre-symptomatic SOD1^{G93A} mice. (B) Male SOD/OXR1 mice display improved motor performance on the accelerating rotarod when compared with SOD/+ males. Values are mean $\pm$ SEM of motor performance (seconds, s) for mice still alive at each respective time point (n = 9–11 per sex per genotype at Days 60–120 and n = 6–11 per sex per genotype at Days 135–165; *P<0.05, **P<0.01, ***P<0.001, Mann-Whitney U-tests). (C) Representative images of Nissl-stained motor neurons in matching lumbar spinal cord cross-sections at Day 135. (D) Motor neuron survival counts in lumbar spinal cord cross-sections at Day 135. Values are mean $\pm$ SEM (n = 3–4 per genotype; *P<0.05, **P<0.01; 1-way ANOVA, with Tukey's post hoc tests). Figure adapted with permission using figures from P. L. Oliver, M. J. Finelli, B. Edwards, E. Bitoun, D. L. Butts, E. B. E. Becker, M. T. Cheeseman, B. Davies, K. E. Davies (2011). "Oxr1 Is Essential for Protection against Oxidative Stress-Induced Neurodegeneration." *PLoS genetics*, 7: e1002338 and K. X. Liu, B. Edwards, S. Lee, M. J. Finelli, B. Davies, K. E. Davies, P. L. Oliver (2015). "Neuron-specific antioxidant OXRF1 extends survival of a mouse model of amyotrophic lateral sclerosis". *Brain*, 138: 1167-81.

Further evidence for the importance of Oxr1 in ALS pathology is provided by a previous study that used an unbiased proteomic approach in a neuronal cell line (Finelli *et al.* 2015). Following co-immunoprecipitation of tagged Oxr1-FL or Oxr1-C, mass spectrometry was used to identify the associated binding proteins of each isoform in the presence or absence of OS (Finelli *et al.* 2015). Oxr1-C had fewer binding partners in non-treated (3) compared with hydrogen peroxide-treated conditions (38). By contrast, Oxr1-FL had a similar number of binding partners in non-treated (16) and treated conditions (13; Finelli *et al.* 2015). Of these proteins, it was revealed that Fus interacted with Oxr1-FL; whereas Fus and Tdp-43 interacted with Oxr1-C (Finelli *et al.* 2015). Furthermore, it was also demonstrated that specific ALS-associated mutations alter Fus and Tdp-43 binding to Oxr1 (Finelli *et al.* 2015). For example, A321G and D169G mutations in Tdp-43 caused a decrease in binding to Oxr1-C; whereas Q331K and M337V mutations did not affect the interaction with Oxr1-C (Finelli *et al.* 2015). Furthermore, *in silico* pathway analysis on Oxr1 binding partners surprisingly revealed that Oxr1 was predicted to affect other cellular functions, including RNA post-transcriptional modification, protein synthesis, and inflammatory response (Finelli *et al.* 2015). Given all of these processes have been implicated in ALS, it is tempting to speculate that Oxr1 could potentially modulate ALS pathology by regulating these processes.

As mentioned previously, OS is known to promote aggregation of proteins, especially RNA-binding proteins that function in the stress granule response under OS conditions (see sections 1.3.3 and 1.3.4). Given that TDP-43 and FUS are mislocalised and found in cytoplasmic aggregates in ALS, and given that Tdp-43 and Fus were found to interact with Oxr1, Finelli *et al.* also investigated whether Oxr1-C affects cellular localization of wild-type and mutant Tdp43 or Fus under OS conditions. First, it was demonstrated that knockdown of all endogenous OXR1 isoforms by ~87.9% resulted in

increased percentage of cells with Fus- and Tdp-43-positive cytoplasmic inclusions (Finelli *et al.* 2015). Second, it was also demonstrated that co-expression of Oxr1-C in cells expressing either wildtype or mutant Fus or Tdp-43 decreased the cytoplasmic aggregation of Fus or Tdp-43 protein (Finelli *et al.* 2015). Importantly, this decrease in cytoplasmic aggregation was only observed for mutant proteins with a preserved binding for Oxr1, such as Tdp-43^{M337V} or Tdp-43^{Q331K} (Finelli *et al.* 2015). This suggests that the interaction between Oxr1 and Tdp-43 or Fus is important for proper cellular localization of Fus and Tdp-43, and overexpression of Oxr1 can decrease Tdp43 and Fus protein aggregation. Furthermore, it was shown that the percentage of cells with stress granules was not affected by knock-down of OXR1 or over-expression of Oxr1-C, suggesting that Oxr1 itself does not affect stress granule formation (Finelli *et al.* 2015). Taken together, these findings strongly support that Oxr1 can modulate ALS pathology *in vivo* and may have therapeutic benefits for the treatment of ALS and related neurodegenerative disorders with SOD1, FUS and TDP-43 pathology.

1.7 Aims of Thesis

Given that loss of *Oxr1* in *bella* mice causes neurodegeneration that is specific to the cerebellum and mutants only survive up to 4 weeks, these mice were not informative regarding the susceptibility of other neuronal populations to loss of *Oxr1* (Oliver *et al.* 2011). It has been proposed that the large size and therefore high metabolic demand of motor neurons renders them more susceptible to OS due to increased reactive ROS generated by mitochondria (Barber, Mead and Shaw 2006). Therefore, it could be postulated that specific loss of Oxr1 in motor neurons will increase their susceptibility to OS and neurodegeneration. In addition, while it has been consistently demonstrated that overexpression of Oxr1 can have beneficial effects in reducing OS, improving ALS-associated cellular pathology and

increasing neuronal survival in the SOD1^{G93A} ALS mouse model, the exact function and importance of Oxr1 in motor neurons and its neuroprotective potential in non-SOD1 related ALS *in vivo* is still not known. Furthermore, while Oxr1 is proving to be an attractive potential therapeutic target, little is known about the transcriptional regulation of Oxr1 expression. Therefore, to address these questions, this project has focused on determining the importance of Oxr1 regulation and its function in motor neurons in different forms of ALS using cellular and mouse models.

My three principal aims were:

- 1) To determine the importance of Oxr1 in motor neurons *in vivo*.
 - Behaviourally and pathologically characterise a conditional mouse mutant where *Oxr1* is selectively deleted in motor neurons.
- 2) To investigate the neuroprotective function of Oxr1 in TDP-43-related ALS *in vivo*.
 - Behaviourally and pathologically characterise a new mouse mutant model of TDP-43 in the presence or absence of increased expression of Oxr1.
 - Utilise primary motor neurons from TDP-43^{M337V} mutant mice and assess whether the manipulation of Oxr1 expression affects ALS-related phenotypes.
- 3) To investigate the transcriptional regulation of Oxr1 and determine whether endogenous Oxr1 expression can be manipulated as a protective strategy.
 - Characterise Oxr1 promoter regions.
 - Develop a luciferase reporter-based cell assay to screen a library of compounds and identify those that are able to activate the Oxr1 promoter.

Chapter 2

Materials and methods

2.1 Ethics statement

All experiments were conducted in adherence to the guidelines set forth by the UK Home Office regulations and with the approval of the University of Oxford Ethical Review Panel.

2.2 Animals and housing

Oxr1^{tm1a(EUCOMM)Wtsi} knockout-first conditional allele mice have LoxP sites flanking (flox) exon 15 and 16 of Oxr1, and were generated by the Wellcome Trust Sanger Institute and maintained on a C57BL/6 background. Transgenic mice with β -actin driving FLPe variant recombinase (B6.SJL-Tg(ACTFLPe)9205Dym/J) (Jackson Laboratory; <https://www.jax.org/strain/003800>) were maintained on a C57BL/6 background. Rosa26 locus lacZ reporter mice (B6;129S4-48Gt(ROSA)26Sortm1Sor/J) (Jackson Laboratory; <https://www.jax.org/strain/003309>) were maintained on a C57BL/6 background and used to detect expression pattern of specific promoter-driven Cre transgene. ChAT-Cre transgenic mice (GENSAT, GM53), in which Cre recombinase is expressed solely in ChAT-expressing motor neurons under the control of the bacterial artificial chromosome (BAC) containing the endogenous *ChAT*-promoter (Gong *et al.* 2007) was maintained on a C57BL/6 background. Breeding schemes paired Oxr1^{tm1a/+} mice with Tg(ACTFLPe) mice to generate progeny with the conditional allele (tm1c), named Oxr1^{tm1c/tm1c} mice. Oxr1^{tm1c/tm1c} mice were then paired with ChAT-Cre transgenic mice to generate litters consisting of control Oxr1^{tm1c/tm1c/+} mice and experimental Oxr1^{tm1c/tm1c}/ChAT-Cre mice, in which Oxr1 expression is ablated in motor neurons.

Tg(Prnp-Oxr1)/+ mice, which were generated by Dr. Ben Davies (Wellcome Trust Centre for Human Genetics) as previously described (Oliver *et al.* 2011), overexpress a HA-tagged Oxr1 transgene driven by the mouse Prnp promoter (Borchelt *et al.* 1996), and were maintained on a C57BL/6 background. Transgenic TDP-43^{M337V} BAC mice, overexpressing a YPET-tagged TDP-43 protein harbouring the M337V mutation, were obtained from Prof. Kevin Talbot (Nuffield Department of Clinical Neuroscience, Oxford) and maintained on a C57BL/6 background. Transgenic TDP-43^{M337V} BAC mice are also available from The Jackson Laboratory as strain JAX#029266 (<https://www.jax.org/strain/029266>). For quantitative behavioural and pathological analyses, breeding schemes paired heterozygous TDP-43^{M337V/+} males with heterozygous TDP-43^{M337V/+}/Oxr1^{Prnp/+} females to produce litters consisting of control non-transgenic (NTg) and Oxr1^{Prnp/+} mice, and experimental homozygous TDP-43^{M337V/M337V} and homozygous TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} animals. Mice from several litters were required to generate each experimental cohort; for quantitative behavioural and pathological analyses, all cohorts were age and sex-matched. For primary motor neuron cultures, breeding schemes paired homozygous TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} males with NTg females to produce litters consisting of heterozygous TDP-43^{M337V/+} and heterozygous TDP-43^{M337V/+}/Oxr1^{Prnp/+} mice. Upon tissue collection at various ages, genotypes for all mouse strains were reconfirmed using fresh tail biopsies. The day of plug was designated as embryonic day 0.5 (E0.5), and the day of birth was designated as postnatal day 0 (P0). Mice received food and water *ad libitum*, and were housed on a 12-hour on/off light cycle.

2.3 Behavioural experiments

2.3.1 Accelerating rotarod test

Cohorts of age-matched, sex-matched mice were tested for time to fall from the accelerating rotarod. Mice were placed on a beam of a rotarod device facing in an orientation opposite to rotation. The rod accelerated from 5-40rpm over a 5-minute period and the time latency to fall from the rod (or two full rotations on the rod without an attempt to continue running) was recorded. Mice were tested for 1 trial each day for 3 consecutive days and an average time for the 3 trials was calculated at each testing age (3, 6, 9, 12, 15 and 18 months).

2.3.2 Grip strength

The four-limb hanging wire test was used to measure muscle strength. Mice were placed in the centre of a metal wire mesh grid, which was then gently inverted such that the mice gripped on in an upside-down position. The grid was held fixed at a height of 40cm above a horizontal surface and the time latency to fall from the grid was recorded over a 2-minute maximum period.

2.4 Histopathology

2.4.1 Tissue Collection and Preparation

For fresh frozen tissue collection, mice were sacrificed by cervical dislocation. For perfused tissue collection, mice were given a lethal intraperitoneal injection of pentobarbitone, followed by transcardial perfusion with 0.9% saline containing 10 U/ml heparin, and fixation with 4% paraformaldehyde (PFA). Perfusions were performed by Prof Peter Oliver. Brains, spinal cord and sciatic nerves were dissected and post-fixed overnight in 4% PFA at 4°C. Tissue was passed through increasing concentrations of sucrose solution over a number of days and frozen in cryopreservation medium O.C.T (VWR) on dry ice.

Hind-limb muscles were freshly dissected and frozen in O.C.T. medium on isopentane in liquid nitrogen. All tissue was stored at -80°C until used. Frozen transverse sections were cut on a cryostat (-18°C ; Leica) at $15\mu\text{m}$ for brain and spinal cord and $10\mu\text{m}$ for muscle. Frozen longitudinal sections were cut $5\mu\text{m}$ for sciatic nerve. Lumbrical muscles were dissected as previously described (Sleigh et al. 2014; dissection steps summarized in Figure 2.1).

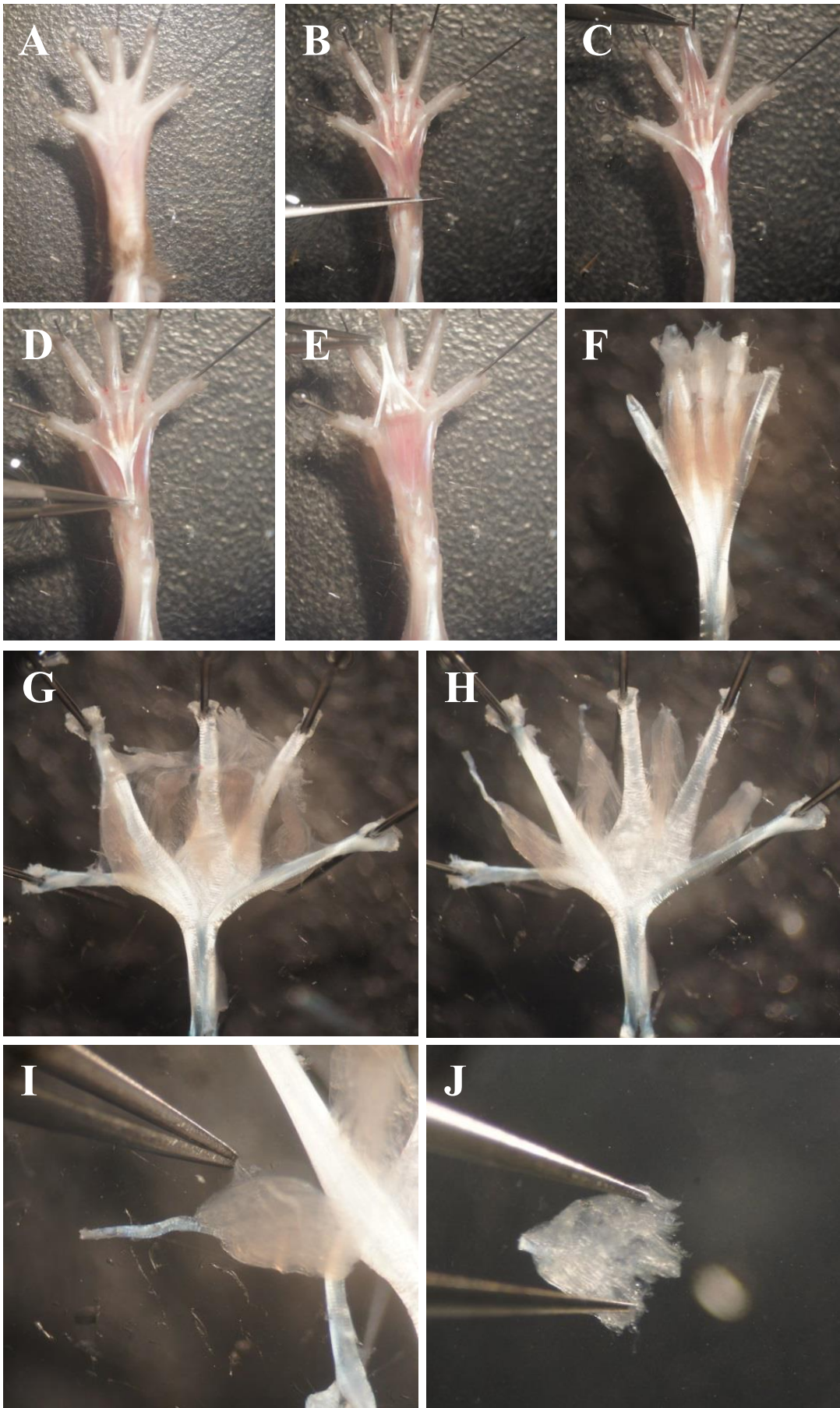


Figure 2.1 Dissection of hind limb lumbrical muscle. The left hind leg was pinned out ventral side up **(A)**, and the remaining plantar skin dissected away revealing the tendons in the foot **(B)**. The overlying flexor digitorum brevis (FDB) tendon was cut transversely close to the ankle, lifted up towards the digits, and removed completely by cutting the distal ends of the tendon at the level of the metatarsophalangeal joints **(C)**. The underlying flexor digitorum longus (FDL) tendon, from which the lumbricals originate, was then visible **(D)**. This tendon was also cut proximally and pulled back towards the digits **(E)**. The tendons, along with connective tissue and the distal ends of the lumbricals, were cut close to the base of the digits and the plantar muscle mass lifted out **(F)**. The FDL tendon was then spread and tautly pinned ventral side up **(G)**. The connective tissue was carefully cleared, revealing the underlying lumbricals between the tendon ends **(H)**. Excess connective tissue was removed by blunt dissection in the regions between the lumbricals and the tendons **(I)**, before fixation. Lumbrical muscles were excised and further connective tissue was removed. Once cleaned up, the distal FDB tendon was disconnected, and the muscle was ready for staining **(J)**. Lumbrical muscles were dissected from an 18-month old $Oxr1^{tm1c/tm1c/+}$ mouse in this figure.

2.4.2 Spinal cord histology

For nissl staining, spinal cord tissue sections were air dried, fixed in 100% ethanol and hydrated with a series of increasing alcohol dilutions for 30 seconds each until washing in distilled water. Sections were subsequently stained for 3 minutes with 0.5% cresyl violet solution and rinsed in distilled water. The sections were then dehydrated with a series of ethanol dilutions (75, 90 and 100%) before being washed in HistoClear (National Diagnostics) for 20 minutes and covered with Histomount (National Diagnostics) and a glass coverslip. All slides were viewed on an Axioskop 2 microscope (Zeiss), images were acquired using an AxioCam HR digital camera (Zeiss), and processed using AxioVision software (Zeiss) and ImageJ (Fiji). For motor neuron counts, all ventral horn motor neurons on at least 24 matched nissl-stained sections of lumbar spinal cord segments (L1-L6) were counted per mouse.

2.4.3 Muscle histology

For haematoxylin and eosin (H&E) stain, fresh muscle sections were placed in haematoxylin for 10 minutes and rinsed in distilled water. Sections were subsequently bleached in 70% ethanol/0.1% hydrochloric acid for 10 seconds, and washed in distilled water for 5 minutes. Sections were then placed in eosin for 10 seconds and washed in distilled water for 5 minutes. Sections were then dehydrated in a series of ethanol dilutions and placed in HistoClear for 20 minutes before being covered with Histomount and a glass coverslip. All slides were viewed on an Axioskop 2 microscope, images were acquired using an AxioCam HR digital camera, and processed using AxioVision software and ImageJ. For centrally nucleated fibre (CNF) analysis, 1 section per mouse was used for CNF counts and divided over the total number of fibres in the image.

2.4.4 Immunohistochemistry (IHC) of CNS

For antigen-retrieval, perfused tissue sections were boiled in sodium citrate buffer for 20 minutes in a microwave and washed in ddH₂O. For experiments using horseradish peroxidase (HRP), endogenous peroxidase was blocked by incubating sections in ~2% hydrogen peroxide solution in methanol for 15 minutes and then washed in ddH₂O. All sections were blocked in 5% normal goat serum/ 0.5% Tween-20 in phosphate buffered saline (PBS) for 1 hour at room temperature (RT). Sections were then incubated with primary antibodies diluted in block solution overnight at 4°C in a dark humidified chamber. After incubation with primary antibody (see table 3 for list of antibodies and dilutions used), sections were washed three times with PBS each for 5 minutes, followed by incubation with appropriate AlexaFluor® secondary antibodies (Invitrogen) at 1:200 for 2 hours at RT in a dark humidified chamber. After washing sections for 5 minutes three times in PBS, sections were covered with Vectashield mounting medium with DAPI (Vector Laboratories) and a glass coverslip, and stored in the dark at 4°C until imaged. For experiments using HRP, sections were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories) at 1:200 for 2 hours at RT. The sections were then washed in PBS three times for 5 minutes each followed by incubation with an avidin-biotinylated peroxidase complex for 1 hour at RT (Vectastain Elite ABC kit - Rabbit). Sections were then washed in PBS three times for 5 minutes each. Sections were then incubated in Imm pact 3, 3'-diaminobenzidine (DAB) solution (Vector Laboratories) for 2 minutes, immediately washed in ddH₂O for 5 minutes, dehydrated in a series of ethanol dilutions, washed in HistoClear for 20 minutes and covered in Histomount and a glass coverslip. Using a reference mouse brain atlas (Paxinos and Franklin 1997) to locate the facial motor nucleus (FMN), TUNEL staining for apoptosis was carried out on coronal sections of fresh-frozen brain with the *In Situ* Cell

Death Detection Kit, TMR Red (Roche) according to the manufacturer's protocol. After final wash with PBS, sections were covered with Vectashield mounting medium with DAPI and a glass coverslip. All slides were viewed on an Axioskop 2 microscope, images were acquired using an AxioCam HR digital camera, and processed using AxioVision software and ImageJ.

Table 2.1 Primary antibodies and dilutions used for IHC

Antibody	Host	Manufacturer	Dilution
Anti – ChAT (<i>Choline acetyltransferase</i>)	Goat	Millipore	1:200
Anti – GFAP (<i>Glial fibrillary acidic protein</i>)	Rabbit	Abcam	1:500
Anti – Iba1 (<i>Ionized calcium-binding adapter molecule 1</i>)	Rabbit	Wako	1:500
Anti – CD68 (<i>Cluster of Differentiation 68</i>)	Rabbit	Millipore	1:200
Anti – TDP-43	Rabbit	ProteinTech	1:2500

2.4.5 Lumbrical muscle IHC and NMJ imaging

Lumbrical muscles were stained as previously described (Sleigh *et al.* 2014). In brief, muscles were placed in a 96-well plate and permeabilised in 2% (v/v) Triton X-100 in PBS for 30 min. Muscles were then blocked in 4% (w/v) BSA and 1% (v/v) Triton X-100 in PBS for 30 min, before incubation with primary antibodies against neurofilament (2H3, 1:50, DSHB) and synaptic vesicles (SV2, 1:100, DSHB) diluted in blocking solution and left overnight at 4°C. The following day, muscles were washed in PBS three times each for 10 minutes before incubation with AlexaFluor® 488 secondary antibody (1:250) and 1.5µg/ml tetramethylrhodamine α -Bungarotoxin (α -BTX; Cambridge Bioscience) in PBS for 2 hours

in the dark. Muscles were then washed three times for 30 min in PBS, and mounted in Mowiol medium (Merck Millipore) on microscope slides with coverslips. To allow the Mowiol medium to set, slides were left at 4°C overnight and subsequently stored in the dark at 4°C until imaged.

For denervation analysis, NMJs were viewed using an Axioskop 2 microscope and were scored as being fully innervated (full overlap between the pre-synaptic nerve terminal and the post-synaptic endplate), partially innervated (>80% of regions of the post-synapse lacking neuronal input) or vacant (no pre-synaptic staining overlaying the endplate). A Leica TCS SP5 confocal microscope equipped with 30mW Argon and HeNe 543 nm lasers and a 63×/1.4NA Plan-Apo oil immersion objective was used to image representative examples of NMJ denervation and also NMJs for endplate analysis. Z-stacks were projected into single images using Leica Application Suite software and resulting ‘collapsed’ images used for all analyses. For all images, nervous tissue is labelled green (2H3 and SV2) and post-synaptic acetylcholine receptors (AChRs) are labelled red (α -Bungarotoxin (α -BTX)). Only NMJs with clearly visible presynaptic axons were scored. To measure NMJ area and intensity, the perimeter circumscribing the postsynaptic staining from ‘collapsed’ Z-stack images (24 sections, 0.5µm step size intervals, 11.6µm total stack depth) was drawn by hand and the enclosed surface area and intensity measured using ImageJ. ≥ 58 NMJs were scored per mouse for denervation analysis and 30-50 NMJs per mouse were scored for NMJ endplate analysis.

2.5 Primary motor neuron culture

To culture primary motor neurons, different steps of previously described protocols were optimised (Brewer and Torricelli 2007; Gingras *et al.* 2007; Taylor, Robinson and Milligan 2007; Wiese *et al.* 2010). Embryonic mice at E13.5 were extracted in sterile

conditions under a dissecting microscope, using small forceps and ice-cold Leibovitz's L15 medium (Invitrogen). Whole spinal cords were dissected, cleared of dorsal root ganglia (Figure 2.2) and transferred to individual 15mL conical centrifuge tubes (BD Falcon™) containing ice-cold L-15 medium. All remaining steps were carried out under a laminar flow hood at RT. To lightly digest tissue, spinal cords were incubated in 1.5ml trypsin solution (Gibco) for 10 minutes at 37°C. Trypsin digestion was then halted by incubating spinal cords in filter-sterilised trypsin inhibitor solution (0.8mg/ml; Sigma) for 5 minutes at RT. Spinal cords were then transferred to fresh 15ml falcon tubes containing 1.5ml filter-sterilised Neurobasal medium (Phenol red; Invitrogen) containing 10% horse serum (heat inactivated; Invitrogen), 1% B27 supplement (Invitrogen) and 1% Glutamax I (Invitrogen), hereinafter referred to as motor neuron growth medium (MNGM). Cells were dissociated by trituration using a 1ml Gilson pipette until the solution appeared homogeneous with no apparent tissue chunks. Care was taken to avoid triturating too vigorously (introducing bubbles) as this decreased motor neuron yield. 4.5ml of MNGM was added to the dissociated spinal cord cell suspension and subsequently passed through a 40µm cell strainer (BD Falcon™).

Purification of motor neurons was performed using density gradient centrifugation. Each layer of the density gradient was prepared as previously described (Brewer and Torricelli 2007), using OptiPrep density 1.32 (Sigma) in MNGM made with Neurobasal (without phenol red) to facilitate the distinction between different layers in subsequent steps. Mixing of the gradient layers was avoided by carefully letting each gradient solution slowly run out of a 5mL pipette, slide down the inner wall of each tube inclined at approximately 45°. To separate spinal cord cells, 6ml of cell suspension was carefully layered on top of the density gradient and was centrifuged at 800×g for 15 minutes at RT in a swinging bucket centrifuge without brake. Motor neurons were collected mostly as a white turbid band at the interface between the medium, which contained cellular debris, and the intermediate size

cells located at the two other interfaces below. Multiple interfaces served as a buffer to retain unwanted cells from the pellet. Some motor neurons were found at the second and third interface however collection of these fractions decreased motor neuron purity. Care was also taken to not collect the pellet as this contained smaller sized cell populations such as microglia.

To wash motor neurons, the motor neuron suspension was made up to 8ml with fresh MNGN in 15ml falcon tubes and centrifuged at 200×g for 3 minutes at RT in a swinging bucket centrifuge. The motor neuron pellet was re-suspended in 1ml of MNGM and cell number was determined. Motor neurons were seeded at a density of 20,000 cells/cm² on sterilised glass coverslips (10mm; Thermo Fisher Scientific) pre-coated in poly-L-ornithine (sigma) in 24-well plates (BD Falcon™). Motor neurons were allowed to attach in a humidified 37°C/ 5% CO₂ incubator for 1 hour, before MNGM was replaced with 500µl/well of fresh MNGM supplemented with 10ng/ml brain derived neurotrophic factor (BDNF; PeproTech), glial derived Neurotrophic Factor (GDNF; PeproTech), neurotrophin-3 (NT-3; PeproTech) and 25ng/ml of ciliary neurotrophic factor (CNTF; PeproTech). The entire procedure from animal sacrifice to motor neuron plating was performed within 4 hours to improve motor neuron yield. After 3-days *in vitro* (DIV), 50% of medium was removed and replaced with fresh MNGM containing BDNF, GDNF, NT-3 and CNTF at final concentrations of 10ng/ml and 25ng/ml, respectively. Cultured motor neurons were allowed to expand for 7 DIV prior to use.

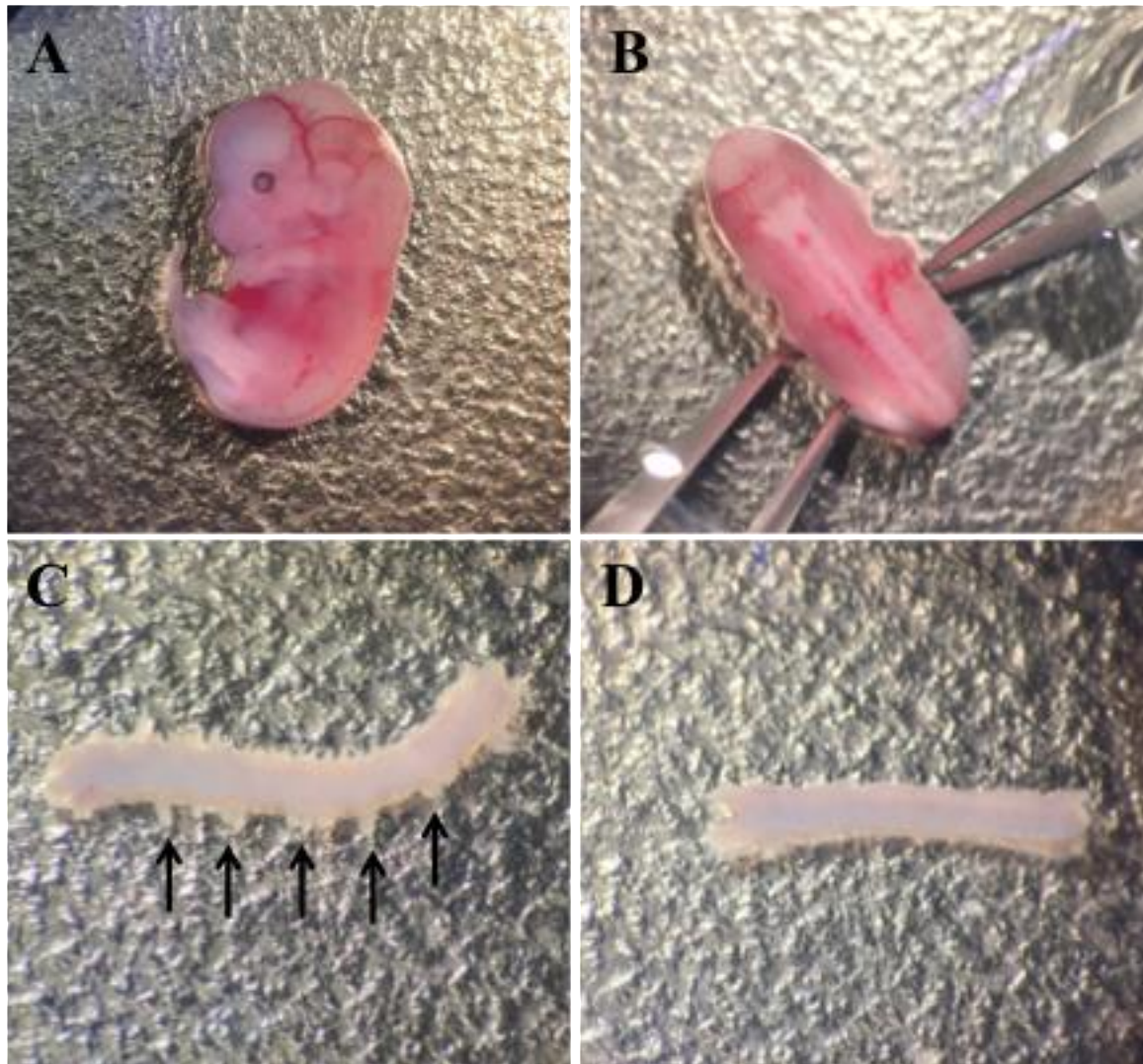


Figure 2.2 Dissection of the spinal cord from mouse embryos. To ensure motor neurons were mature enough to be cultured, only embryos between E12 to E14 (**A-B**) were collected. Embryos were faced dorsal side down, decapitated and entrails were removed in order to visualise the spinal column. The entire spinal cord was removed by cutting along both sides of the spinal column one vertebrae at a time. Dorsal root ganglia (**C, arrows**) and associated meninges were removed with a pair of tweezers, to obtain a ‘shaved’ spinal cord (**D**).

2.5.1 Immunocytochemistry (ICC) and motor neuron imaging

To examine the effect of OS on SG formation, motor neurons were treated with 0.5mM sodium arsenite (Sigma) in growth media 60 minutes prior to fixation. All motor neurons were fixed for 10 minutes in 4% paraformaldehyde in PBS (pH 7.2). Motor neurons were then washed in PBS three times for 15 minutes, before being removed and placed in a dark humidified chamber. Motor neurons were blocked and permeabilised for 1 hour by incubation with PBS containing 5% normal goat serum and 0.5% Triton X-100. Motor neurons were then incubated with primary antibodies diluted in block solution overnight at 4°C in a dark humidified chamber (see table 4 for list of antibodies and dilutions used). The following day, motor neurons were washed in PBS three times for 15 minutes and incubated with appropriate AlexaFluor® secondary antibodies for 2 hours at RT. Motor neurons were then washed in PBS three times for 15 minutes, mounted on glass slides using Vectashield mounting medium with DAPI and stored in the dark at 4°C until imaged.

All primary motor neurons were imaged using a Leica TCS SP5 confocal microscope. For all images, motor neurons are labelled green (SMI32), and protein (TDP-43, GFP) and stress granules (G3BP1) are labelled red. All Images were processed in ImageJ and only cells positive for SMI32 were analysed. To calculate cytoplasmic localisation of proteins, the perimeters circumscribing both the motor neuron and then the nucleus were drawn by hand and the signal intensity in each of the enclosed areas was measured. Between 45-60 motor neurons were used for protein localisation analysis. For stress granule analysis, ≥ 25 randomly selected motor neurons were used to assess successful formation and quantity of stress granules per cell. Only stress granules with a diameter $> 0.5\mu\text{m}$ were counted. Data analysed for each experiment was collected from independent cultures using ≥ 2 litters.

Table 2.2 Primary antibodies and dilutions used for ICC

Antibody	Host	Manufacturer	Dilution
Anti – SMI32 (<i>Neurofilament H non-phosphorylated</i>)	Mouse	Biolegend	1:1000
Anti – TDP-43	Rabbit	ProteinTech	1:200
Anti – GFP (<i>Green fluorescent protein</i>)	Rabbit	Invitrogen	1:200
Anti – G3BP1 (<i>GTPase Activating Protein (SH3 Domain) Binding Protein 1</i>)	Rabbit	ProteinTech	1:500

2.6 Protein extraction, co-immunoprecipitation and western blotting

Tissues were homogenized using a Precellys 24 homogeniser (Bertin Instruments), in appropriate volumes of ice-cold RIPA buffer (150mM NaCl, 50mM Tris (pH=7.5), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100 and 1% protease inhibitor cocktail (Sigma)). After 30 minutes incubation on ice, samples were centrifuged at 14,324×g for 20 minutes at 4°C. The supernatant (soluble fraction) was collected and kept on ice. When required, the pellet (insoluble fraction) was re-suspended in urea buffer (7M urea, 2M thiourea and 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) in 30mM Tris buffer, pH 8.0), sonicated for 10 seconds at 42% amplitude and centrifuged at 14,324×g for 20 minutes at 4°C. The supernatant comprising the insoluble protein fraction was collected. For equal loading, soluble protein concentrations were quantified using the Pierce™ BCA protein assay kit (Thermo Scientific) according to manufacturer's instructions. Samples were boiled for 5 min at 100°C in equal volumes of loading buffer (5% β-mercaptoethanol in 2×Laemmli buffer; Bio-Rad).

For co-immunoprecipitation, protein was extracted using a mild immunoprecipitation buffer (150mM NaCl, 50mM Tris (pH=7.5), 1% CHAPS and 1% protease inhibitor cocktail), to preserve protein-protein interactions. 1mg of protein was added to 4µg of primary antibody (anti-TDP-43; ProteinTech) and left overnight at 4°C on a spinning wheel. The following day, 50µl G-Sepharose beads (Sigma) were added and left for 2 hours at 4°C on a spinning wheel. G-Sepharose beads were washed three times with immunoprecipitation buffer and boiled for 5 min at 100°C in 50µl of loading buffer to re-suspend immunoprecipitated proteins. As a negative control, non-specific IgG was added instead of primary antibody.

For OxyBlot, protein was extracted using 50mM dithiothreitol (DTT) in ice-cold RIPA buffer, to prevent protein oxidation. Remaining steps were performed using the OxyBlot Protein Oxidation Detection Kit (S7150; Merck) according to manufacturer's instructions.

Protein samples were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred to a methanol-activated polyvinylidene difluoride (PVDF) membranes (Amersham). Membranes were subsequently blocked with 5% skim milk powder in PBST (PBS, 2% Tween-20) for 1 hour at RT, before incubation with appropriate primary antibody diluted in block solution overnight at 4°C (see table 5 for list of antibodies and dilutions used). After washing 3 times with PBST, membranes were incubated with the appropriate HRP-conjugated secondary antibodies (Amersham, 1: 5,000), and blots were developed using the ECL or ECL Plus detection system (Amersham). When required, the integrated optical density of each band was measured in ImageJ and expression normalized to β -actin levels in the same samples for comparative expression assessment.

Table 2.3 Primary antibodies and dilutions used for western blots

Antibody	Host	Manufacturer	Dilution
Anti – HA (HA-tag)	Rabbit	Sigma	WB – 1:1000 CoIP – 1:500
Anti – Oxr1	Rabbit	(Oliver <i>et al.</i> 2011)	WB – 1:500 CoIP – 1:250
Anti – GFP	Rabbit	Invitrogen	1:1000
Anti – TDP-43	Rabbit	ProteinTech	1:1000
Anti – GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	Mouse	Biolegend	1:1000
Anti – β -actin	Mouse	Abcam	1:1000

2.7 RNA extraction and microarray

Immediately after mice were sacrificed, spinal cords were dissected and lumbar regions were isolated and frozen at -80°C until used (*Performed by Prof. Peter Oliver*). Using a Precellys 24 homogeniser, lumbar spinal cords were homogenised in 200 μ l of Trizol reagent (Invitrogen) according to manufacturer's instructions. Total RNA was further purified from contaminating genomic DNA using the RNeasy Mini kit as per the manufacturer's instructions with on-column DNase digestion (Qiagen). Care was taken to keep RNA samples on ice when possible and all RNA was eluted in RNase-free ddH₂O. For all extractions, RNA concentration and integrity was validated using a 4200 TapeStation (Agilent) to confirm RIN scores were ≥ 7.7 and A260/A280 and A60/A230 ratios ≥ 1.8 .

Microarray experiments including data analysis were performed by Cambridge Genomic Services, University of Cambridge. In brief, a species-specific Gene 2.1 ST Array Plate (Affymetrix) in combination with WT PLUS amplification kit (Affymetrix) was used

according to the manufacturer's instructions. Successfully amplified samples were labelled using the GeneChip WT terminal labelling kit (Affymetrix) using the in-line hybridization control. Plate arrays were processed on the GeneTitan instrument (Affymetrix) using the GeneTitan Hybridization, Wash and Stain kit (Affymetrix). Finally, plates were scanned using the array specific parameters provided by Affymetrix. After scanning, files were loaded in R using the oligo package (Bioconductor; Carvalho & Irizarry 2010). Data quality was assessed and summarised in a technical quality control report. Variation within biological replicates was investigated using clustering methods. Raw data was processed using the Robust Multichip Analysis (RMA) method (Irizarry *et al.* 2003). Data was background corrected, normalised using quantile and summarized. Comparisons were performed using the Limma package and the results corrected for multiple testing using False Discovery Rate (FDR; (Benjamini and Hochberg 1995).

2.8 Construction of reporter plasmids

A 419 bp fragment and a 1159 bp fragment, both containing the conserved promoter sequence of mouse *Oxr1-C* (NM_001130164), was amplified by PCR using mouse genomic DNA as the template and the following primers (sense; 419 bp fragment): 5'-GAGAGGTACCCTGTGAGCAAACAGGGAAGTG-3' (underlined nucleotides indicate Kpn1 site), (sense; 1159 bp fragment): 5'-GAGAGGTACCCGGTCCGATATGGCTAGTAAGC-3' (underlined nucleotides indicate Kpn1 site), and (antisense): 5'-GAGACTCGAGTTCCTTCCCAGGTCACATTAAGC-3' (underlined nucleotides indicate Xho1 site). PCR was performed using Platinum™ Pfx DNA Polymerase (Thermo Scientific) under the following conditions: pre-denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute and then final extension at 72°C

for 5 min. The conserved promoter fragment of Oxr1-C was then cloned into both pGL4.10[*luc2*] and pGL4.22[*luc2CP/Puro*] vectors (Promega) using standard molecular cloning methods. Sequencing (*performed by Beckman Coulter Genomics*) was used to identify successful constructs containing the mouse Oxr1-C promoter in the 5'-flanking region of the *firefly* luciferase-coding sequence, and were designated as pGL4.10/mOxr1-Cp and pGL4.22/mOxr1-Cp. The conserved promoter sequence of human *OXR1-C* (NW_004078037.1) was previously cloned into the pGL4.10[*luc2*] by Kevin Liu and is designated as pGL4.10/hOxr1-Cp.

For mutant reporter plasmids, all mutations were generated by site-directed mutagenesis using a Quickchange Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer's instructions, with primer sequences listed in Table 5. Mutagenesis of hOXR1-C promoter sequence was previously carried out by Kevin Liu using primers previously described (Liu 2014). cDNA for the transcription factor constructs were previously cloned in to pCDNA3 vectors by Kevin Liu (Liu 2014).

Table 2.4 Site-directed Mutagenesis Primers

Primer name	Sequence: 5' – 3'
FoxL1 mut F	GGACTATCTGTTCAATGGCGACTACTTAGTGATTAGC
FoxL1 mut F	GCTAATCACTAAGTAGTCGCCATTGAACAGATAGTCC
Pbx1 mut F	GCTCTCTGTCATGTCGGTCAAATCATTGTTGC
Pbx1 mut R	GCAACAATGATTTGACCGACATGACAGAGAGC
Nkx2.5 mut F	GGGTGCAGTGCAACCTAACTTCCGCTTGGGAAACACATTC
Nkx2.5 mut R	GAATGTGTTTTCCCAAGCGGAAGTTAGGTTGCACTGCACCC
Sp1 mut F	GGGAAACACATTCTTAACTCCCCAGCTTTACAG
Sp1 mut R	CTGTAAAGCTGGGGAGTTAAGAATGTGTTTCCC

NFκB mut F	CCTAACTTGAGCTTGTTAAACACATTCTTCCC
NFκB mut R	GGGAAGAATGTGTTTAAACAAGCTCAAGTTAGG
Gata3 mut F	GGATACTACTTAGTGAGGAGCTCTCTGTCATG
Gata3 mut R	CATGACAGAGAGCTCCTCACTAAGTAGTATCC
Pbx1 (2) mut F	GCAAATTTATCTCTGGTAGAGGAAGAATGC
Pbx1 (2) mut R	GCATTCTTCCTCTACCAGAGATAAATTTGC
FoxM1 mut F	GTTGCTAGCAAACCTGGGTTGTCAGTGTAGCAGGGC
FoxM1 mut R	GCCCTGCTACAGTGACAACCCAGTTTGCTAGCAAC

2.9 Maintenance of cell lines

Neuro2a (N2A) cells were maintained routinely in 75cm² or 175cm² flasks (BD Falcon™) containing Dulbecco's Modified Eagles Medium (DMEM-Glutamax; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), and 1% Penicillin-Streptomycin solution (Invitrogen) in a humidified 37°C/ 5% CO₂ incubator. Cells were passaged by following the mammalian cell tissue culture protocol (Abcam) approximately every 3 days or when cells reached 90% confluency.

2.10 Transfection

24 hours prior to transfection, N2A cells were seeded in white opaque 96-well plates (BD Falcon™) at a density of approximately 0.5×10^5 cells per well. On the day of transfection, cells reached ~60% confluency. Cells were transfected with DNA constructs using FuGene 6 (Roche Applied Science) diluted in Opti-MEM (Invitrogen), according to the manufacturer's instructions. All experiments requiring a Dual-Glo luciferase assay, a pRL-TK control reporter vector (Promega) that expressed *Renilla* luciferase (*RLuc*) driven

by the TK promoter was also co-transfected. In each well, N2A cells were transfected with 40ng of reporter plasmid or DNA construct and at a 6:1 ratio of Fugene (µl): DNA (µg).

2.11 Dual-Glo Luciferase assay

24 hours after transfection, N2A cells were lysed and assayed for *firefly* and *Renilla* luciferase activity using the Dual-Glo Luciferase assay system (Promega), according to manufacturer's instructions. Briefly, cells were incubated in luciferase reagents for 30 minutes before light emission from each sample was measured using a FLUOstar Optima plate reader (BMG Labtech). Wells on a plate were read for either 5, 10 or 15 seconds, depending on the individual experiment. In all experiments, each condition was performed in triplicate wells and data was compiled from several individual cultures.

2.12 Stable cell line generation

A kill curve was generated to determine the optimal antibiotic concentration to use to select positive cell clones (2.5µg/ml puromycin; Sigma). 24 hours before transfection, N2A cells were seeded in a 24 well plate at a density of 0.1×10^5 cells per well. On the day of transfection, cells reached ~50% confluency. Cells were transfected with pGL4.22/mOxr1-Cp or a control vector designated pGL4.22/Empty using FuGene 6 diluted in Opti-MEM, according to the manufacturer's instructions. Transfected N2As were cultured for 24 hours, followed by treatment with puromycin (2.5µg/ml) for 7 days. Culture medium was changed every 2-3 days in order to remove floating dead cells. Surviving cells were washed in PBS before being incubated in 100µl Trypsin for 30 seconds followed by 900µl of medium. To keep stable polyclonal colonies, 500µl of cell suspension was frozen in freezing medium (10% dimethyl sulfoxide (DMSO), 50% FBS in DMEM) and stored at -80°C. The remaining cell suspension was serially diluted (1:1000, 1:500, 1:200, 1:100 and

1:5) in 63cm² dishes and cultured while maintaining puromycin selection pressure (2.5µg/ml) for 10–14 days or until single cells had formed colonies consisting of ~20 cells. Culture medium was changed every 2-3 days.

On the day of colony picking, culture medium was aspirated and single monoclonal colonies were rapidly picked using a 10µl pipette and placed in separate wells of a 24 well plate. Cell colonies were then immediately trypsinised and split into duplicate 24 well plates (1 plate to be used in assay to identify positive clonal cells and 1 plate to expand positive clonal cells). While maintaining puromycin selection pressure (1.25µg/ml), all monoclonal cell colonies were cultured for 14-21 days or until cells has reached ~70% confluency. Using 1 of the duplicate plates, cells were eppendorfed in 1x squishing buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 25 mM NaCl, 200µg/ml Proteinase K). DNA was extracted by incubating tubes in a PCR machine at 65°C for 30 minutes followed by 95°C for 2 minutes to inactivate the proteinase K. To identify positive cell lines which has integrated the reporter plasmid, DNA was amplified by PCR using the following primers; RVprimer3: 5'-d(CTAGCAAAATAGGCTGTCCC)-3' (Promega) and Luc2R2: 5'-CTTTTGCAGCCCTTTCTTGTC-3'. PCR was performed using REDTaq DNA Polymerase (Sigma) under the following conditions: pre-denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 2 minutes and then final extension at 72°C for 5 min. Once positive cell lines were identified, cells from the second duplicate plate were transferred to 75cm² flasks and cultured to high confluence. A small proportion of each cell line was also transferred to white opaque 96-well plates, cultured for 24 hours and tested for *firefly* luciferase (*FLuc*) expression using the Luciferase assay system (Promega), according to manufacturer's instructions. A FLUOstar Omega plate reader (BMG Labtech) was used to pipette 25µl of luciferase substrate per well and immediately measure luminescence from

each sample. Plates were shaken for 3 seconds at 500 rpm before being read. Cell lines stably expressing *FLuc* were then expanded to 175cm² flasks before being frozen and stored at -80°C.

2.13 Small compound screen

Two libraries of compounds were screened for Oxr1-C promoter activation: 80 compounds were chosen from a kinase inhibitor library and 80 from an epigenetic modifier library. All compounds were obtained from Prof. Angela Russell (Organic Chemistry, University of Oxford). All compounds were supplied as a 10mM solution in 100% DMSO and stored at -20°C. Optimal DMSO concentration for the compound screen was determined by testing stable cell lines for luciferase expression and cytotoxicity in various doses of DMSO (0.1-2.0). Cytotoxicity was measured using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) according to manufacturer's instructions.

For the compound screen, N2A cells were seeded in white opaque 96-well plates at a density of approximately 0.5×10^5 cells per well in 95µl of medium. On the day of treatment, cells reached ~60% confluency and were treated with 5µl of test compounds dissolved at a final concentration of 10µM in 0.3% DMSO. All compounds were tested in triplicate and cells treated with 0.3% DMSO only were used as a negative control. Following 24 hours incubation, *FLuc* expression was measured as previously described (2.12 *Stable cell line generation*).

Counter screens were carried out for compounds found to activate the Oxr1-C promoter. Cell proliferation was measured using the cell proliferation assay kit (2210; Merck), according to manufacturer's instructions. To test for compound stabilisation/inhibition of *FLuc*, Quantilum® recombinant *FLuc* (0.08µg/ml; Promega) was mixed with adenosine 5' triphosphate disodium salt (ATP; Sigma) at a concentration close

to its K_m (10 μ M) in assay buffer (5mg/ml BSA, 25mM HEPES, 5mM DTT, 5mM MgCl₂, 1mM EDTA, pH7.8 in ddH₂O), and kept on ice. Each compound was serially diluted at final concentrations of 0.3nM, 0.95nM, 3nM, 9.5nM, 30nM, 95nM, 0.3 μ M, 0.95 μ M, 3 μ M, 9.5 μ M and 30 μ M in assay buffer with DMSO at a final concentration of 2.7%. 10 μ l of each compound dilution was added in triplicate wells of a white opaque 96-well plate. 40 μ l of Luciferase/ATP mix was dispensed in all wells and incubated for 15 minutes on ice. 40 μ l of Beetle Luciferin (Promega) was added to all wells at a final concentration close to its K_m (10 μ M), and luminescence was immediately measured using A FLUOstar Omega plate reader. 3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl] benzoic acid (PTC-124), a known stabiliser/inhibitor of *FLuc* was used as a positive control.

2.14 Statistical analysis - Results were analysed using Prism (GraphPad Software, Inc.) with the appropriate statistical tests. Data are presented as mean $\pm$ SEM (standard error of mean), with n indicating the number of independent samples used in each group for comparison.

Chapter 3

Oxr1 is not essential for motor neuron survival *in vivo*

3.1 Introduction

One of the biggest questions in the field of neurodegenerative disease is why certain neuronal populations are more vulnerable to cellular stress and degeneration than others. For example, it is still unclear why mutations in C9ORF72, SOD1, TDP-43 and FUS cause the selective vulnerability of motor neurons to degenerate in ALS, despite the mutant protein being expressed throughout the CNS (Rosen *et al.* 1993; Kabashi *et al.* 2008; Vance *et al.* 2009; DeJesus-Hernandez *et al.* 2011). Furthermore, the pattern and pathology associated with motor neuron degeneration is similar in fALS and sALS, indicating that selective motor neuron vulnerability is independent of the genetic cause of disease (Shaw *et al.* 1997). OS appears to be a common etiological factor in ALS and other neurodegenerative diseases characterised by selective neuronal vulnerability, including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD; Lin & Beal 2006). Therefore, understanding the mechanisms of selective neuronal vulnerability and identifying factors that render these cells more or less vulnerable to OS is of great importance, especially for the development of better therapeutic strategies.

It has been demonstrated consistently that Oxr1 imparts a protective function against OS and OS-induced cell death in a variety of organisms, ranging from yeast and nematodes to flies and mice (Volkert, Elliott and Housman 2000; Oliver *et al.* 2011; Kobayashi *et al.* 2014; Sanada *et al.* 2014). More specifically, overexpressing Oxr1 in cells seems to confer protection against OS, while losing Oxr1 expression renders cells more vulnerable to OS and OS-induced cell death. This is exemplified in the *bella* mouse mutant, which harbours a

spontaneous deletion of the *Oxr1* gene. These mice display increased levels of oxidative DNA damage and degeneration specifically in cerebellar GCs (Oliver *et al.* 2011). The importance of *Oxr1* to GC survival was further supported by *in vitro* studies demonstrating that cultured GCs from *bella* mice were more vulnerable to OS-induced cell death than wild-type GCs (Oliver *et al.* 2011), supporting that loss of *Oxr1* increases a cell's vulnerability to OS.

Oxr1 is a highly conserved protein expressed not only in cerebellar GCs, but throughout the entire CNS, suggesting it plays an important role in additional neuronal populations (Volkert, Elliott and Housman 2000; Durand *et al.* 2007; Finelli *et al.* 2016). In support of this, it has been demonstrated previously that OXR1 is upregulated in the spinal cord of ALS patients and in pre-symptomatic mouse models of ALS and HD (Oliver *et al.* 2011; Liu 2014). In addition, OXR1 is also differentially expressed in the posterior cingulate cortex of PD patients without dementia (Stamper *et al.* 2008). Together, these studies implicate OXR1 in the neurodegenerative disease process and suggest that OXR1 could even serve as an early biomarker for ALS. More recent studies have also demonstrated the neuroprotective potential of *Oxr1* in extending the lifespan and improving ALS-associated phenotypes in the SOD1^{G93A} mouse model of ALS (Liu *et al.* 2015). Furthermore, *in vitro* studies have shown that *Oxr1* interacts with and regulates the localisation of the well-known ALS proteins, TDP-43 and FUS (Finelli *et al.* 2015). Taken together, these studies begin to build a strong argument for a protective function of *Oxr1* in ALS. Despite its specific expression in motor neurons in the spinal cord (Liu *et al.* 2015), the importance of *Oxr1* expression to motor neuron survival is yet to be determined.

It has been proposed that the selective vulnerability of neurons to OS can be the result of intrinsic OS, high demand for ROS as signalling molecules, chronic inflammatory response, low ATP generation, mitochondrial dysfunction, deficient DNA damage repair,

calcium dysregulation and glutamate hyperactivity (Wang and Michaelis 2010). Besides the molecular and cellular mechanisms described above, the physical size of neurons may, to some degree, determine neuronal vulnerability to OS. Possible reasons for this include the high demand for energy and mitochondrial activity, dependence on long-distance axonal transport, high content of neurofilaments which tend to form aggregates, and a relatively large surface area for increased exposure to oxidants in the extra-cellular environment (Wang and Michaelis 2010). As motor neurons are larger in size (50-60µm), with axons projecting over longer distances (up to 1m), it could be expected that they may be more vulnerable to OS (Shaw and Eggett 2000; Cleveland and Rothstein 2001). Indeed, *in vivo* studies whereby the antioxidant protein SOD1 is knocked out in mice has demonstrated that motor neurons are more vulnerable to OS and models share many commonalities with ALS (Reaume *et al.* 1996).

Given its antioxidant function in other cells, I hypothesize that *Oxr1* plays a similarly important role in regulating motor neuron survival *in vivo* and that loss of *Oxr1* in motor neurons may cause a motor neuron degenerative phenotype. *Bella* mice display no motor neuron death in the spinal cord and die by P26 (Oliver *et al.* 2011). Therefore, this model was uninformative on the role of *Oxr1* in motor neurons. To circumvent this, the Oliver group previously generated a motor neuron-specific *Oxr1* conditional knockout mouse line. Given that mice expressing solely the *Oxr1*-C TLDC domain-containing isoform exhibit no phenotypic abnormalities (Oliver *et al.* 2011), it was important that expression of all known *Oxr1* isoforms, including *Oxr1-FL* and *Oxr1-C*, was ablated. Therefore, two essential exons, 15 and 16, in the TLDC domain were targeted for deletion using the previously described Cre-LoxP system for gene targeting (Figure 3.1A; Skarnes *et al.* 2011). Briefly, *Oxr1^{tm1a(EUCOMM)Wtsi}* knockout-first conditional allele (*tm1a*) mice were obtained from the Wellcome Trust Sanger Institute. This particular allele introduces a floxed promoter-driven

neo cassette and an IRES-lacZ trapping element into the intronic region between exons 14 and 17 of *Oxr1*, truncating the Oxr1 protein by 101 amino acids (Figure 3.1B). Importantly, mice homozygous for this insertion, *Oxr1*^{tm1a/tm1a}, displayed a phenotype identical to *bella* mutants (Oliver *et al.* 2011; Finelli *et al.* 2016). Heterozygous *Oxr1*^{tm1a/+} mice were then crossed with a transgenic FLPe mouse line (Flp), in which the human β -actin promoter drives the expression of FLPe variant recombinase that removes both the IRES-lacZ trapping element and the floxed promoter-driven neo cassette. The offspring of this cross contain the conditional allele (*tm1c*) in which exons 15 and 16 of *Oxr1* are flanked by LoxP sites, recognisable by Cre recombinase (Figure 3.1B). Mice homozygous for the *tm1c* allele (*Oxr1*^{tm1c/tm1c}) express a functional Oxr1 protein and are therefore viable and are indistinguishable from wild-type mice.

To specifically delete *Oxr1* in motor neurons, the conditional-ready *Oxr1* knockout mouse line (*Oxr1*^{tm1c/tm1c}) was crossed to a transgenic line in which Cre recombinase is conditionally expressed in motor neurons under the control of the bacterial artificial chromosome (BAC) containing the endogenous promoter for choline acetyltransferase (ChAT; Figure 3.1B; Gong *et al.* 2007). ChAT is the enzymatic machinery required to generate acetylcholine and is expressed in cholinergic neurons, including motor neurons (Dobransky and Rylett 2005). Firstly however, the specificity of Cre expression to ChAT-positive motor neurons was confirmed by crossing the *ChAT*-cre mouse line with a lacZ reporter (R26RlacZ) mice (Soriano 1999). The progeny of this cross, R26RlacZ/*ChAT*-cre mice, are expected to co-express lacZ in neurons that express *ChAT*-driven-Cre. It was demonstrated that Cre recombinase was expressed in ChAT-expressing motor neurons in the spinal cord and facial motor nucleus with very high specificity (Figure 3.2A-B). In addition, Cre recombinase expression was also observed in ChAT-expressing neurons in the pedunculo pontine tegmental nucleus, and medial habenula in the brain (Peter Oliver,

unpublished data). Next, deletion of *Oxr1* in ChAT-expressing motor neurons was confirmed by *in situ* hybridization using a probe specific to the deleted exons. It was demonstrated that the expression level of *Oxr1* in *Oxr1^{tm1c/tm1c}/ChAT-cre* mice was barely detectable compared to *Oxr1^{tm1c/tm1c}/+* mice (Figure 3.2C; Peter Oliver, unpublished data). Together, these data confirm that *Oxr1* expression is ablated specifically in ChAT-expressing motor neurons of *Oxr1^{tm1c/tm1c}/ChAT-cre* mice, providing a novel and valuable model to study selective motor neuron vulnerability to the loss of *Oxr1*.

3.2 Aims of Chapter 3

The aim of this chapter was to determine whether *Oxr1* is essential for motor neuron survival and function, and whether specific loss of *Oxr1* *in vivo* would cause phenotypes similar to those observed in other motor neuron degenerative diseases, such as ALS. In order to achieve this, I characterised the motor behaviour of *Oxr1^{tm1/tm1c}/ChAT-cre* mice and *Oxr1^{tm1c/tm1c}/+* littermates over a time-course and pathologically examined key components of the neuromuscular system. Generation of experimental mice had begun prior to the start of this study. Therefore, this work focuses on the behaviour and pathology of aged mice.

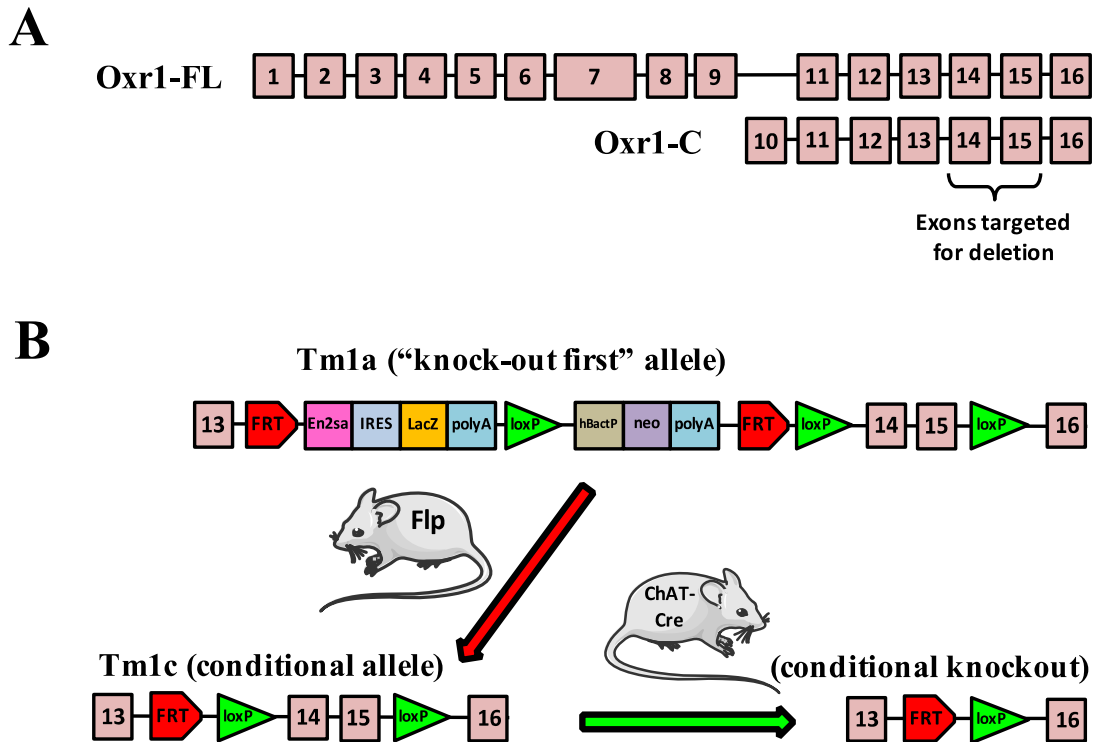


Figure 3.1 Generation of motor neuron-specific *Oxr1* conditional knock-out mice. (A) Schematic of *Oxr1*-FL and *Oxr1*-C isoforms, showing the locations of exons 14 and 15, which are targeted for the conditional construct to knock-out all isoforms of *Oxr1* (not to scale). **(B)** Schematic of “knock-out first” allele (*tm1a*), which introduces a floxed promoter-driven neo cassette and an IRES-lacZ trapping cassette into the intronic region between exons 13 and 16 of *Oxr1*, disrupting gene function. Crossing the *tm1a* mouse line with a β -actin driven FLPe mouse line (Flp) converts the knock-out first allele into a conditional allele (*tm1c*), restoring gene activity. Mice expressing *tm1c* crossed with the *ChAT* promoter-driven Cre mouse line (*ChAT*-Cre) will generate a conditional knock-out mouse line, in which exons 14 and 15 of *Oxr1* are deleted in *ChAT*-expressing motor neurons. The frameshift mutation subsequently leads to nonsense mediated decay of the truncated *Oxr1* transcript. FRT: flippase recognition target sites, En2sa: mouse *engrailed 2* exon 2 splice acceptor, IRES: internal ribosome entry site, lacZ: *lacZ* reporter gene, polyA: SV40 polyadenylation signal, LoxP: Locus of X-over P1 Cre recombinase target sites, hBactP: autonomous human β -actin promoter, neo: neomycin resistance gene, and ChAT: Choline acetyltransferase.

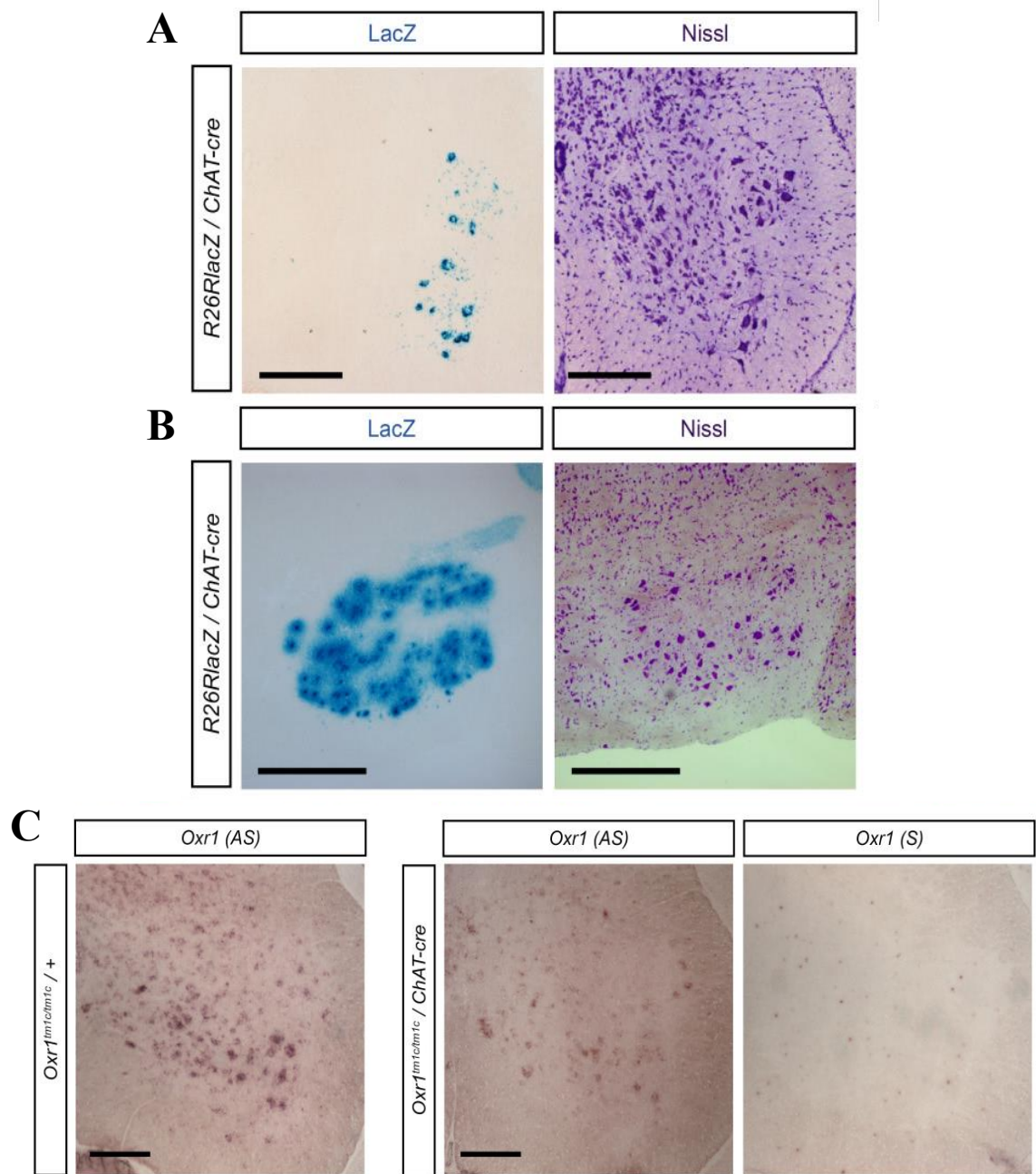


Figure 3.2 *Oxr1* expression in ChAT-expressing motor neurons is specifically deleted in *Oxr1^{tm1c/tm1c}/ChAT-cre* mice. Representative images of adjacent sections stained for nissl and LacZ to show specific Cre activity in ChAT-expressing motor neurons in the spinal cord **(A)** and facial motor nucleus **(B)**. Scale bars: 500 μ m. **(C)** *In situ* hybridization with anti-sense labelling shows loss of *Oxr1* expression in spinal motor neurons of *Oxr1^{tm1c/tm1c}/ChAT-cre* mice compared to *Oxr1^{tm1c/tm1c}/+* mice. Scale bar: 250 μ m. LacZ staining was performed by Arran Babbs, nissl staining was performed by Peter Oliver and Kevin Liu, *in situ* hybridization was performed by Peter Oliver.

3.3 Results

3.3.1 Specific loss of *Oxr1* in motor neurons does not cause motor dysfunction in mice.

Given that constitutive loss of *Oxr1* in *bella* mice leads to severe ataxic gait, failure to gain weight and death by P26, motor coordination, muscle strength and weight of $Oxr1^{tm1c/tm1c}/ChAT-Cre$ mice was analysed. To assess motor coordination, I used the accelerating rotarod to measure latency to fall for $Oxr1^{tm1c/tm1c}/+$ and $Oxr1^{tm1c/tm1c}/ChAT-Cre$ mice. I found no significant difference in rota rod performance for both male and female $Oxr1^{tm1c/tm1c}/ChAT-Cre$ mice compared to littermate $Oxr1^{tm1c/tm1c}/+$ control mice (Figure 3.3A-B). I also assessed muscle strength using the four-limb hanging wire test, but found no significant difference in hang time for $Oxr1^{tm1c/tm1c}/+$ and $Oxr1^{tm1c/tm1c}/ChAT-Cre$ mice (Figure 3.3C-D). These results suggest that loss of *Oxr1* in motor neurons does not impair motor coordination or muscle strength in mice at 18 months of age. Body weight has been shown previously to be a simple and reliable measure for the disease “onset” and progression in the $SOD1^{G93A}$ ALS mouse model (Zhou *et al.* 2007). Interestingly, for both males and females, I found a significant decrease (~17% and ~23%, respectively) in weight of $Oxr1^{tm1c/tm1c}/ChAT-Cre$ mice compared to $Oxr1^{tm1c/tm1c}/+$ mice (Figure 3.3E-F), suggesting loss of *Oxr1* in motor neurons affects body weight in mice.

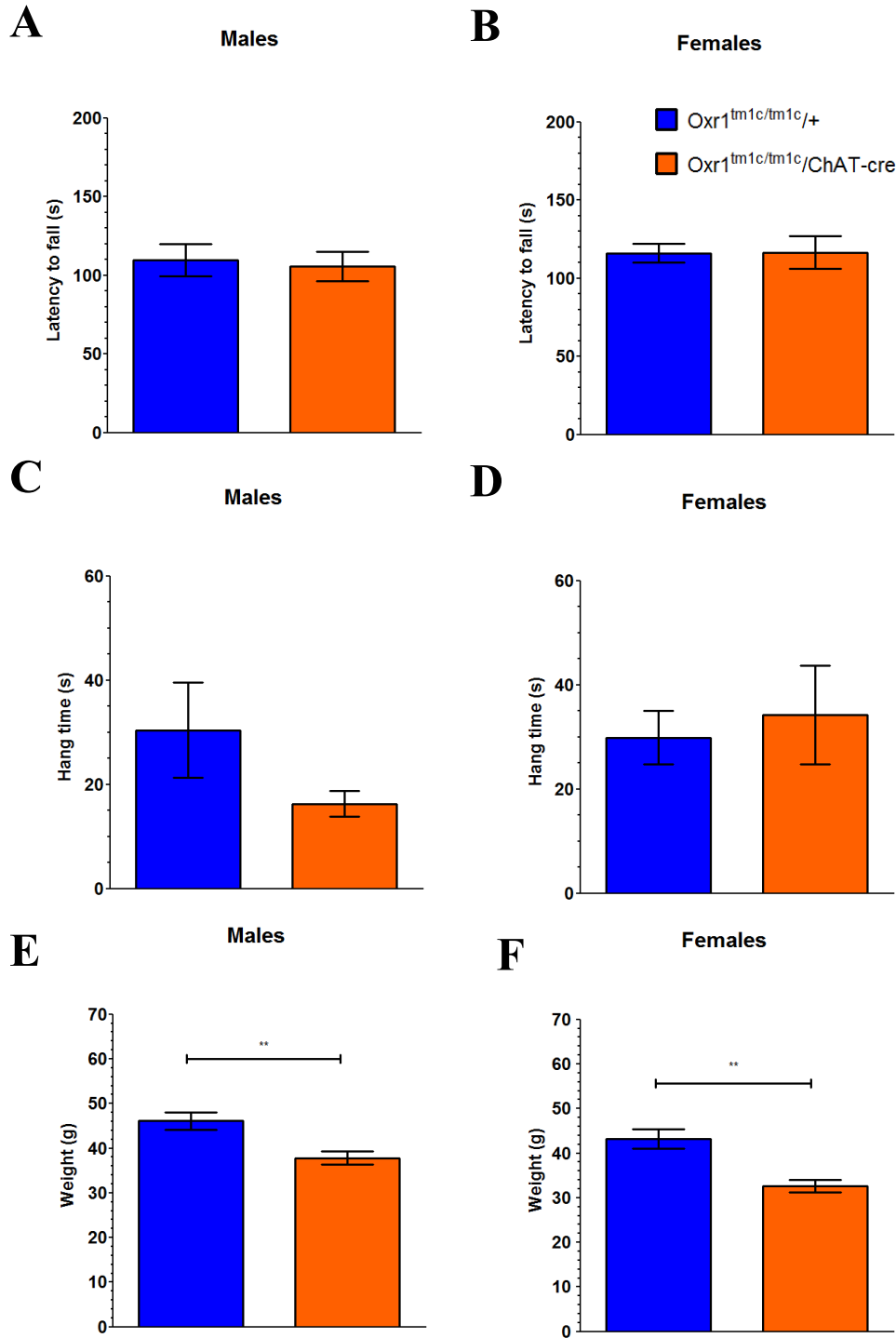


Figure 3.3 Specific loss of *Oxr1* in motor neurons does not affect motor function or muscle strength in $Oxr1^{tm1c/tm1c/ChAT-cre}$ mice at 18 months of age. Rotarod analysis shows no difference in motor coordination for male (A) and female (B) $Oxr1^{tm1c/tm1c/ChAT-cre}$ mice when compared with $Oxr1^{tm1c/tm1c/+}$ mice. Grip strength shows no difference in muscle strength for male (C) and female (D) $Oxr1^{tm1c/tm1c/ChAT-cre}$ mice when compared with $Oxr1^{tm1c/tm1c/+}$ mice. Both male (E) and female (F) $Oxr1^{tm1c/tm1c/ChAT-cre}$ mice showed a significant decrease in weight when compared with $Oxr1^{tm1c/tm1c/+}$ mice. Values are mean $\pm$ SEM (n=11-16 for A and B; n=7-10 for C, D, E and F; **p<0.005, Unpaired student's t-test).

3.3.2 Specific loss of *Oxr1* in motor neurons does not cause motor neuron degeneration.

Given that deletion of *Oxr1* in *bella* mice causes neurodegeneration specifically in the granule cell layer of the cerebellum, and mutants do not survive past P26 (Oliver *et al.* 2011), it was not possible to study the vulnerability of motor neurons to loss of *Oxr1* in aged animals. Therefore, using anti-ChAT stained spinal cord sections from the lumbar enlargement of the conditional mutants and littermate controls, I analysed the number of ChAT-positive motor neurons. I observed no significant difference in the number of ChAT-positive motor neurons in the spinal cord between *Oxr1^{tm1c/tm1c}/ChAT-Cre* mice and littermate controls at 18 months of age, suggesting specific loss of *Oxr1* does not lead to motor neuron degeneration in the spinal cord (Figure 3.4A&C).

As previously mentioned, *Oxr1* was also deleted in ChAT-expressing motor neurons in the facial motor nucleus of *Oxr1^{tm1c/tm1c}/ChAT-Cre* mice. Therefore, to determine whether this specific population was affected by deletion of *oxr1*, TUNEL staining for apoptotic cells was carried out. I observed no obvious signs of apoptosis of motor neurons in the facial motor nucleus between genotypes (Figure 3.4B); cerebellar sections from a *bella* mutant was used as a positive control (data not shown). This suggests that selective loss of *Oxr1* does not lead to motor neuron degeneration in the brainstem by 18 months of age either.

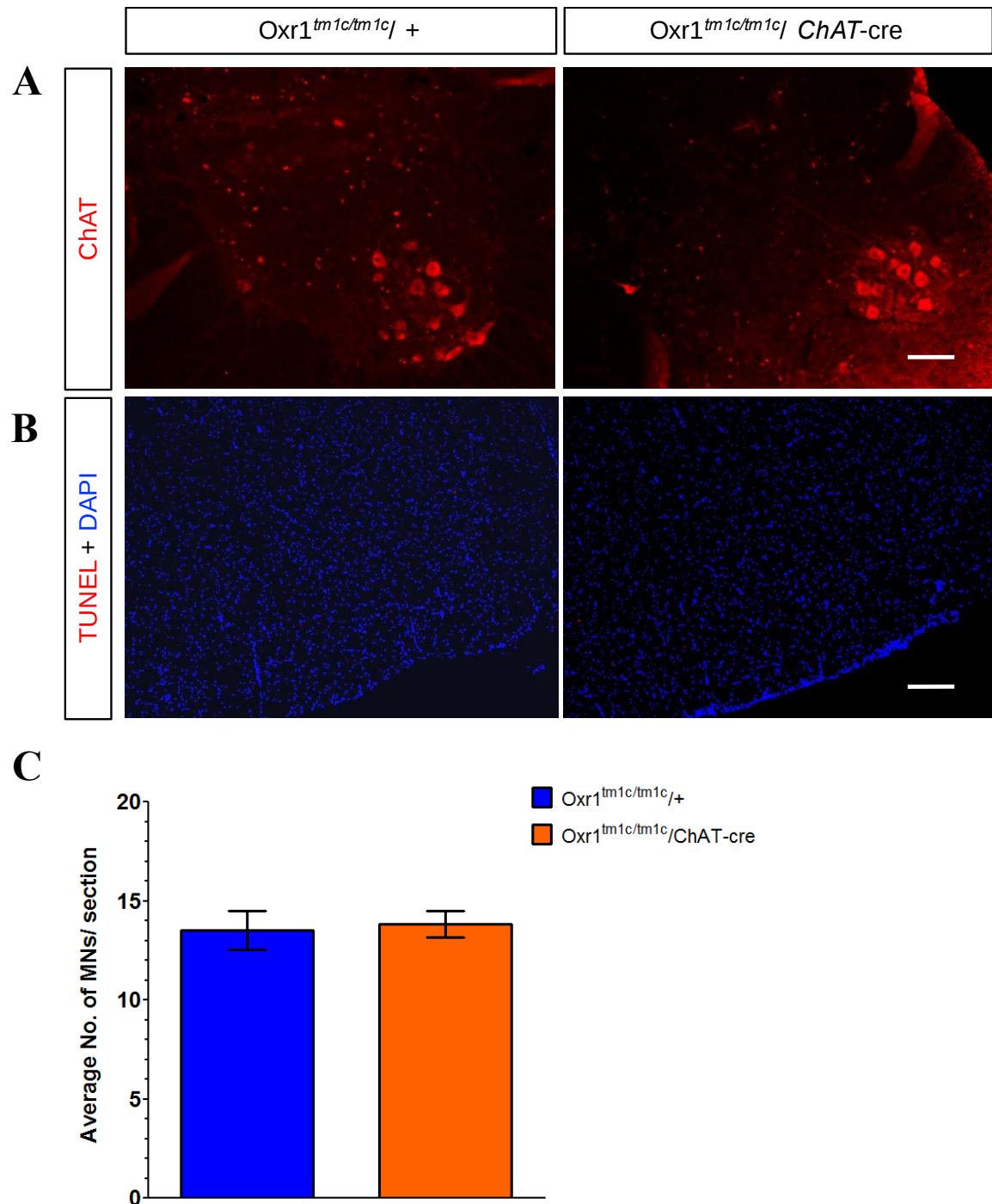


Figure 3.4 Specific loss of *Oxr1* in motor neurons does not cause motor neuron degeneration in $Oxr1^{tm1c/tm1c}/ChAT\text{-}cre$ mice by 18 months of age. (A) Representative images of ChAT-stained lumbar spinal cord sections from $Oxr1^{tm1c/tm1c}/+$ and $Oxr1^{tm1c/tm1c}/ChAT\text{-}cre$ mice. (B) Images of TUNEL-stained brain sections of the facial motor nucleus from $Oxr1^{tm1c/tm1c}/+$ and $Oxr1^{tm1c/tm1c}/ChAT\text{-}cre$ mice. (C) Graph shows the average number of ChAT-positive motor neurons counted per spinal cord section. Values are mean $\pm$ SEM (For A&C; n=4, for B; n=1, Mann-Whitney U test). Scale bars: 100 μ m.

3.3.3 Specific loss of *Oxr1* in motor neurons causes microgliosis.

It is well-established that OS can activate neuroinflammatory pathways (Koutsilieris *et al.* 2002; Mittal *et al.* 2014). Given that constitutive loss of *Oxr1* results in astrogliosis and microgliosis in the cerebellum and spinal cord of *bella* mice (Liu *et al.* 2015), and *Oxr1* has been suggested to modulate neuroinflammatory pathways and the immune response (Liu *et al.* 2015; Finelli and Oliver 2017), I investigated whether specific loss of *Oxr1* in motor neurons induces a neuroinflammatory response. Spinal cord sections from 18-month-old *Oxr1*^{tm1c/tm1c}/+ and *Oxr1*^{tm1c/tm1c}/*ChAT*-Cre mice were immunostained for GFAP - an astrocyte-specific marker that can indicate the occurrence of astrogliosis - and Iba1, a marker for microglial activation (Trias *et al.* 2013). I observed no increased intensity of GFAP in the ventral horns of lumbar spinal cord sections from *Oxr1*^{tm1c/tm1c}/*ChAT*-Cre mice compared to *Oxr1*^{tm1c/tm1c}/+ mice (Figure 3.5A&C), suggesting that loss of *Oxr1* specifically in motor neurons does not cause astrogliosis at 18 months of age. Interestingly however, I did observe increased intensity of Iba1 in the lumbar spinal cord of *Oxr1*^{tm1c/tm1c}/*ChAT*-Cre mice (Figure 3.5B), and quantitative analysis revealed a significant ~25% increase in the number of Iba1-positive cells (Figure 3.5E). I also observed a 10% increase in Iba1 staining intensity, although this did not reach significance (Figure 3.5D). Together, these data suggest that loss of *Oxr1* specifically in motor neurons causes some induction of microgliosis in the spinal cord.

The inflammatory response present in the spinal cord of ALS mouse models is usually preceded by motor axon pathology and the presence of monocytes/macrophages surrounding the peripheral nerve fibres (Dadon-Nachum, Melamed and Offen 2011). Therefore, to address whether a similar inflammatory response was also evident in the motor axon, sciatic nerve sections were stained for Iba1 and CD68, a marker for monocytes/macrophages (Hulette, Downey and Burger 1992). I observed a non-significant

increase in CD68 and Iba1-positive cells in the sciatic nerve of *Oxr1*^{tm1c/tm1c}/*ChAT*-Cre mice (Figure 3.6A-B), suggesting loss of *Oxr1* specifically in motor neurons may result in increased inflammation in the motor axon. However, additional data and quantification of cell numbers are needed to confirm this.

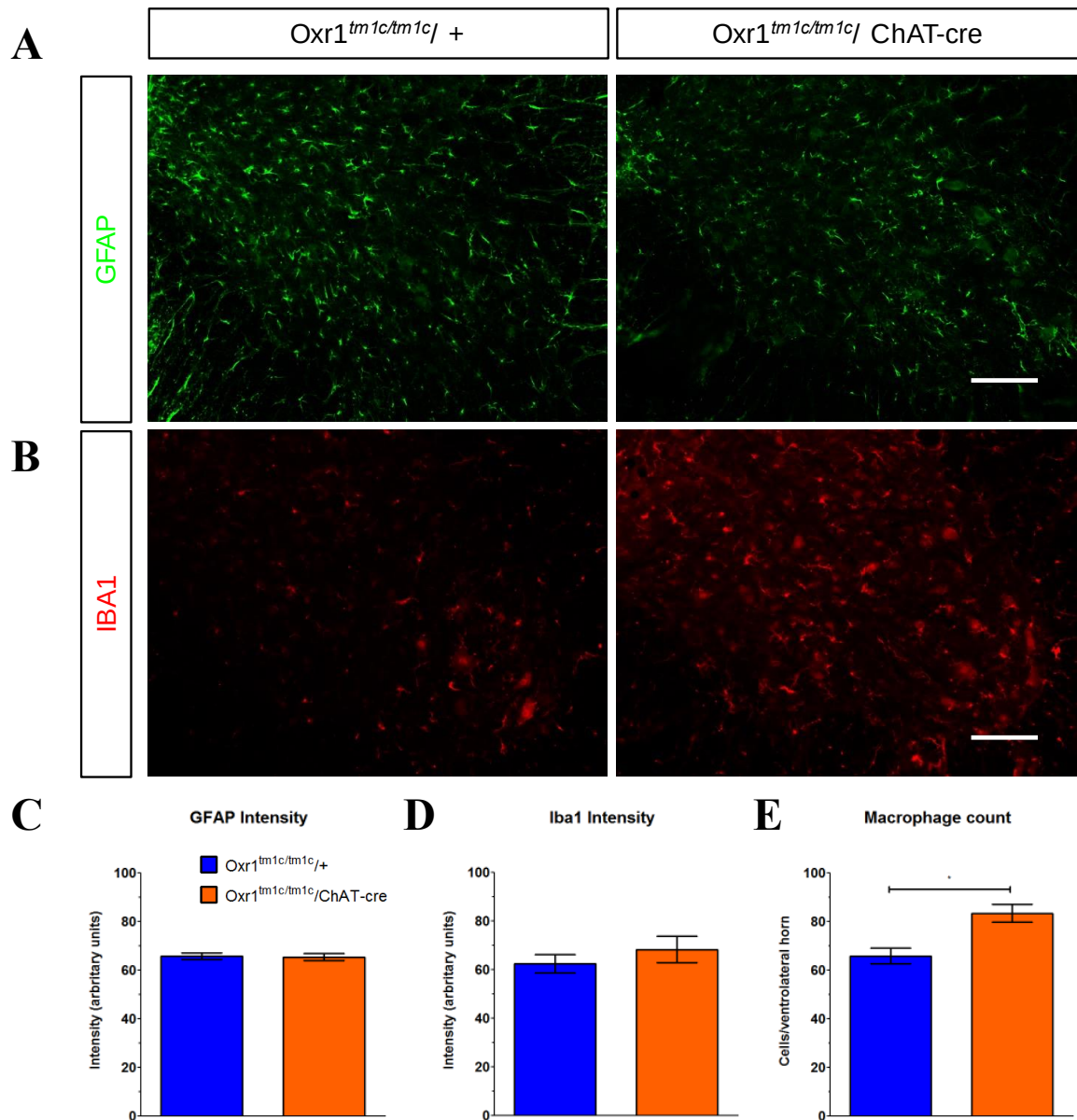


Figure 3.5 Specific loss of *Oxr1* in motor neurons causes an inflammatory response in the spinal cord *Oxr1^{tm1c/tm1c}/ChAT-cre* mice by 18 months of age. Representative images of lumbar spinal cord sections stained with GFAP (**A**) and Iba1 (**B**) from *Oxr1^{tm1c/tm1c}/+* and *Oxr1^{tm1c/tm1c}/ChAT-cre* mice. Quantification of GFAP staining intensity in the ventral horns (**C**) revealed no increase in astrogliosis in *Oxr1^{tm1c/tm1c}/ChAT-cre* mice. Quantification of Iba1 staining intensity in the ventral horns (**D**) and macrophage/microglial number (**E**) revealed increased microgliosis in spinal cord of *Oxr1^{tm1c/tm1c}/ChAT-cre* mice. Values are mean $\pm$ SEM (n=3-5, *p<0.05, Unpaired student's t-test). Scale bar: 100µm.

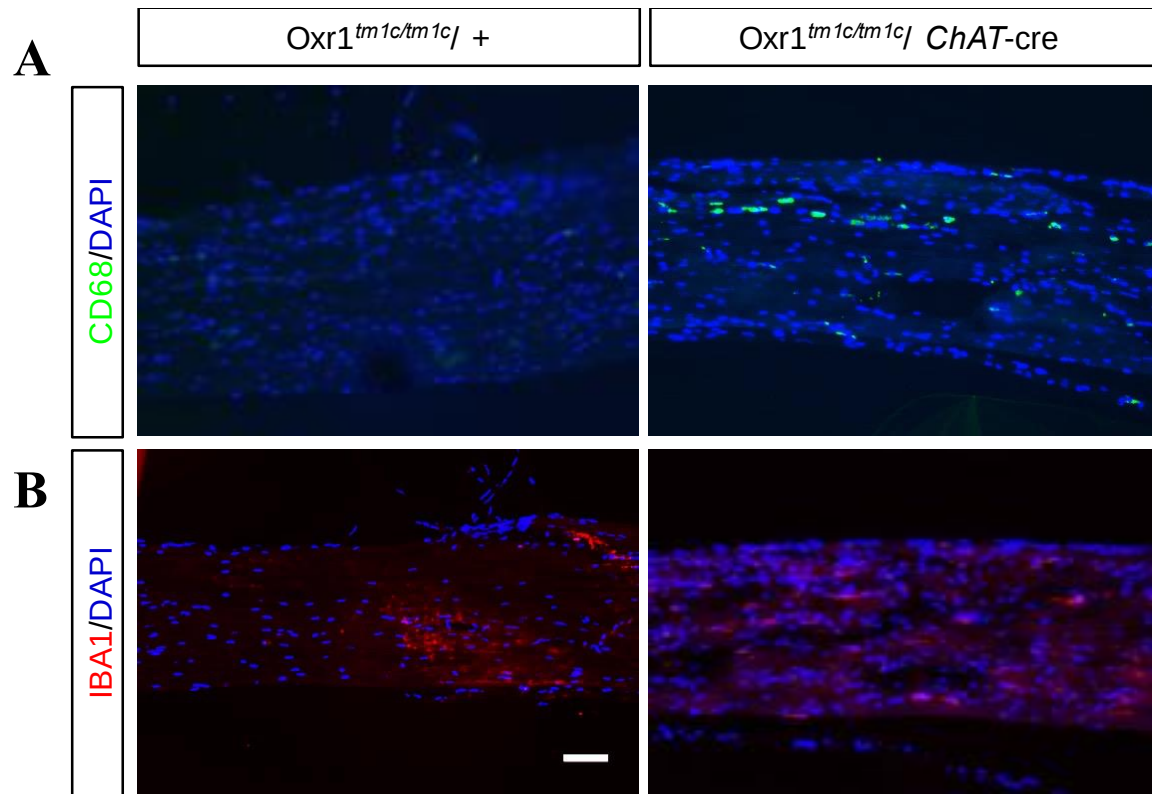


Figure 3.6 Limited evidence of inflammatory response associated with loss of Oxr1 in motor neurons in the sciatic nerve of Oxr1^{tm1c/tm1c}/ChAT-cre mice by 18 months of age.

Images of sciatic nerve sections stained with CD68 (A) and Iba1 (B) from Oxr1^{tm1c/tm1c}/+ and Oxr1^{tm1c/tm1c}/ChAT-cre mice (n=1). Scale bar: 100μm

3.3.4 Specific loss of *Oxr1* in motor neurons does not cause denervation at the NMJ.

A number of studies have demonstrated that OS contributes to cytoskeletal defects and impaired axonal transport - both cellular functions important for NMJ maintenance (Parakh *et al.* 2013). Moreover, it has been demonstrated that presynaptic nerve terminals at the NMJ are sensitive to ROS, and under OS conditions, neurotransmitter releasing machinery can be compromised (Pollari *et al.* 2014). Together, this suggests that OS has a detrimental effect on the structure and function of the NMJ. Importantly, temporal analysis of neuromuscular pathology in ALS mouse models indicate that motor neuron degeneration begins distally at the NMJ, markedly earlier than the onset of any behavioural symptoms, and proceeds towards the soma in a retrograde “dying-back” manner (Fischer *et al.* 2004; Rocha *et al.* 2013). Therefore, despite observing no motor defect or motor neuron degeneration in *Oxr1^{tm1c/tm1c}/ChAT-cre* mice, I wanted to assess whether selective loss of *Oxr1* in motor neurons caused denervation at the NMJ.

To do this, I dissected deep lumbrical muscles located in the hind-paw. Typically, larger muscles, such as the gastrocnemius, are used in research to assess pathological phenotypes associated with the NMJ. However, lumbrical muscles possess a major technical advantage, in that they can be whole-mounted and the entire innervation pattern visualised without sectioning (Sleigh *et al.* 2014). Importantly, this permits a more precise assessment of NMJ phenotypes, such as denervation, by avoiding sectioning artefacts and inconsistent fixation. After staining lumbrical muscles for markers of presynaptic nerve terminals (SV2 and 2H3) and post-synaptic AChRs located on the motor endplate (α-BTX), I analysed the number of NMJs that were fully innervated, partially innervated or fully denervated (vacant). I observed no significant difference in the innervation of lumbrical NMJs between *Oxr1^{tm1c/tm1c}/+* and *Oxr1^{tm1c/tm1c}/ChAT-cre* mice by 18 months of age (Figure 3.7A&B), suggesting specific loss of *Oxr1* in motor neurons does not elicit denervation at the NMJ.

3.3.5 Specific loss of *Oxr1* in motor neurons does not cause atrophy of hind limb muscle groups.

Progressive muscle atrophy is the major manifestation of ALS and other degenerative diseases caused by motor neuron loss (Kiernan *et al.* 2011; Bowerman *et al.* 2017). As previously mentioned, the first signs of disease appear in muscle, with denervation of the NMJ occurring prior to motor neuron death (Fischer *et al.* 2004; Moloney, de Winter and Verhaagen 2014). Interestingly, pathological studies using an inducible motor neuron-specific *Sod1* knockout mouse model, revealed significant muscle atrophy, despite *Sod1* expression being unaltered in skeletal muscle (Bhaskaran *et al.* 2017). This suggests that elevated OS in the motor neurons alone can cause muscle atrophy in mice. Therefore, to determine whether loss of *Oxr1* specifically in motor neurons causes muscle atrophy, transverse sections from the four-major hind limb muscle groups were stained with H&E, in order to visualise individual muscle fibres. I observed no obvious changes in the number or morphology of muscle fibres in gastrocnemius, tibialis anterior (TA), extensor digitorum longus (EDL) or soleus muscle in either *Oxr1*^{tm1c/tm1c/+} and *Oxr1*^{tm1c/tm1c/ChAT-cre} mice at 18 months of age (Figure 3.8A-D). This suggests loss of *Oxr1* in motor neurons does not cause atrophy in hind limb muscle.

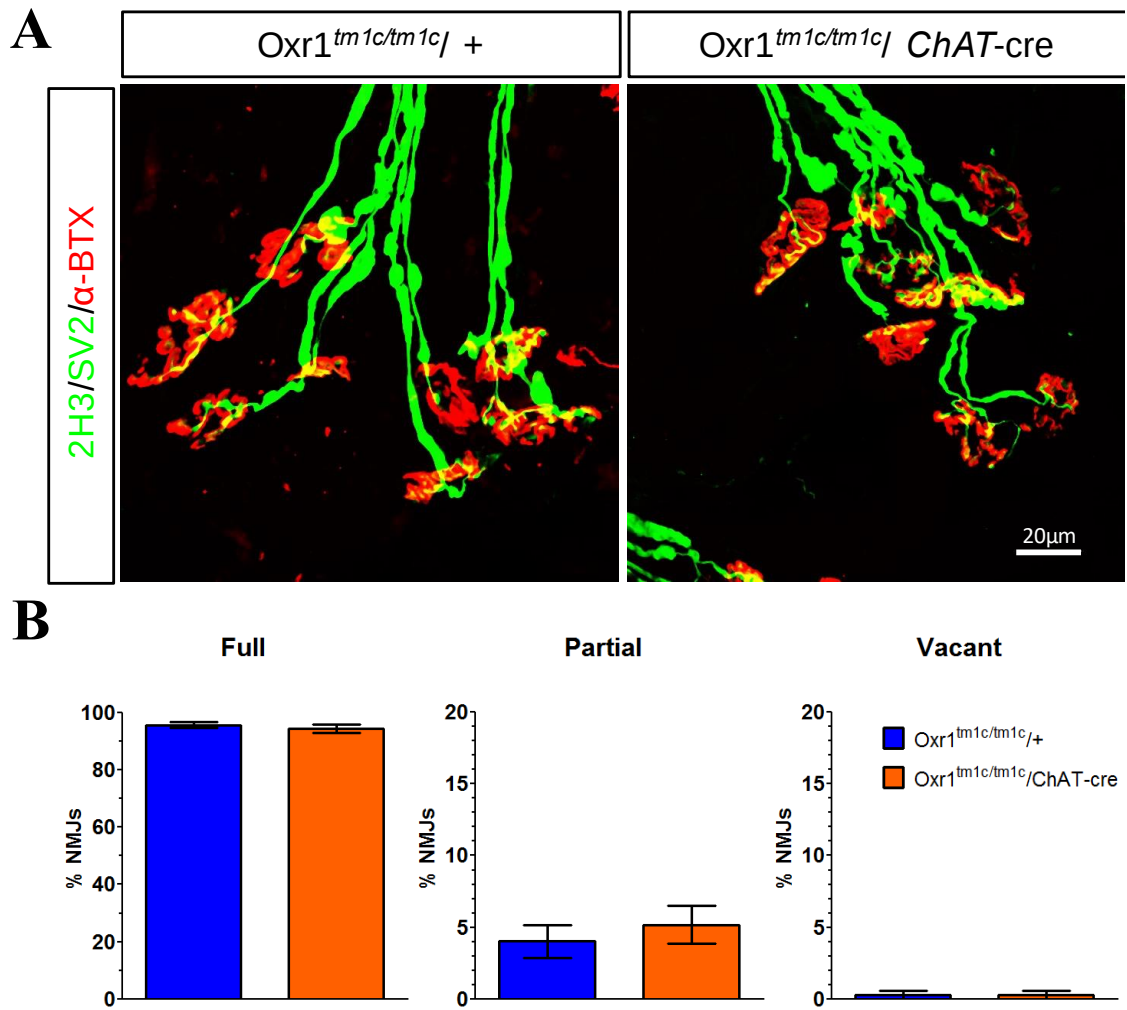


Figure 3.7 Specific loss of *Oxr1* in motor neurons does not cause denervation at the neuromuscular junction in $Oxr1^{tm1c/tm1c}/ChAT\text{-}cre$ mice by 18 months of age. (A) Representative images of neuromuscular junctions in lumbrical muscle from $Oxr1^{tm1c/tm1c}/+$ and $Oxr1^{tm1c/tm1c}/ChAT\text{-}cre$ mice. **(B)** Denervation analysis revealed there were no differences in the percentage of fully or partially innervated and vacant NMJs in $Oxr1^{tm1c/tm1c}/ChAT\text{-}cre$ mice compared to $Oxr1^{tm1c/tm1c}/+$ mice. Values are mean $\pm$ SEM (n=6, Unpaired student's t-test). Scale bar: 20 μ m.

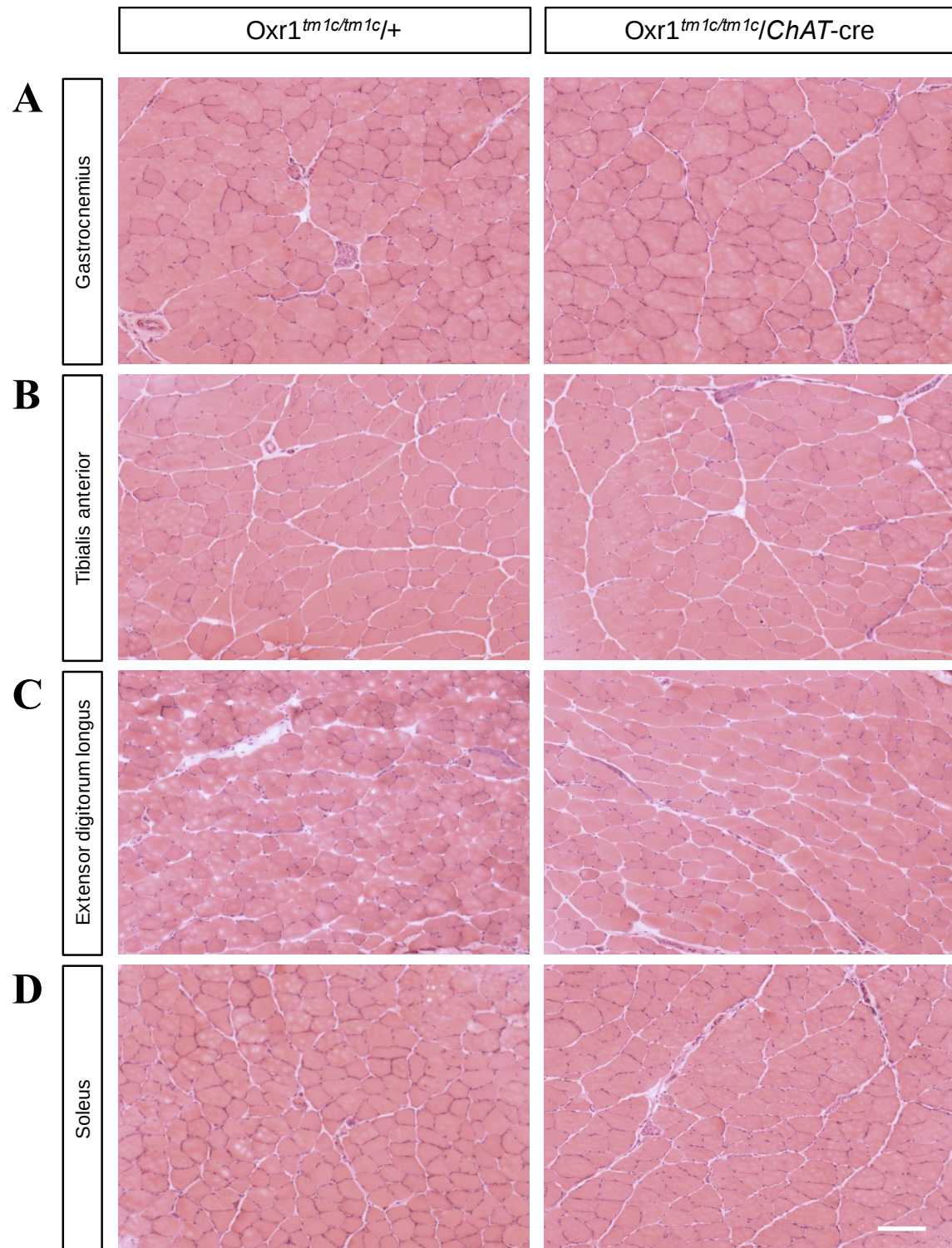


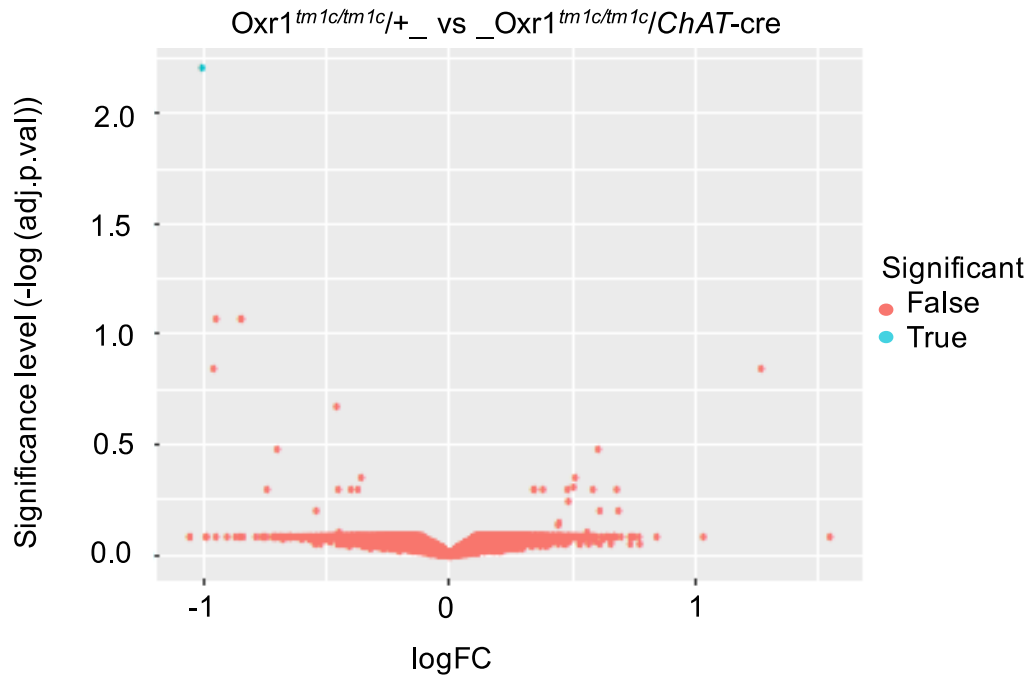
Figure 3.8 Specific loss of *Oxr1* in motor neurons does not cause hind limb muscle atrophy in *Oxr1^{tm1c/tm1c/ChAT-cre}* mice by 18 months of age. Representative images of haematoxylin and eosin (H&E) stained gastrocnemius (**A**), tibialis anterior (**B**), extensor digitorum longus (**C**) and soleus (**D**) muscle fibres from *Oxr1^{tm1c/tm1c/+}* and *Oxr1^{tm1c/tm1c/ChAT-cre}* mice. (n=3). Scale bar: 100µm.

3.3.6 Specific loss of *Oxr1* in motor neurons does not alter the transcriptome in the spinal cord of mice.

Previous studies have demonstrated that reducing expression of *Oxr1* leads to changes in expression of other genes potentially acting downstream, such as catalase, Gpx, HO-1, p21 and peroxiredoxin 4 (Prdx4; Jaramillo-Gutierrez et al. 2010; Yang et al. 2014; Yang et al. 2015). Furthermore, increasing expression of *Oxr1* in the SOD1^{G93A} mouse model of ALS was able to rescue deregulated transcriptional pathways (Liu *et al.* 2015). This suggests that altering the levels of *Oxr1* can affect the expression of other genes. Additionally, given that I observed subtle microgliosis in the spinal cord of *Oxr1*^{tm1c/tm1c}/*ChAT*-cre mice, I wanted to investigate whether loss of *Oxr1* in motor neurons alters the transcriptional pathways in the spinal cord of *Oxr1*^{tm1c/tm1c}/*ChAT*-cre mice. To this end, RNA was purified from the lumbar spinal cord of 18-month-old *Oxr1*^{tm1c/tm1c}/*ChAT*-cre mice and *Oxr1*^{tm1c/tm1c}/+ littermates. Microarray transcriptome analysis was then undertaken externally (Cambridge Genomic Services).

There was no obvious clustering based on genotype and no samples were identified as biological or technical outliers, therefore all samples were included in the subsequent bioinformatic analysis. Microarray analysis revealed that out of 16,671 transcripts, only one gene, phospholipase D family member 5 (PLD5), passed the stringent threshold for False Discovery Rate (FDR; Figure 3.9A&B). I observed a 2-fold decrease in PLD5 expression in *Oxr1*^{tm1c/tm1c}/*ChAT*-cre mice compared to controls. Furthermore, in order to confirm the microglial response observed in the spinal cord of *Oxr1*^{tm1c/tm1c}/*ChAT*-cre mice, I was also able to look for changes in genes known to be involved in these pathways. While I observed a 1.2-fold increase in *Iba1* expression in *Oxr1*^{tm1c/tm1c}/*ChAT*-cre mice, this was not found to be significant. More detailed analysis of this microarray data investigating additional microglial markers would be useful in the future to confirm this inflammatory response. It

was also shown that there were no changes in the expression of genes associated with the OS response, such as *Gpx1*, *Ho-1*, *Prdx4*. Together, this suggests that loss of *Oxr1* in motor neurons does not cause any significant genome-wide expression changes in the spinal cord of *Oxr1^{tm1c/tm1c}/ChAT-cre* mice at 18 months of age.

A**B**

Transcript ClusterID	seqname	start	stop	logFC	Adj. P. Val
Pld5	chr1	175962306	176275312	-1.01	0.01
Fh1	chr1	175600374	175625635	-0.85	0.09
Rgs7	chr1	175059076	175492545	-0.85	0.09
A330094K24Rik	chr18	77729681	77733470	-0.95	0.09
Chml	chr1	175682237	175688353	-0.96	0.14
Uts2	chr4	150997097	151001810	1.26	0.14
Zcchc10	chr11	53324679	53333301	-0.46	0.21
H2-K1	chr17	33996012	34000333	0.60	0.33
Opn3	chr1	175662421	175692776	-0.70	0.33
Erg4	chr6	85511122	85513589	-0.36	0.44

Figure 3.9 Specific loss of *Oxr1* in motor neurons does not cause any overt transcriptomic changes in the spinal cord of *Oxr1^{tm1c/tm1c/ChAT-cre}* mice by 18 months of age. (A) Volcano plot of microarray data showing gene expression changes between *Oxr1^{tm1c/tm1c/+}* and *Oxr1^{tm1c/tm1c/ChAT-cre}* mice. Dots represent genes whose expression was unaltered (red; false) and differentially expressed (green; true). X-axis represents the fold change (log2 scale), y-axis represents the level of significance ($-\log_{10}(\text{adj.p.value})$). **(B)** A summary table showing the top 10 genes that were differentially expressed according to adjusted p value (adj.p.val).

3.4 Discussion

In this chapter, a mouse model in which *Oxr1* is specifically deleted in ChAT-expressing motor neurons was characterised in detail. The conditional knockout *Oxr1^{tm1c/tm1c}/ChAT-cre* mice displayed no differences in survival, motor function or muscle strength. Surprisingly, weight loss appeared to be a clear feature of *Oxr1^{tm1c/tm1c}/ChAT-cre* mice, however it is currently unclear why this occurs. Nonetheless, in agreement with these behavioural observations, I found limited pathology in key components of the neuromuscular system. More specifically, *Oxr1^{tm1c/tm1c}/ChAT-cre* mice displayed no motor neuron loss and no muscle denervation or atrophy compared to age-matched controls. Therefore, this work suggests that specific loss of *Oxr1* in motor neurons alone does not increase motor neuron vulnerability to degeneration *in vivo*.

Overproduction or failed removal of ROS can lead to an increase in OS in all neurons, yet, the vulnerability of neuronal populations to OS varies from one region of the CNS to another. For example, the hippocampal regions cornu ammonis 1 (CA1) and CA3 are composed of morphologically similar pyramidal neurons, yet, when exposed to OS-generating agents, pyramidal neurons in the CA1 region are more vulnerable to OS-induced cell death, while those in the CA3 region mostly survive (Wilde *et al.* 1997; Vornov, Park and Thomas 1998). Constitutive deletion of *Oxr1* in *bella* mutants leads to OS, and a subsequent increase in OS-induced DNA damage in the granule cell layer of the cerebellum (Oliver *et al.* 2011). Furthermore, *bella* mutants display increased degeneration specifically in the granule cell layer, despite high levels of expression in other brain regions such as the cortex (Oliver *et al.* 2011; Finelli *et al.* 2016). This suggests that while the cerebellum from *bella* mice show some signs of OS due to the loss of *Oxr1*; it is clearly limited *in vivo*, likely due to the relatively small proportion of neurons affected (~1–2% of all GCs) in end-stage mutants (Oliver *et al.* 2011). While I did not assess the OS status of motor neurons using 8-

OHdG or other oxidative DNA damage markers, I show here that specific loss of *Oxr1* in motor neurons does not alter the expression of other antioxidant genes in the spinal cord. This suggests there is no obvious signs of an OS-response, thereby implying that there is no OS in the spinal cord of *Oxr1^{tm1c/tm1c}/ChAT-cre* mice. Future studies measuring damage to DNA, proteins or lipids would be needed to confirm the absence of OS in these conditional knockout mice.

It was hypothesised that deletion of *Oxr1*, combined with age-related stress might result in motor neuron degeneration in *Oxr1^{tm1c/tm1c}/ChAT-cre* mice. However, I found this not to be the case, even when mice were aged to 18 months. This suggests that specific loss of *Oxr1* does not increase the vulnerability of motor neurons to degeneration, which is likely a reflection of the limited, if any, signs of OS in the spinal cord of *Oxr1* conditional knockout mice. This raises the question: why are *Oxr1*-depleted GCs more vulnerable to OS-induced degeneration than *Oxr1*-depleted motor neurons? One possible explanation for this could simply be that cerebellar GCs have an inherent increased vulnerability to OS, compared to motor neurons. In support of this hypothesis, it has been demonstrated that after 14 days *in vitro*, wild-type cerebellar GCs are highly sensitive to increased oxygen tension (20%) and displayed increased cell death compared to cells grown in oxygen tension similar to that in the body (5%; X. Wang et al. 2009). Importantly, this observation was not recapitulated in wild-type cerebral cortical neurons, suggesting that GCs are more vulnerable to OS (Wang *et al.* 2009b). This differential response to OS between GCs and cortical neurons is consistent with other studies investigating neuronal vulnerability to OS (Scorziello *et al.* 2001; Kaur, Aschner and Syversen 2007). Unfortunately, to the best of my knowledge, a study directly comparing the vulnerability of wild-type cerebellar GCs and wild-type motor neurons to OS has not been carried out. Therefore, it is difficult to determine with certainty which neuronal population is more vulnerable to OS.

Another explanation to the differential vulnerability between *Oxr1*-depleted GCs and *Oxr1*-depleted motor neurons could be that *Oxr1* plays a more important role in the cerebellum than in spinal motor neurons. This is somewhat supported by comparisons made between primary GCs and primary cortical neurons from *bella* mice (Oliver *et al.* 2011). After 14-days *in vitro* and hydrogen peroxide treatment, a significant 80% increase in apoptosis was observed in mutant GCs compared to wild-type controls; whereas a much smaller increase in apoptosis (~20%) was seen in mutant cortical neurons (Oliver *et al.* 2011). This suggests that *Oxr1* levels play a more important role for neuronal survival in the cerebellum, consistent with the specificity of neurodegeneration seen in *bella* mice. Of note, I only investigated the effect of *Oxr1* loss on the vulnerability of motor neurons to degeneration *in vivo*. Therefore, it would be worthwhile to determine whether cultured primary motor neurons from *Oxr1^{tm1c/tm1c}/ChAT-cre* mice display a phenotype more similar to *bella* cortical neurons than *bella* GCs under OS conditions. Additionally, it should also be considered that other TLDc proteins such as *Ncoa7* or *Tbc1d24* could be functionally compensating for the loss of *Oxr1* in motor neurons, but not in GCs. This is supported by the lack of *Ncoa7* expression in GCs (Finelli *et al.* 2016).

Finally, the differential vulnerability of *Oxr1*-depleted GCs and *Oxr1*-depleted motor neurons could also be explained by the differences in model design between the *bella* mutant and the conditional knockout. As *Oxr1* is constitutively deleted in *bella* mutant mice, cerebellar glial cells would also be *Oxr1* deficient. Cerebellar glial cells are known to play important roles in cerebellar development and function (Buffo and Rossi 2013). Therefore, it could be postulated that loss of *Oxr1* in these glial cells could lead to intracellular OS and dysfunction, potentially contributing to GC degeneration and the overall phenotype of *bella* mice. By contrast, in *Oxr1^{tm1c/tm1c}/ChAT-cre* mice, glial cells surrounding motor neurons express *Oxr1*. Therefore, it could be expected that normal glial function is preserved, thereby

contributing to the health and survival of *Oxr1*-depleted motor neurons. This hypothesis is somewhat supported by *in vivo* studies using a conditional neuron-specific SOD1^{G37R} mutant mouse model of ALS. In contrast to observations in transgenic lines overexpressing the same ALS-associated SOD1 mutation in a constitutive manner (Wong *et al.* 1995), it was demonstrated that neuron-specific expression does not increase motor neuron vulnerability to degeneration and mice do not exhibit any motor defects up to 18 months of age (Pramatarova *et al.* 2001). This suggests that expression of mutant SOD1^{G37R} and its toxic effect on the cell is required in other cell types, such as glial or muscle cells, for the development of a motor neuron degenerative phenotype. Additional studies in transgenic mutant SOD1 mouse models that constitutively express mutant SOD1 protein, showed that selective loss of mutant SOD1 expression in astrocytes or microglia slowed the progression of disease-associated phenotypes, including motor neuron survival (Boillee *et al.* 2006; Wang *et al.* 2009a; Wang, Gutmann and Roos 2011). This further exemplifies that stressed or dysfunctional glial cells can progress the development of degenerative phenotypes. Perhaps generating a mouse model in which *Oxr1* is conditionally deleted in motor neurons and surrounding glia would elicit enough stress for a degenerative phenotype to develop.

Glial cells, such as astrocytes and microglia, play important roles in maintaining overall CNS homeostasis, providing trophic support to neurons, clearing synapses of released neurotransmitters, re-establishing ionic gradients near the synapse, mediating immune responses in the brain, and reducing OS (Dringen, Gutterer and Hirrlinger 2000; Park, Zhang and Gibson 2001). When regional homeostasis in the CNS is disturbed because of OS or other neurodegenerative conditions, astrocytes and microglia become activated. This activation is often termed astrogliosis and microgliosis, and can also be taken as indications of neuronal toxicity or death (Hall, Oostveen and Gurney 1998). In this chapter, I demonstrate that loss of *Oxr1* in motor neurons results in subtle microgliosis in the spinal

cord of the conditional knockout mice, despite displaying no motor neuron death. Furthermore, I observed no significant astrocytic response. While the cause and consequence of this response is still undetermined, it is in part agreement with observations in end-stage *bella* mice, demonstrating that loss of *Oxr1* resulted in microgliosis and astrogliosis in the cerebellum and spinal cord (Liu *et al.* 2015). It should be considered however, that this neuroinflammatory response in *bella* mice was clearly more prominent in the cerebellum, with minimal detection of reactive astrocytes and microglia in the spinal cord. The discrepancy between these two models in astroglial response could again be due to the fact that glial cells in *bella* mice lack *Oxr1* expression, whereas in glial cells in *Oxr1^{tm1c/tm1c}/ChAT-cre* mice do not (Zhang *et al.* 2014). Further supporting the notion that glial cells can modulate the development and/or progression of neurodegenerative phenotypes.

While these glial responses can be beneficial to neurons, the continuous or repeated activation of astrocytes and microglia under conditions of chronic inflammatory stress can lead to the increased production of ROS and other neurotoxic mediators (D'Ambrosi, Cozzolino and Carri 2017). Given that I observe no motor neuron loss in the conditional knockout, it could be hypothesised that these reactive microglia are still functioning to protect and support the surrounding motor neurons, resembling a more M2/protective phenotype (Geloso *et al.* 2017). It would be worthwhile in the future to characterise the phenotype of these reactive microglia. A possible approach would be to use laser capture microdissection (LCM) or fluorescent activated cell sorting (FACS) to isolate microglia from spinal cord tissue (Longbrake *et al.* 2007; Galatro *et al.* 2017). Then, expression of both pro-inflammatory markers such as IL-1 β and TNF- α , as well as anti-inflammatory markers such as IL-4 and TGF- β could be tested (West *et al.* 2011; Liao *et al.* 2012; Hu *et al.* 2015; Tang and Le 2016). There is emerging evidence that suggests neuron-glial crosstalk

may play a role in selective neuronal vulnerability to OS (Wang and Michaelis 2010). Therefore, depending on the phenotype of these reactive microglia, it could provide another possible explanation as to why I observe no motor neuron loss in *Oxr1^{tm1c/tm1c}/ChAT-cre* mice.

Given that *Oxr1* does not possess any catalytic activity like other antioxidant enzymes (Oliver *et al.* 2011), recent studies have attempted to dissect the pathways downstream of OXR1, to identify how *Oxr1* confers protection against OS and cell death. For example, using the HeLa cell line, transcriptomic analysis was carried out after the expression of OXR1 was knocked down in cells by 85% (Yang *et al.* 2015). Interestingly, combining data from non-treated and hydrogen peroxide treated cells, it was revealed that more than 700 genes were differentially expressed in OXR1-depleted cells compared to wild-type cells, including antioxidant genes such as GPX2, HO-1, p21 and PRDX4, genes of the p53 signalling pathway involved in cell-cycle arrest, such as cyclin D, and genes involved in apoptosis, such as cytochrome-C and caspase-9 (Yang *et al.* 2015). The altered expression of antioxidant genes is somewhat supported by studies utilising *Oxr1* knockdown in the mosquito and *Oxr1* deletion in mice (Jaramillo-Gutierrez *et al.* 2010; Oliver *et al.* 2011). Taken together, these knockdown studies suggest that *Oxr1* can function in the OS response pathway by directly or indirectly regulating the expression of other antioxidant enzymes, and can also modulate the expression of genes involved in the cell cycle and apoptosis.

Here, I demonstrate that loss of *Oxr1* in motor neurons did not result any significant changes to the expression of such genes, suggesting limited, if any, OS in this tissue at that time-point. Although this is in agreement with the lack of motor neuron loss and muscle denervation or atrophy observed in these mice, it should be considered that spinal cord homogenates were used to generate this data. Therefore, transcriptomic data generated is

representative of a mix of neuronal and glia cells unaffected by *Oxr1* deletion, in addition to motor neurons lacking *Oxr1*. Thus, if there were any small expression changes specific to *Oxr1*-depleted motor neurons, it is entirely possible they could have been masked. In order to address the issue of cellular heterogeneity in homogenate samples in the future, LCM or FACS could be used to isolate *Oxr1*-depleted motor neurons from the spinal cord, as similarly described (Hedlund *et al.* 2010; Schaller *et al.* 2017). Subsequent transcriptional analysis could then potentially provide more insight as to how specific loss of *Oxr1* does not increase motor neuron vulnerability to OS and degeneration. Neurodegenerative disease often presents with pathology, such as OS, in highly specific neuronal populations, despite widespread or ubiquitous expression of the mutated gene(s) involved (Saxena and Caroni 2011). Therefore, multiple examples of selective neuronal vulnerability and resistance to OS, such as *bella* and *Oxr1^{tm1c/tm1c}/ChAT-cre* mice, will be vital in the future to understand why certain neurons are targeted while others are spared.

Chapter 4

The neuroprotective function of Oxr1 in TDP-43 related ALS

4.1 Introduction

Mutations in TDP-43 have been reported by several groups and account for approximately 5% of fALS cases, <1% of sALS cases and even rare familial cases of FTD (Kabashi *et al.* 2008; Sreedharan *et al.* 2008; Corrado *et al.* 2009; Daoud *et al.* 2009). Despite their disparate etiology, comparisons between fALS and sALS have revealed >95% of all ALS cases share a common pathological signature, namely the cytoplasmic mislocalisation of TDP-43 and the formation of aggregates containing hyper-phosphorylated, truncated and ubiquitinated TDP-43 protein (Neumann *et al.*, 2006). Moreover, TDP-43 aggregation and neuropathology has been identified in a wider spectrum of distinct neurodegenerative disorders collectively known as TDP-43 proteinopathies (Kwong *et al.* 2008). Interestingly, TDP-43 proteinopathies are not limited to the CNS as TDP-43-positive cytosolic muscle aggregates have been identified in familial and sporadic inclusion body myositis (Salajegheh *et al.* 2009). Therefore, studies that aim to elucidate the physiological functions of TDP-43 and the molecular mechanisms underlying TDP-43 proteinopathies are of great clinical importance.

TDP-43 is a ubiquitously expressed DNA/RNA-binding protein that functions in diverse cellular processes such as modulating transcription, regulating RNA splicing and stability, and microRNA biogenesis (Lagier-Tourenne, Polymenidou and Cleveland 2010; Fiesel and Kahle 2011). In the CNS, TDP-43 binds over 6000 RNA targets, regulates the expression of 600 mRNA species and the alternative splicing of over 950 species, exemplifying its crucial function in RNA metabolism (Polymenidou *et al.* 2011). While TDP-43 is predominantly found in the nucleus, ~30% can be found in the cytoplasm under

normal physiological conditions (Barmada *et al.* 2010). However, under conditions of cellular stress such as OS, proteasome inhibition and heat shock, TDP-43 is recruited to the cytoplasm where it functions in the stress granule response (Colombrita *et al.* 2009; Dewey *et al.* 2011; McDonald *et al.* 2011). This response involves the formation of cytoplasmic protein complexes that regulate mRNA expression, processing and sequester mRNAs redundant for cell survival (Anderson and Kedersha 2002; Kedersha and Anderson 2007). This not only further supports a role for TDP-43 in RNA metabolism, but also suggests its function is particularly important under conditions of cellular stress.

To date, genetic studies have revealed 53 mutations in TDP-43, however not all of these are associated with ‘pure’ ALS and/or predicted to be pathogenic (Buratti 2015; <http://alsod.iop.kcl.ac.uk>). One ALS-associated pathogenic mutation, A90V, is found between the bipartite nuclear localisation signal (Figure 4.1), which is important for the normal localisation of the protein (Winton *et al.* 2008; Chiang *et al.* 2012). Another pathogenic mutation, D169G, is located in one of the two RNA-recognition motif (RRM) domains (Figure 4.1; Kabashi *et al.* 2008). However, the remaining mutations are located in the glycine-rich C-terminal domain (Figure 4.1; Buratti 2015). This region, involved in protein-protein interactions (Buratti *et al.* 2005), is also necessary for regulating the splicing inhibitory activity of TDP-43 for certain RNA transcripts (D’Ambrogio *et al.* 2009), and influences the solubility and cellular localization of TDP-43 (Ayala *et al.* 2008). This suggests that this domain is particularly important for normal TDP-43 function and can lead to neurodegeneration when disrupted.

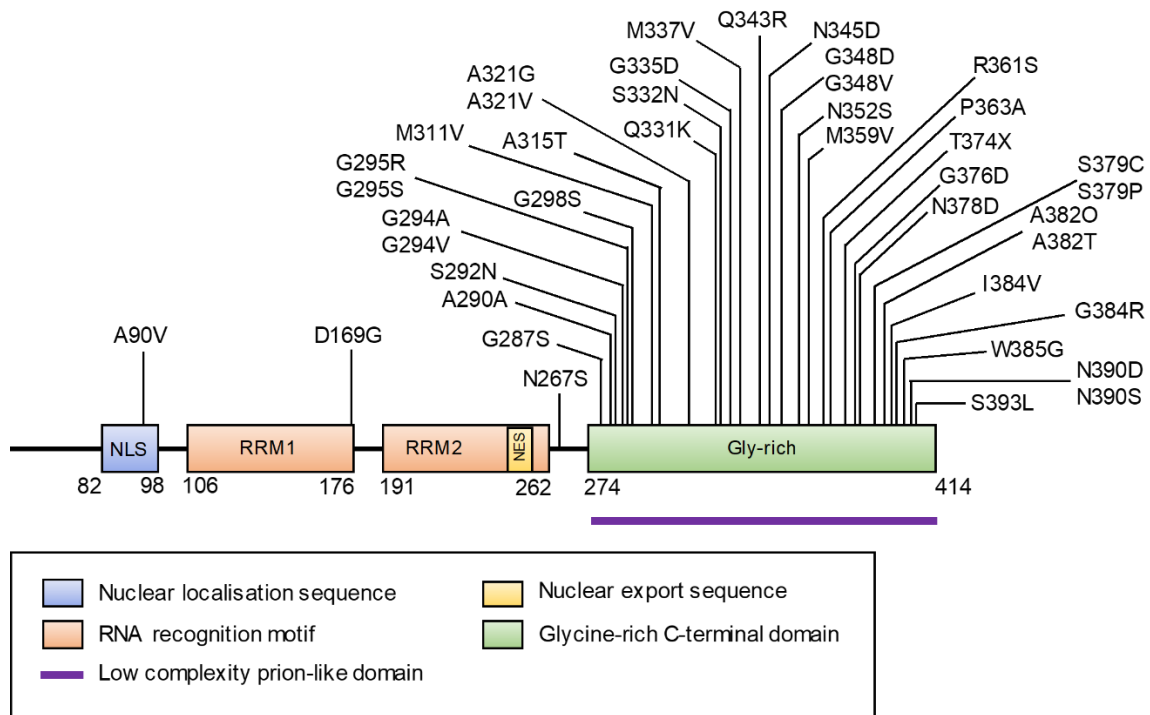


Figure 4.1 Schematic representation of TDP-43. Diagram shows structure and functional domains of TDP-43, including two RNA recognition motifs, a nuclear localisation sequence, a nuclear export sequence and a glycine-rich domain of low complexity that possesses prion-like activity (Gitler and Shorter 2011). The localisation of pathogenic ALS-associated mutations are annotated in their corresponding residues. Sequence data was obtained from Uniprot (domain prediction; <http://www.uniprot.org>). A complete list of TDP-43 mutations can be found at <http://alsod.iop.kcl.ac.uk>.

The M337V mutation, a disease-associated mutation in the glycine-rich C-terminal domain (Figure 4.1) is frequently used for *in vitro* studies as it recapitulates some characteristic features of ALS. For example, culturing primary motor neurons from BAC transgenic mice that contain a single copy of the human genomic TARDBP locus carrying the M337V mutation revealed that TDP-43^{M337V} is mislocalised to the cytoplasm more frequently than wild-type TDP-43 and spontaneously developed cytoplasmic aggregates under basal conditions (Mutihac *et al.* 2015). This is supported by studies using iPSC-derived motor neurons from ALS patients, demonstrating that TDP-43 protein was predominantly found in the soluble fraction in iPSC-derived motor neurons from controls, whereas it was mainly found in detergent-resistant insoluble fractions from cells carrying the TDP-43^{M337V} mutation (Bilican *et al.* 2012; Egawa *et al.* 2012). Furthermore, iPSC-derived TDP-43^{M337V} motor neurons formed cytosolic aggregates similar to those seen in post-mortem tissue from ALS patients (Egawa *et al.* 2012), and increased cell death compared to control motor neurons (Bilican *et al.* 2012). Taken together, these studies support that the M337V mutation in TDP-43 is toxic to motor neurons and suggests that patient-derived TDP-43^{M337V} neurons recapitulate key biochemical aspects of TDP-43 pathology in ALS.

The toxicity of TDP-43^{M337V} has also been demonstrated *in vivo* using a variety of animal models of ALS. In *C. elegans*, expression of wild-type human TDP-43 in neurons causes moderate motor defects, whereas human TDP-43^{M337V} expression causes premature death, severe motor dysfunction, motor neuron degeneration accompanied by hyperphosphorylation, truncation, and ubiquitination of TDP-43 protein that accumulates in detergent-insoluble aggregates (Liachko, Guthrie and Kraemer 2010). This is supported by observations in a rat model of TDP-43-related ALS whereby inducing expression of TDP-43^{M337V} by postnatal day 10 (P10) resulted in motor dysfunction, motor neuron loss, axonal

and NMJ degeneration, muscle atrophy, neuroinflammation, TDP-43 aggregation in spinal motor neurons and premature death by 2 months of age (Zhou *et al.* 2010). Taken together, these studies further support that the M337V mutation in TDP-43 is toxic to motor neurons and when modelled *in vivo* can recapitulate many of the features observed in human ALS. Nevertheless, it remains undetermined whether toxicity to motor neurons from mutations in TDP-43 is mediated through a gain of toxic function, a loss of function, or a combination of both.

Expression of TDP-43^{M337V} has been shown to cause alterations in markers of OS, which is an early feature of ALS. For example, expression of mutant TDP-43 in a motor neuron-like cell line results in mitochondrial dysfunction and nuclear accumulation of Nrf2, an indicator and modulator of OS (Duan *et al.* 2010). Interestingly, it has also been demonstrated that mutant TDP-43 inhibits antioxidant signalling pathways and can promote OS. For example, using a motor neuron-like cell line expressing hTDP-43^{M337V}, it was observed that the expression of Nrf2 and a downstream antioxidant enzyme NQO1 was decreased compared to control cells expressing wild-type hTDP-43 (Tian *et al.* 2017). This is somewhat supported by studies using ALS patient fibroblasts harbouring a TDP-43^{M337V} mutation. It was observed that the levels of GSH, a marker of OS, were decreased in TDP-43^{M337V} patient fibroblasts compared to controls (Moujalled *et al.* 2017). Taken together, these studies support that mutant TDP-43 inhibits antioxidant response pathways and increases the susceptibility to OS-induced damage and cell death.

As mentioned previously, OS regulates the cellular localisation of TDP-43, which, when relocated to the cytoplasm, plays an important role in the formation of stress granules (Colombrita *et al.* 2009). However, prolonged OS has also been shown to promote aberrant mislocalisation and aggregation of TDP-43. For example, after exposing N2A cells to hydrogen peroxide, immunoblots and immunocytochemical staining revealed that there was

increased TDP-43 and phosphorylated TDP-43 (pTDP-43) protein in cytosolic fractions and increased cytoplasmic aggregates of TDP-43, compared to non-treated controls (Ayala *et al.* 2011a). This suggests OS can induce TDP-43 mislocalisation and aggregation. This is somewhat supported by studies demonstrating that depletion of GSH levels in motor neuron-like cells induced pathology similar to that of mutant TDP-43, including cytoplasmic mislocalisation and aggregation of insoluble TDP-43 (Iguchi *et al.* 2012; Chen *et al.* 2018b). In agreement, depletion of the antioxidant protein OXR1 in HeLa cells, followed by exposure to hydrogen peroxide resulted in increased cytoplasmic aggregation of TDP-43, further supporting increased OS can induce TDP-43 mislocalisation and aggregation (Liu 2014). Finally, treatment of fibroblast-like cells with HNE was shown to cause phosphorylation and cytosolic mislocalisation of TDP-43 (Kabuta *et al.* 2015). As previously mentioned, CSF HNE levels are increased in ALS patients and therefore could potentially contribute to TDP-43 aggregation (Smith *et al.* 1998). Taken together these studies build a strong argument for the role of OS in the cytoplasmic mislocalisation and aggregation of TDP-43.

It is also well established that OS regulates the solubility of TDP-43. For example, it was demonstrated *in vitro* that OS promotes TDP-43 cross-linking via cysteine oxidation and disulphide bond formation within and surrounding the RRM2 domain, leading to decreased TDP-43 solubility (Cohen *et al.* 2012). Moreover, increased levels of cross-linked TDP-43 species have been observed in FTD brains, supporting that aberrant TDP-43 cross-linking is a prominent pathological feature in neurodegenerative disease (Cohen *et al.* 2012). In addition to cysteine oxidation, lysine acetylation is another post-translational modification promoted by OS that is known to control TDP-43 solubility and aggregation. It was demonstrated that acetylation of key lysine residues in the RRM domains of TDP-43 impaired its RNA binding function and promoted the aggregation of insoluble,

hyperphosphorylated TDP-43 (Cohen *et al.* 2015). Importantly, acetylated TDP-43 aggregates are found in ALS patient spinal cords, therefore these data suggest that OS promotes aberrant TDP-43 acetylation, which could contribute to the formation of insoluble TDP-43 aggregates found in human ALS (Cohen *et al.* 2015). OS is clearly a key contributory factor in the mislocalisation and aggregation of insoluble TDP-43 protein, which is observed in the majority of ALS cases and cases of FTD with TDP-43 pathology. Therefore, the identification of factors that reduce OS and/or ameliorate the mislocalisation of TDP-43 would be an important avenue to pursue for the design of novel therapeutic strategies for ALS and other TDP-43 proteinopathies.

It has been demonstrated previously by the Oliver group that the short isoform of Oxr1, Oxr1-C, binds to TDP-43 (Finelli *et al.* 2015). Furthermore, certain ALS-associated mutations in TDP-43 (D169G and A321G; Figure 4.1) significantly decreased binding to Oxr1-C, whereas other mutations (M337V and Q331K; Figure 4.1) did not (Finelli *et al.* 2015). In addition, it was also demonstrated under OS conditions that overexpression of Oxr1-C in cells expressing specific TDP-43 mutants improved the cytoplasmic mislocalisation and aggregation of TDP-43 (Finelli *et al.* 2015). Importantly though, only mutants with a preserved binding for Oxr1-C (TDP-43^{M337V} and ^{Q331K}) could relocate to the nucleus when cells were co-expressed with Oxr1-C (Finelli *et al.* 2015). This not only suggests that binding of TDP-43 with Oxr1-C was important and necessary for the proper cellular localisation of TDP-43, but also begins to support the hypothesis that Oxr1 has potential to reduce TDP-43 pathology.

Interestingly, previous work by the Oliver group investigated the therapeutic potential of Oxr1 in SOD1-related ALS (Liu *et al.* 2015). To study whether overexpression of Oxr1 was neuroprotective *in vivo*, the Oliver group generated a transgenic mouse, in which the mouse prion protein (*mPrnp*) promoter drives overexpression of mouse *Oxr1-FL*

cDNA with a C-terminal HA-tag ([Tg(*mPrnp-Oxr1*]; hereon referred to as *Oxr1^{Prnp/+}*; Borchelt et al. 1996). It was demonstrated that HA-tagged *Oxr1* protein was expressed as early as embryonic Day 13.5 and also expressed specifically in neurons in the brain and spinal cord (Liu *et al.* 2015). Furthermore, adult *Oxr1^{Prnp/+}* mice show a consistent 5-fold increase in *Oxr1* protein expression in the brain and spinal cord compared to non-transgenic (NTg) controls (Liu *et al.* 2015). *Oxr1^{Prnp/+}* mice were crossed with *SOD1^{G93A}* mice (Gurney *et al.* 1994) and offspring were tested for longitudinal behavioural and pathological parameters (Liu *et al.* 2015). Importantly, adult *Oxr1^{Prnp/+}* mice presented no behavioural defects or pathology, and microarray analysis revealed that the expression of *Oxr1* was the only gene altered in the spinal cord compared to NTg controls (Liu *et al.* 2015). This supports that *Oxr1^{Prnp/+}* mice are a good model to investigate the neuroprotective effects of *Oxr1*. It was also demonstrated that neuronal overexpression of *Oxr1* in the *SOD1^{G93A}* mouse model of ALS extended survival and delayed spinal cord and muscle pathogenesis (Liu *et al.* 2015). Moreover, *Oxr1* overexpression delayed early *SOD1^{G93A}* ALS-induced alterations to the transcriptome, including the activation of neuroinflammatory pathways in the spinal cord (Liu *et al.* 2015). These findings support the notion that increasing expression of *Oxr1* is sufficient to modulate the pathogenesis and disease progression of a mouse model of ALS.

A number of groups have attempted to generate mouse models of ALS expressing either wild-type or mutant TDP-43 using either BAC technology or cDNA-derived overexpression of the transgene using ubiquitously expressed or neuronal-specific promoters (see Table 1.2, Chapter 1; Węgorzewska *et al.*, 2009; Shan *et al.*, 2010; Stallings *et al.*, 2010; Tsai *et al.*, 2010; Wils *et al.*, 2010; Xu *et al.*, 2010; Xu *et al.*, 2011). While some models have successfully recapitulated certain phenotypes associated with human ALS, such as motor dysfunction, none have succeeded in modelling all features of the disease. Specifically, adult or end-stage mutant mice rarely present with motor neuron degeneration

and/or TDP-43 positive aggregates in motor neurons (see Table 1.2, Chapter 1). In addition, disease phenotypes observed are highly variable between different models, which is likely a reflection of the choice of promoter, level and timing of transgene expression and pathways not relevant to human ALS, such as non-specific toxicity from TDP-43 overexpression (McGoldrick *et al.* 2013). Therefore, it is critical to generate new animal models for TDP-43 proteinopathies.

In this context, the Talbot group (Nuffield Department of Clinical Neurosciences, University of Oxford) generated transgenic mice in which a BAC construct containing an 80kb genomic fragment consisting of the entire human TDP-43 locus (either TDP-43^{WT} or TDP-43^{M337V}) and its regulatory regions was specifically inserted into the mouse Rosa-26 locus in a single copy (Mutihac *et al.* 2015; Gordan *et al.*, unpublished work). As a result, homozygous TDP-43^{M337V} mice expresses hTDP-43 at low levels (<0.5-fold increase of endogenous Tdp-43), and could therefore be more suitable in modelling human ALS than models which grossly over-express TDP-43 protein (up to 5-fold increase of endogenous Tdp-43; Janssens *et al.* 2013). The Talbot group also planned to generate either TDP-43^{WT} or TDP-43^{M337V} mice on a null *Tardbp* background, however no live offspring were produced (Gordon *et al.*, 2019). This may be due to the relatively low levels of expression, or perhaps more likely, a failure for human TDP-43 to functionally replace the mouse gene (Wu *et al.*, 2010). Nonetheless, in contrast to mice expressing human TDP-43^{WT}, expression of human TDP-43^{M337V} in this mouse model, leads to progressive motor impairment by 9-12 months of age, loss of NMJ integrity at 12 months of age and a ~50% reduction in survival by 2 years of age (Gordon *et al.*, 2019). In addition, primary motor neurons expressing human TDP-43^{M337V} show mislocalisation and aggregation of TDP-43, and a defect in stress granule dynamics (Gordon *et al.*, 2019). Therefore, this model provides a valuable tool to study both behavioural and cellular aspects of TDP-43 dysfunction.

4.2 Aims of Chapter 4

Given that Oxr1 can improve mislocalisation and aggregation of mutant TDP-43^{M337V} *in vitro* (Finelli *et al.* 2015), I aimed to investigate the neuroprotective function of Oxr1 in TDP-43-related ALS *in vivo*. In order to do this, I first crossed the transgenic mouse line overexpressing Oxr1 in neurons (Oxr1^{Prnp/+}) with the new BAC transgenic mouse model of a human TDP-43 mutation (M337V; Gordon *et al.*, 2019). To determine whether overexpression of Oxr1 can improve the pathogenesis and disease progression in the mutant TDP-43^{M337V} ALS mouse model, I then carried out longitudinal behavioural and pathological analysis on experimental mice.

4.3 Results

4.3.1 Oxr1 is not upregulated in TDP-43^{M337V/M337V} mice by 18 months of age.

In this chapter, I investigate whether Oxr1 is a genetic modifier of TDP-43 pathology *in vivo* by crossing two different mouse lines. Specifically, mice heterozygous for the TDP-43^{M337V} BAC transgene and the Oxr1 transgene (TDP-43^{M337V/+}/Oxr1^{Prnp/+}) were crossed with TDP-43^{M337V/+} mice to generate experimental progeny with four genotypic combinations (NTg, Oxr1^{Prnp/+}, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Prnp/+}). Using whole tissue lysates from either brain or spinal cord samples, I immunoblotted with anti-GFP to recognize Ypet-tagged hTDP-43^{M337V} and anti-TDP-43 to recognize both human and mouse species. I confirmed that TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} mice express the human Ypet-tagged TDP-43^{M337V} protein (~70kDa; Figure 4.2A&B). As expected, I also confirmed that Oxr1^{Prnp/+} and TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} mice also express HA-tagged Oxr1-FL in both brain and spinal cord samples (Figure 4.2A&B). In addition, expression of human Ypet-tagged TDP-43^{M337V} in heterozygous TDP-43^{M337V/+} mouse brain was approximately half that observed in homozygous TDP-43^{M337V/M337V} mouse

brain (Figure 4.2C). Importantly, TDP-43 mutant mice express human Ypet-tagged TDP-43^{M337V} at low levels compared to endogenous Tdp-43 (Figure 4.2A-C), which supports the use of TDP-43^{M337V/M337V} mice as a mouse model of ALS based on low-level expression of human TDP-43^{M337V}. Together, these data confirm that the progeny from this mouse cross express the correct transgenes and can therefore be used to investigate the neuroprotective function of Oxr1 in TDP-43-related ALS.

Given that isoforms of Oxr1 are up-regulated in post-mortem spinal cord samples of ALS patients as well as in the pre-symptomatic SOD1^{G93A} mouse model of ALS (Oliver *et al.* 2011), I also investigated whether Oxr1 is upregulated in TDP-43^{M337V/M337V} mice by 18 months of age. In both brain and spinal cord, I observed no obvious differences in expression of Oxr1 in TDP-43^{M337V/M337V} mice compared to NTg controls by 18 months of age (Figure 4.2A&B), suggesting Oxr1 is not upregulated in the CNS of these mutant mice.

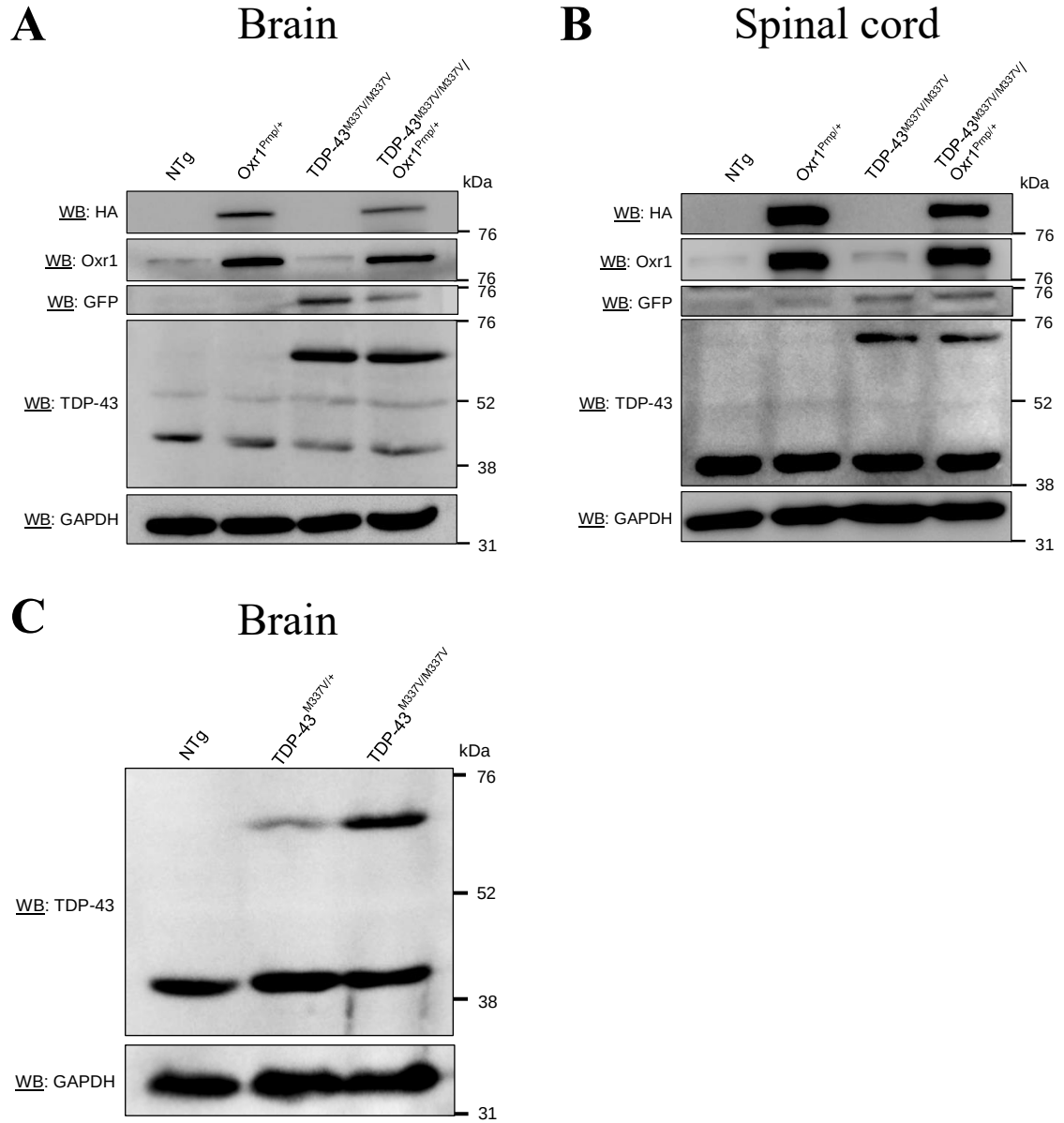


Figure 4.2 Oxr1 is not upregulated in the brain or spinal cord of TDP-43^{M337V/M337V} mice by 18 months of age. Western blot confirmation of stable transgene overexpression of mouse HA-tagged Oxr1 in Oxr1^{Pmp/+} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice and human Ypet-tagged TDP-43^{M337V} (~70kDa) in TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice from (A) brain and (B) spinal cord samples at 18 months of age. (C) Human Ypet-tagged TDP-43^{M337V} is expressed at low levels (<1-fold) in the brains of homozygous TDP-43^{M337V/M337V} mice, and even more so (<0.5-fold) in heterozygous TDP-43^{M337V/+} mice, relative to the endogenous TDP-43 (~43kDa).

4.3.2 Overexpression of *Oxr1* improves motor defects in TDP-43^{M337V/M337V} mice.

Given that other mouse models of TDP-43^{M337V} present with reduced body weight, progressive motor dysfunction and decreased lifespan (Xu *et al.* 2011; Arnold *et al.* 2013), I first monitored TDP-43^{M337V/M337V} mice by body weight and behavioural tests commonly used to monitor motor coordination (rotarod) and muscle strength (grip strength). Cohorts used for testing, including sample size/sex/genotype/age group can be found in Table 4.1. Both male and female TDP-43^{M337V/M337V} mice were phenotypically normal at birth and demonstrated no difference in weight, motor coordination and muscle strength up to 6 months of age (Figure 4.3A - F).

At 9 months of age, male TDP-43^{M337V/M337V} mice display a significant decrease in motor coordination and muscle strength compared to NTg controls (Figure 4.3C & E). While this decrease in motor coordination and muscle strength is progressive, the difference between male TDP-43^{M337V/M337V} mice and NTg littermates was not significant between 12 and 18 months of age (Figure 4.3C & E). This suggests that there is a window for the onset of motor dysfunction in adult TDP-43^{M337V/M337V} mice, however this is not exacerbated at later time points. Interestingly, between 12 and 18 months of age, male NTg mice display increased body weight compared to TDP-43^{M337V/M337V} mice (Figure 4.3A). As body weight may affect performance in behavioural motor tests (McFadyen *et al.* 2003), I hypothesize that the loss of significance between TDP-43^{M337V/M337V} and NTg mice in motor coordination and muscle strength tests from 12-18 months of age could be a result of the increased weight of male NTg mice.

Between 9 and 18 months of age, female TDP-43^{M337V/M337V} mice show a slight decline in motor coordination, however this does not reach significance when compared to NTg littermates (Figure 4.3D). Similar to observations in male mice, female TDP-43^{M337V/M337V} mice at 9 months of age present decreased muscle strength compared to NTg

littermates, however this did not reach significance from 12-18 months of age (Figure 4.3F). Furthermore, from 9-18 months of age, there were no significant differences in body weight between female TDP-43^{M337V/M337V} and NTg mice (Figure 4.3B). Together, these data suggest that even though there was a brief decrease in muscle strength in female TDP-43^{M337V/M337V} mice compared to NTg littermates, their motor coordination remained largely unaffected and showed no significant weight loss, indicative of a subtle disease phenotype. Therefore, for the remainder of this chapter, the majority of pathological analysis was focused on male mice.

In parallel, I also assessed TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice for the same parameters in order to determine whether neuronal overexpression of Oxr1 improves motor dysfunction in TDP-43^{M337V/M337V} mice. For both males and females, I observed no differences in body weight between TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice across all time-points (Figure 4.3A & B). Interestingly, I observed that male TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice display a significant improvement in motor coordination compared to TDP-43^{M337V/M337V} littermates at 9, 12 and 15 months of age (Figure 4.3C). In addition, between 12-18 months of age, male TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice show increased muscle strength compared to male TDP-43^{M337V/M337V} littermates (Figure 4.3E). Similarly, female TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice display a significant improvement in motor coordination and muscle strength from 12-18 and 9-12 months of age, respectively as compared to female TDP-43^{M337V/M337V} littermates (Figure 4.3D & F). Therefore, taken together, these data suggest that overexpression of Oxr1 in neurons improves motor defects in TDP-43^{M337V/M337V} mice.

Table 4.1 Motor function testing cohorts

		Male	Female
NTg	3 months	10	15
	6 months	11	16
	9 months	11	15
	12 months	11	14
	15 months	11	13
	18 months	11	13
Oxr1^{Prnp/+}	3 months	7	11
	6 months	8	13
	9 months	7	12
	12 months	6	11
	15 months	6	11
	18 months	3	10
TDP-43^{M337V/M337V}	3 months	20	18
	6 months	19	19
	9 months	16	19
	12 months	17	17
	15 months	16	16
	18 months	11	14
TDP-43^{M337V/M337V}/ Oxr1^{Prnp/+}	3 months	22	22
	6 months	22	22
	9 months	23	21
	12 months	22	20
	15 months	21	18
	18 months	17	16

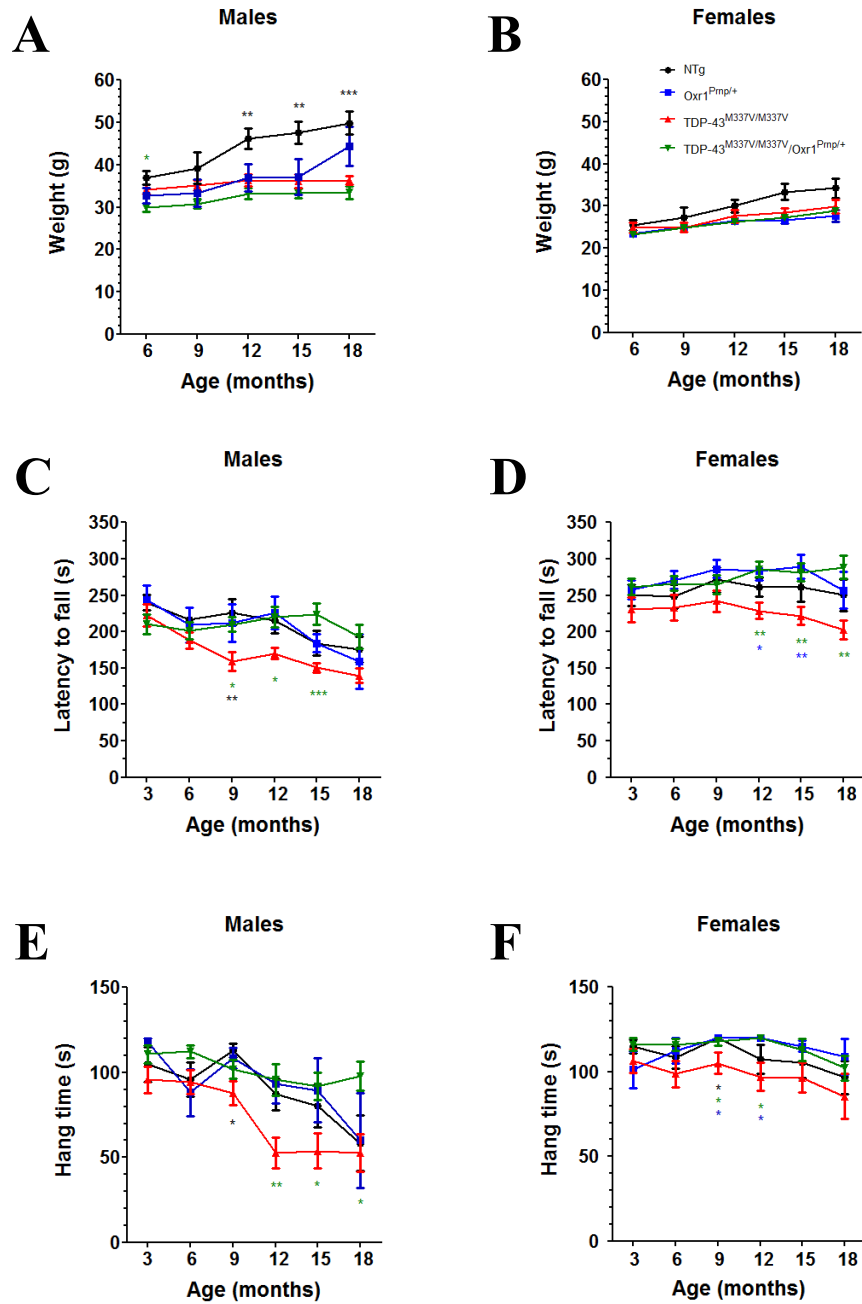


Figure 4.3 Overexpression of Oxr1 improves motor function and muscle strength in TDP-43^{M337V/M337V} mice. TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice show no significant difference in weight compared to TDP-43^{M337V/M337V} mice for both male (A) and female (B) cohorts. From 9-12 months onwards, rotarod analysis shows a significant improvement in motor coordination for both male (C) and female (D) TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice compared to male and female TDP-43^{M337V/M337V} mice, respectively. Grip strength reveals a significant improvement in muscle strength for both male (E) and female (F) TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice compared to male and female TDP-43^{M337V/M337V} mice, respectively. Values are mean $\pm$ SEM (see supplementary Table 1 for sample size /sex/genotype/time point; *p<0.05, **p<0.005, ***p<0.001; one-way ANOVA, with Dunnett's multiple comparison test comparing each group to TDP-43^{M337V/M337V} mice).

4.3.3 TDP-43^{M337V/M337V} mice display no signs of lower motor neuron degeneration or cytoplasmic aggregation of TDP-43.

Motor neuron degeneration is the hallmark feature of ALS (Kiernan *et al.* 2011). Furthermore, using mutant TDP-43^{M337V} rodent models, it has been demonstrated that overexpression of TDP-43^{M337V} can cause lower motor neuron degeneration (Zhou *et al.* 2010; Huang *et al.* 2012; Arnold *et al.* 2013). Therefore, using nissl-stained spinal cord sections from the lumbar enlargement of male experimental mice, I determined if expression of human TDP-43^{M337V} would result in lower motor neuron degeneration in TDP-43^{M337V/M337V} mice, and if so, whether overexpression of Oxr1 in neurons could rescue any motor neuron loss observed. I found no significant differences in the number of motor neurons between the different genotypes (Figure 4.4A & C), suggesting expression of TDP-43^{M337V} does not cause motor neuron degeneration in mice at 18 months of age.

Cytoplasmic mislocalisation and aggregation of TDP-43 occurs in >95% of ALS cases (Neumann *et al.* 2006; Turner *et al.* 2017), and a rat model expressing TDP-43^{M337V} has presented with TDP-43 aggregation in spinal motor neurons (Zhou *et al.* 2010). Therefore, using spinal cord sections stained for TDP-43, I determined if expression of human TDP-43^{M337V} would result in cytoplasmic TDP-43 aggregation in TDP-43^{M337V/M337V} mice, and if so, whether overexpression of Oxr1 in neurons could ameliorate any TDP-43 aggregation observed. Here, I found TDP-43^{M337V/M337V} mice show no significant increase in the number of motor neurons with TDP-43 aggregates compared to NTg littermates (Figure 4.4B & D). This suggests that expression of TDP-43^{M337V} is not sufficient to induce aberrant TDP-43 mislocalisation and aggregation in mice by 18 months of age.

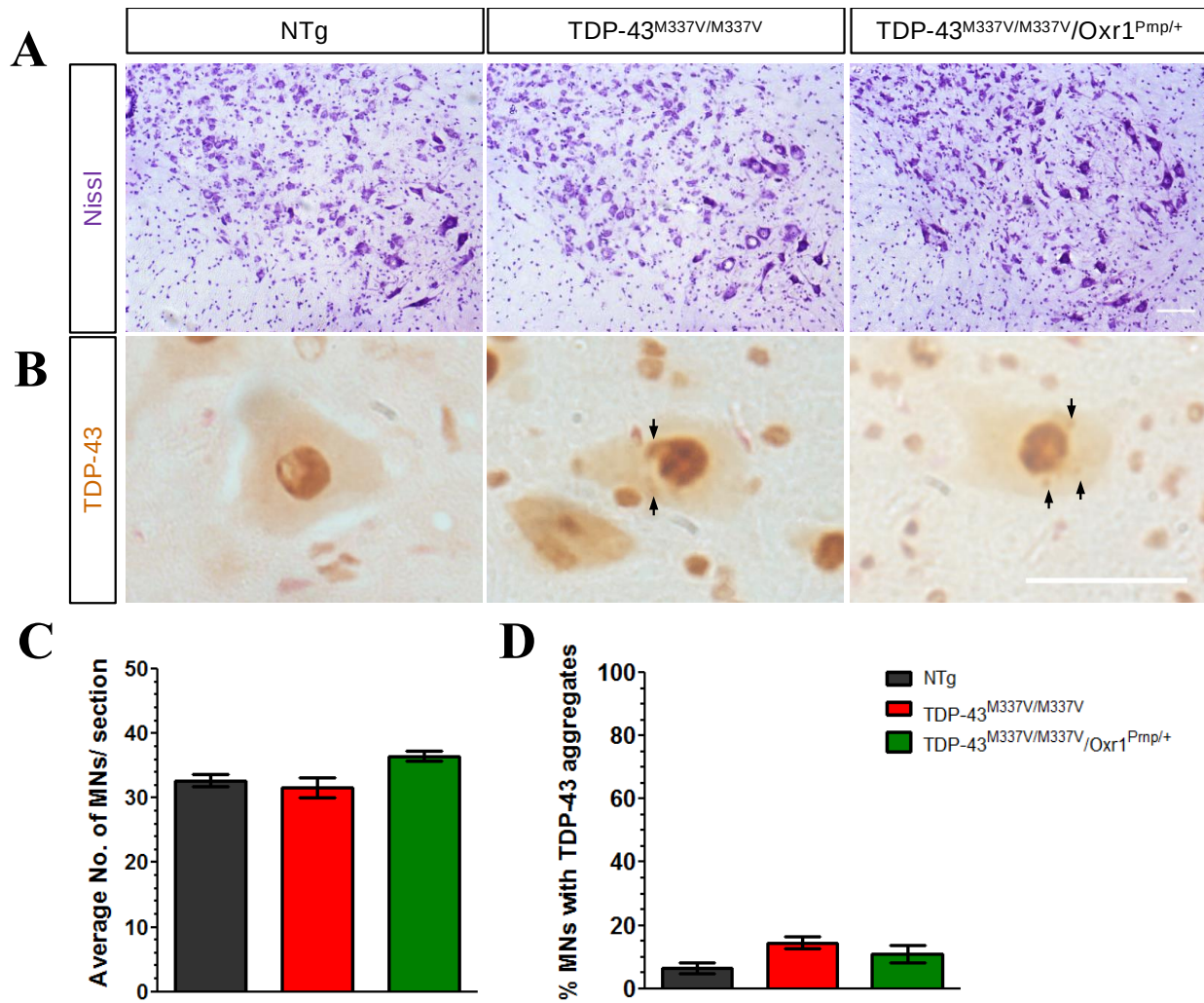


Figure 4.4 No motor neuron loss or cytoplasmic aggregation of TDP-43 in spinal cord of male TDP-43^{M337V/M337V} mice. (A) Representative images of nissl-stained spinal cord sections from NTg, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice. (B) Representative images of spinal motor neurons stained with anti-TDP-43 from NTg, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice. (C) Motor neuron counts from nissl-stained spinal cord sections reveal no motor neuron loss in TDP-43^{M337V/M337V} mice compared to NTg littermates. (D) Motor neuron counts containing TDP-43 aggregates reveal no significant increase in TDP-43 aggregation in motor neurons from TDP-43^{M337V/M337V} mice compared to NTg littermates. (C-D) Values are mean $\pm$ SEM (C) n=4; (D) n=3-4; Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test). (A) Scale bar: 100 μ m (B) Scale bar: 50 μ m.

4.3.4 Neuroinflammation is observed in lumbar spinal cords from TDP-43^{M337V/M337V} mice.

Neuroinflammation, characterised by astrogliosis and microgliosis, is a common feature of ALS and has been shown to play an active role in driving disease progression in patients and mouse models of ALS expressing TDP-43^{M337V} (Nagy, Kato and Kushner 1994; Turner *et al.* 2004; Xu *et al.* 2011; Arnold *et al.* 2013). Furthermore, neuroinflammation has been observed in the earliest stages of disease, prior to any signs of motor neuron degeneration (Alexianu, Kozovska and Appel 2001; Gerber *et al.* 2012). Therefore, to investigate whether astrogliosis and microgliosis could be observed in male TDP-43^{M337V/M337V} mice, lumbar spinal cord sections were stained for GFAP and Iba1. Despite the data being variable between biological replicates, I observed that the ventral horns of TDP-43^{M337V/M337V} mice display significantly increased staining intensity for GFAP (~56%; Figure 4.5A & C) and Iba1 (~31%; Figure 4.5B & D), compared to NTg littermates. This suggests that expression of TDP-43^{M337V} causes astrogliosis and microgliosis in male mice by 18 months of age.

It has been demonstrated previously, using quantitative immunohistochemical staining, that overexpression of Oxr1 in neurons of SOD1^{G93A} mice decreased astrogliosis and microgliosis in the spinal cord, suggesting that Oxr1 decreases pathological neuroinflammation (Liu *et al.* 2015). Therefore, in parallel, I determined whether neuronal overexpression of Oxr1 could ameliorate the neuroinflammation observed in TDP-43^{M337V/M337V} mice. While I observed a ~16% decrease in GFAP staining intensity (Figure 4.5C), and a ~25% decrease in Iba1 staining intensity (Figure 4.5D) in TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} compared to TDP-43^{M337V/M337V} littermates, this did not reach significance. Therefore, these data suggest that neuronal overexpression of Oxr1, does not reduce astrogliosis and microgliosis in the spinal cord of male TDP-43^{M337V/M337V} mice.

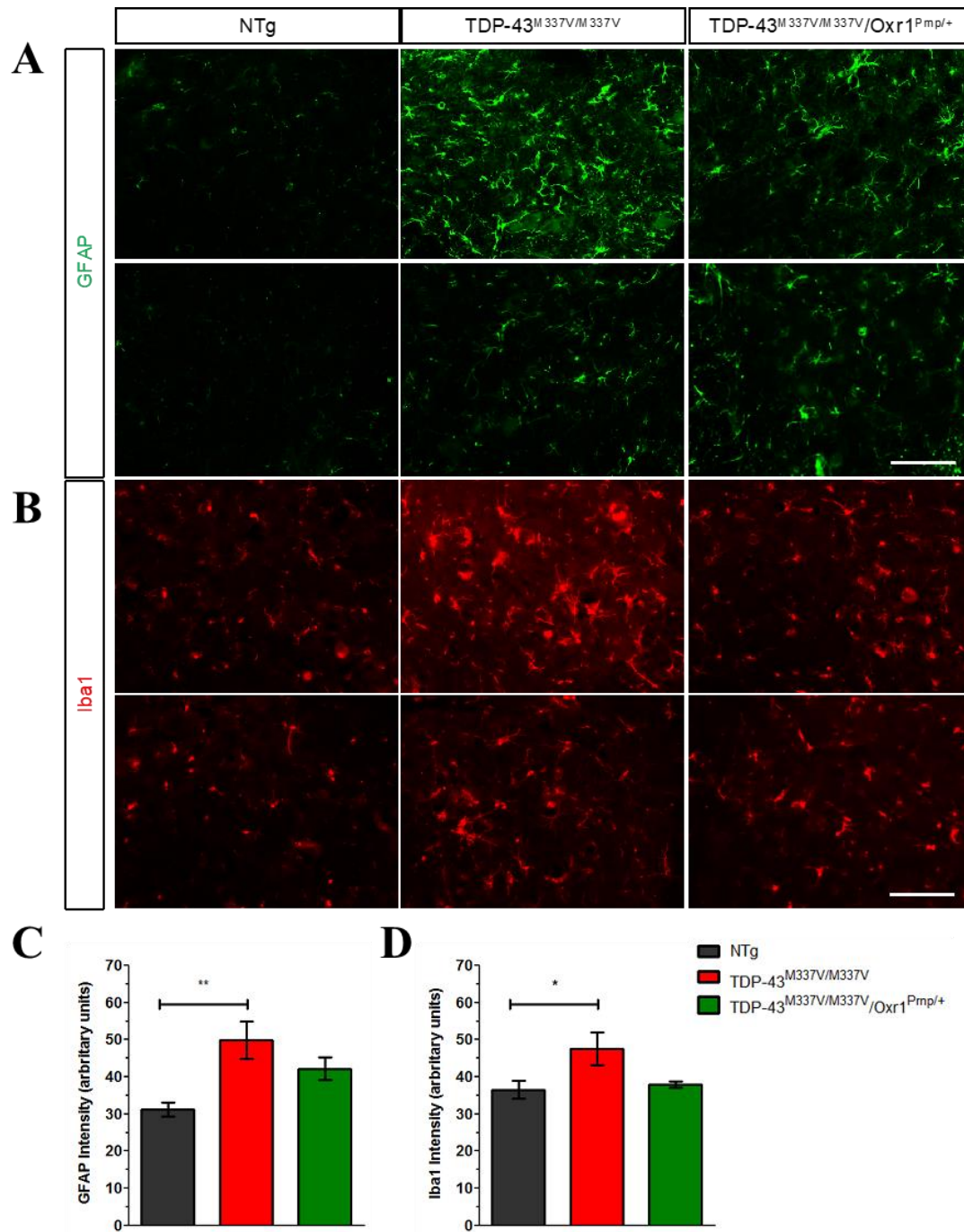


Figure 4.5 Neuroinflammation observed in lumbar spinal cords from male TDP-43^{M337V/M337V} mice at 18 months of age. (A) Representative images of spinal cord sections stained with anti-GFAP from NTg, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice. (B) Representative images of spinal cord sections stained with anti-Iba1 from NTg, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice. (C) GFAP signal intensity from spinal cord sections reveals increased astrogliosis in TDP-43^{M337V/M337V} mice. (D) Iba1 signal intensity from spinal cord sections reveals increased microgliosis in TDP-43^{M337V/M337V} mice. (C-D) Values are mean $\pm$ SEM (n=3-4; *p<0.05, **p<0.005; One-way ANOVA with Dunnett's multiple comparison test comparing each group to TDP-43^{M337V/M337V} mice). Scale bars: 100 μ m.

4.3.5 Overexpression of Oxr1 reduces muscle denervation and NMJ degeneration in TDP-43^{M337V/M337V} mice

Muscle denervation and degeneration of the NMJ occurs in the early stages of ALS, prior to motor neuron loss (Fischer *et al.* 2004; Moloney, de Winter and Verhaagen 2014; Cappello and Francolini 2017). In addition, it has been demonstrated previously that rodent models expressing mutant TDP-43^{M337V} presented with muscle denervation and abnormal NMJ morphology (Zhou *et al.* 2010; Huang *et al.* 2012). Therefore, I determined if expression of human TDP-43^{M337V} would result in NMJ pathology in TDP-43^{M337V/M337V} mice, and if so, whether overexpression of Oxr1 in neurons could rescue any NMJ pathology observed. To this end, hind-paw lumbrical muscles stained with antibodies for presynaptic nerve terminals (SV2 and 2H3) and post-synaptic AChRs located on the motor endplate (α -BTX), I analysed the number of NMJs that were fully innervated, partially innervated or fully denervated (vacant; Figure 4.6A) in experimental mice.

I observed that male TDP-43^{M337V/M337V} mice display a significant ~21% decrease in the number of fully innervated NMJs at 18 months of age compared to NTg littermates (Figure 4.6C). In addition, male TDP-43^{M337V/M337V} mice display a significant increase in the number of partially innervated NMJs (~14%; Figure 4.6D) and vacant NMJs (~10%; Figure 4.6E), compared to NTg littermates. This suggests that expression of TDP-43^{M337V} causes lumbrical muscle denervation in male mice by 18 months of age. Interestingly, I also observed a significant ~11% increase in the number of fully innervated NMJs in male TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice compared to TDP-43^{M337V/M337V} littermates (Figure 4.6C). In addition, I also found a significant decrease in the number in partially innervated and vacant NMJs compared to TDP-43^{M337V/M337V} littermates (7-8%; Figure 4.6D & E). This suggests that neuronal overexpression of Oxr1 improves lumbrical muscle denervation in TDP-43^{M337V/M337V} mice.

NMJ post-synaptic endplate area usually increases over time in to adulthood due to muscle fibre growth. However, during the progression of ALS, muscle fibres degenerate/regenerate, which causes alterations in the size of post-synaptic motor endplates (Cappello and Francolini 2017). In addition, lack of neuronal signal and subsequent AChR dispersal can lead to decreased staining intensity of the motor endplate, which can be used as a secondary indicator of muscle denervation (Sleigh *et al.* 2014). Therefore, I also investigated the area and staining intensity of post-synaptic endplates of lumbrical NMJs from experimental mice (Figure 4.6B). Interestingly, I observed that the area of post-synaptic endplates was significantly decreased in TDP-43^{M337V/M337V} mice compared to NTg littermates at 18 months of age (Figure 4.6F), suggesting that expression of hTDP-43^{M337V} results in motor endplate degeneration in male TDP-43^{M337V/M337V} mice. While I observed a decrease in motor endplate (α -BTX) staining intensity in TDP-43^{M337V/M337V} mice compared to NTg controls, this did not reach significance (Figure 4.6G). Interestingly, TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice displayed a significant increase in post-synaptic endplate area compared to TDP-43^{M337V/M337V} littermates (Figure 4.6F). Therefore, this suggests that neuronal overexpression of Oxr1 reduces motor endplate degeneration in male TDP-43^{M337V/M337V} mice.

To confirm these findings are similar between genders, I also carried out the same NMJ analysis on 18-month old female TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice. Similar to what was observed in male mice, I found that female TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice have a significant increase in fully innervated NMJs and significant decrease in vacant NMJs, compared to female TDP-43^{M337V/M337V} littermates (Figure 4.7A, C, D, E). In addition, the post-synaptic motor endplate area is significantly increased in female TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice compared to female TDP-43^{M337V/M337V} littermates (Figure 4.7B, F, G). Therefore, taken together, these data support

that overexpression of Oxr1 improves lumbrical muscle denervation and NMJ degeneration in TDP-43^{M337V/M337V} mice.

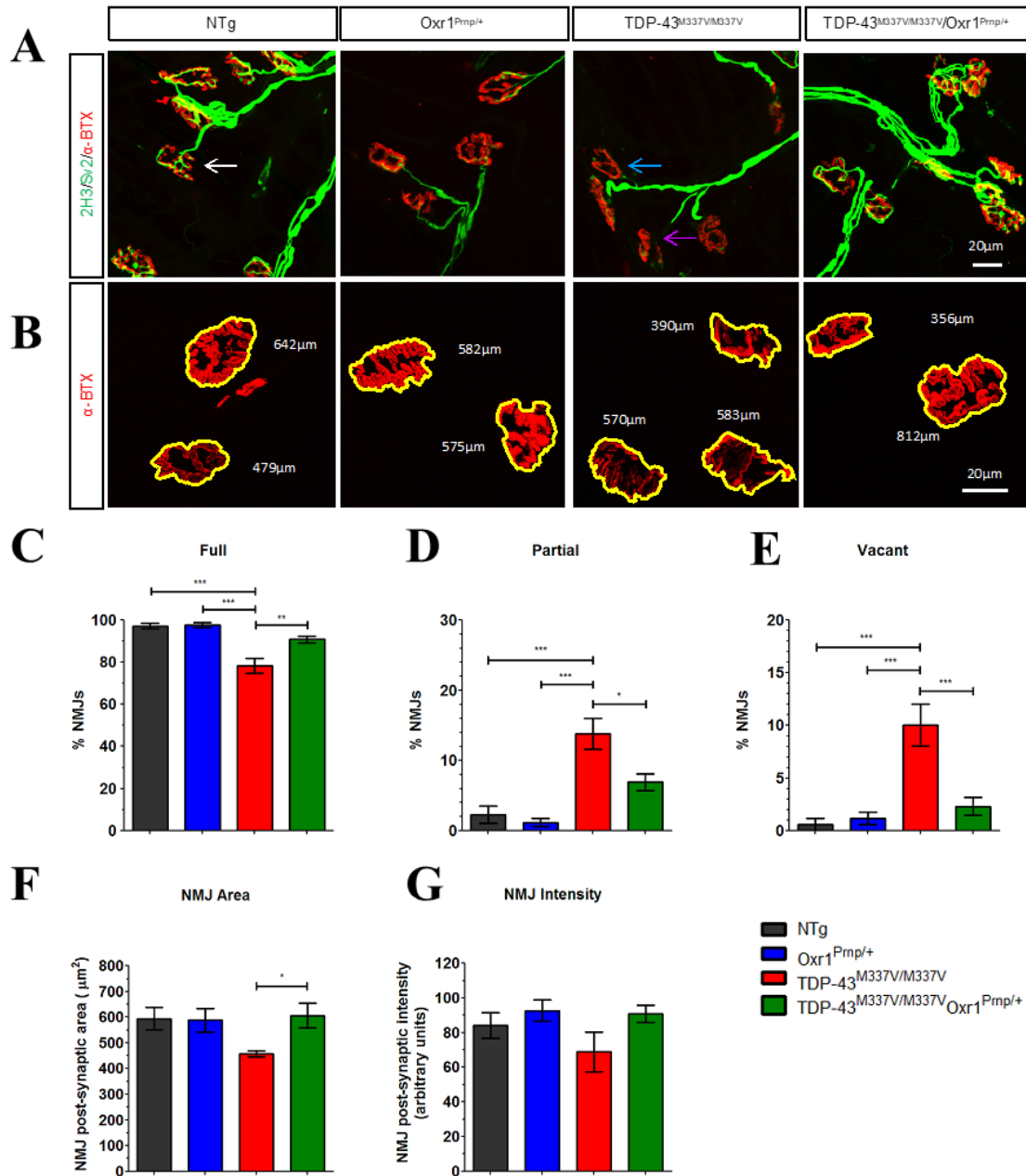


Figure 4.6 Overexpression of Oxr1 improves muscle denervation and NMJ degeneration. (A) Representative images showing lumbrical NMJs from NTg, Oxr1^{Pmp/+}, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice at 18 months of age. Levels of denervation were assessed by determining the overlap between pre-synaptic (2H3/SV2) and post-synaptic (α-BTX) staining. NMJs were scored as fully innervated (white arrow), partially innervated (blue arrow), or vacant (purple arrow). (B) Representative images of motor endplates used to measure post-synaptic area and average fluorescence intensity. (C-E) Denervation analysis reveals a significant decrease in fully innervated and a significant increase in partially innervated and vacant NMJs in TDP-43^{M337V/M337V} mice, which is rescued by overexpression of Oxr1. (F-G) Motor endplate area and fluorescence intensity is reduced in TDP-43^{M337V/M337V} mice, and is rescued by overexpression of Oxr1. Values are mean ± SEM (n=3-6; *p<0.05, **p<0.005, ***p<0.001; One-way ANOVA with Dunnett's multiple comparison test comparing each group to TDP-43^{M337V/M337V} mice). Scale bars: 20μm.

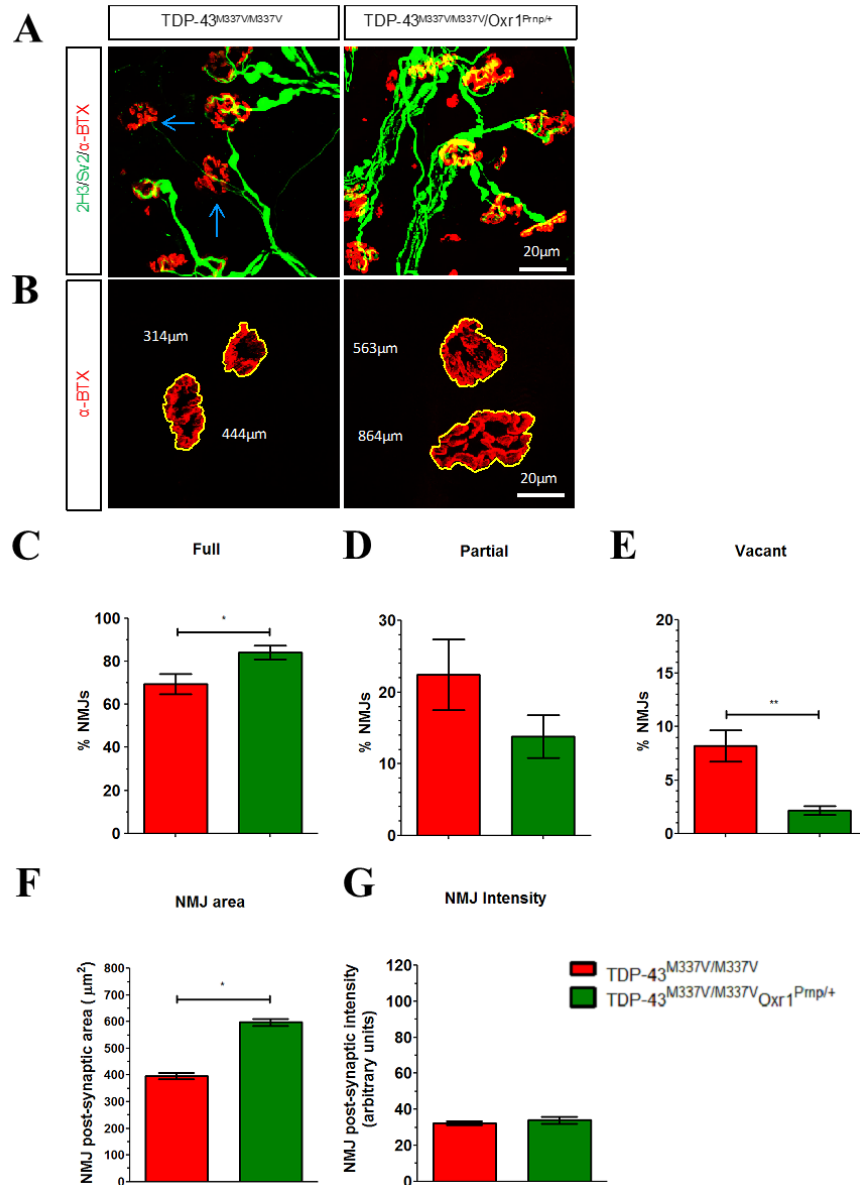


Figure 4.7 Overexpression of Oxr1 improves muscle denervation and NMJ degeneration. (A) Representative images showing lumbrical NMJs from NTg, Oxr1^{Pmp/+}, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V/Oxr1^{Pmp/+}} mice at 18 months of age. Levels of denervation were assessed by determining the overlap between pre-synaptic (2H3/SV2) and post-synaptic (α-BTX) staining. NMJs were scored as fully innervated (white arrow), partially innervated (blue arrow), or vacant (purple arrow). (B) Representative images of motor endplates used to measure post-synaptic area and average fluorescence intensity. (C-E) Denervation analysis reveals a significant decrease in fully innervated and a significant increase in partially innervated and vacant NMJs in TDP-43^{M337V/M337V} mice, which is rescued by overexpression of Oxr1. (F-G) Motor endplate area and fluorescence intensity is reduced in TDP-43^{M337V/M337V} mice, and is rescued by overexpression of Oxr1. Values are mean ± SEM (n=3-6; *p<0.05, **p<0.005, ***p<0.001; One-way ANOVA with Dunnett's multiple comparison test comparing each group to TDP-43^{M337V/M337V} mice). Scale bars: 20μm.

4.3.6 Overexpression of Oxr1 improves muscle pathology observed in TDP-43^{M337V/M337V} mice.

Muscle atrophy is another hallmark feature of ALS that occurs concomitantly with muscle denervation and NMJ degeneration loss (Fischer *et al.* 2004; Kiernan *et al.* 2011; Moloney, de Winter and Verhaagen 2014). Furthermore, it has been demonstrated that expression of mutant TDP-43 can cause muscle atrophy and pathology in rodents (Zhou *et al.* 2010; Huang *et al.* 2012). Therefore, in order to determine if expression of hTDP-43^{M337V} would result in muscle pathology in TDP-43^{M337V/M337V} mice, I stained hind limb muscle sections from male experimental mice with H&E in order to visualise individual muscle fibres. I also tested whether neuronal overexpression of Oxr1 could rescue any muscle pathology observed. In ALS, muscles that are largely composed of fast-twitch muscle fibres appear to be more vulnerable to degeneration; whereas muscles with a high proportion of slow-twitch fibres are generally affected at later stages of the disease and usually to a lesser extent (Frey *et al.* 2000; Fischer *et al.* 2004; Hegedus, Putman and Gordon 2007). Given that tibialis anterior muscles from C57BL6J mice contain a high proportion of fast-twitch muscle fibres, compared to gastrocnemius and soleus muscles (Augusto, Padovani and Campos 2004), I chose to analyse this muscle group. I observed no obvious signs of muscle atrophy in tibialis anterior muscle in TDP-43^{M337V/M337V} mice compared to NTg littermates. However, I found that there was a significant increase in the number of centrally nucleated fibres in TDP-43^{M337V/M337V} mice compared to NTg littermates (Figure 4.8A & B). Given that centrally nucleated fibres are indicative of muscle fibre degeneration and subsequent regeneration, these data suggest that expression of human TDP-43^{M337V} causes muscle pathology in male mice at 18 months of age. Furthermore, the number of centrally nucleated fibres was significantly decreased in TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} mice, compared to TDP-

43^{M337V/M337V} mice, suggesting that overexpression of Oxr1 improves muscle pathology in TDP-43^{M337V/M337V} mice.

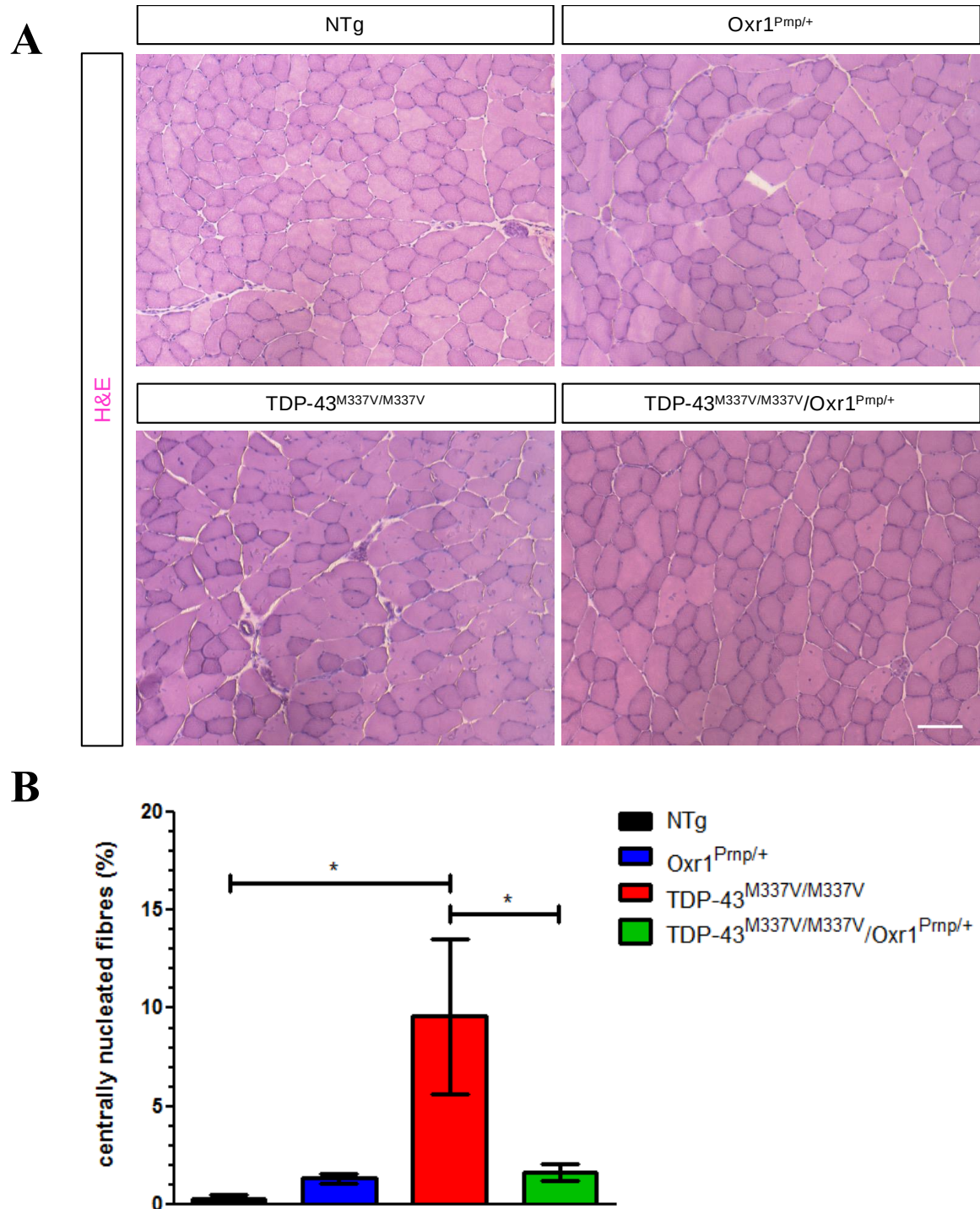


Figure 4.8 Overexpression of Oxr1 improves muscle pathology in TDP-43^{M337V/M337V} mice at 18 months of age. (A) Representative images of H&E-stained tibialis anterior muscle fibre cross sections from NTg, Oxr1^{Pmp/+}, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice at 18 months of age. (B) The percentage of centrally nucleated fibres per section is significantly increased in TDP-43^{M337V/M337V} mice, which is rescued by overexpression of Oxr1. Values are mean $\pm$ SEM (n=3-5, *p<0.05, One-way ANOVA with Dunnett's multiple comparison test comparing each group to TDP-43^{M337V/M337V} mice). Scale bar: 100 μ m.

4.3.7 Protein oxidation is not a prominent feature in TDP-43^{M337V/M337V} mice by 18 months of age.

ROS can react with side chains of lysine, arginine, proline, and threonine of protein, leading to protein oxidation, which can be measured through indirect detection of carbonyls (Dalle-Donne *et al.* 2003). Elevated protein carbonyl levels have been detected in the motor cortex and spinal cord of ALS patients, implicating protein oxidation as a pathological feature of the disease (Shaw *et al.* 1995; Ferrante *et al.* 1997a). Expression of mutant TDP-43^{M337V} has been shown to be associated with increased markers for OS, inhibition of antioxidant response pathways and increased susceptibility to OS-induced damage and cell death (Duan *et al.* 2010; Moujalled *et al.* 2017; Tian *et al.* 2017). Therefore, I investigated whether expression of hTDP-43^{M337V} would result in increased protein oxidation in TDP-43^{M337V/M337V} mice, and also tested whether neuronal overexpression of Oxr1 could reduce any aberrant protein oxidation observed. To assess the overall level of oxidative modification of proteins in the spinal cord of 18-month old mice, I carried out western blot analyses using the OxyBlot Protein Oxidation Detection Kit. I observed no obvious difference in the level of protein carbonyls between genotypes (Figure 4.9A & B), suggesting that protein oxidation is not a prominent feature in TDP-43^{M337V/M337V} mice.

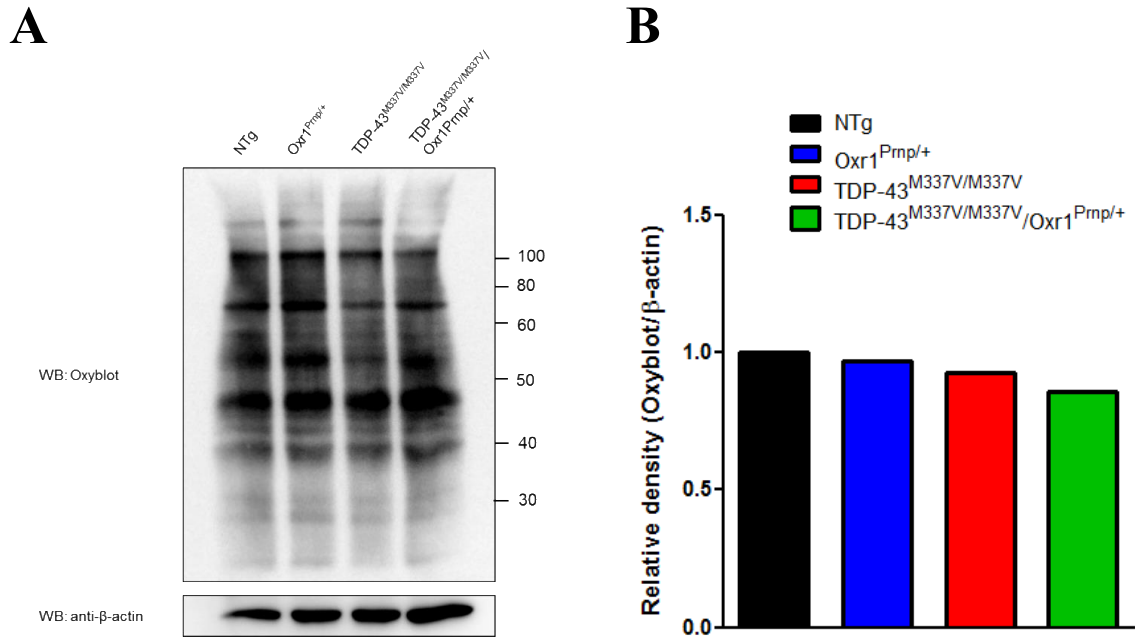


Figure 4.9 Widespread protein oxidation is likely not a prominent feature in TDP-43^{M337V/M337V} mice. (A) OxyBlot analysis was to detect the level of carbonyl groups introduced into protein side chains as a result of protein oxidation. Spinal cord lysates from 18-month old NTg, Oxr1^{Pmp/+}, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice were analysed. (B) Levels of oxidized protein was quantified by normalization to the levels of β-actin, and reveals no obvious genotypic differences (n=1).

4.3.8 The transcriptome is unaltered in the spinal cord of TDP-43^{M337V/M337V} mice.

It is well established that TDP-43 plays a vital role in RNA metabolism and regulates the expression of approximately 600 mRNAs in the CNS (Lagier-Tourenne, Polymenidou and Cleveland 2010; Polymenidou *et al.* 2011). Furthermore, using transcriptomics, it has been demonstrated that mutations in TDP-43 caused profound gene expression changes in the mouse cortex (Igaz *et al.* 2011). Therefore, to investigate whether expression of hTDP-43^{M337V} would lead to similar global gene expression changes in the spinal cord of TDP-43^{M337V/M337V} mice, and whether neuronal overexpression of Oxr1 could restore and transcriptional changes. To this end, RNA was purified from the lumbar spinal cord of littermate mice of all four genotypic combinations. Microarray transcriptome analysis was then undertaken externally (Cambridge Genomic Services). Even though I observed little to no pathology in the spinal cord of symptomatic 18-month-old TDP-43^{M337V/M337V} mice, I decided to use this time point for expression analysis in order to detect potential changes in ‘pre-pathogenic’ pathways, such as inflammation. Comparing the TDP-43^{M337V/M337V} expression profile to that of the NTg revealed that out of 20,572 transcripts, none were significantly differentially expressed (Figure 4.10A). This suggests that expression of TDP-43^{M337V} does not alter the transcriptome in the spinal cord of mice at 18 months of age. I also observed a non-significant 1.25-fold increase in TDP-43 expression in TDP-43^{M337V/M337V} mice compared to NTg, supporting that hTDP-43^{M337V} is expressed at relatively low levels in this model. In addition, I observed that Oxr1 was the only gene significantly differentially expressed between TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} and TDP-43^{M337V/M337V} mice (Figure 4.10B), suggesting overexpression of Oxr1 does not alter any transcriptional pathways in TDP-43^{M337V/M337V} mice.

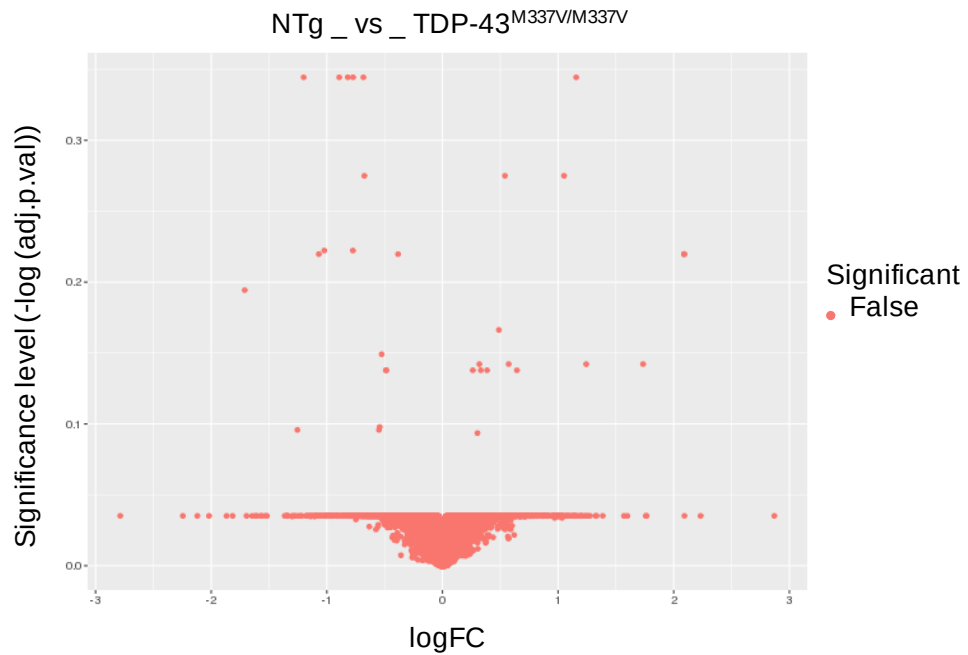
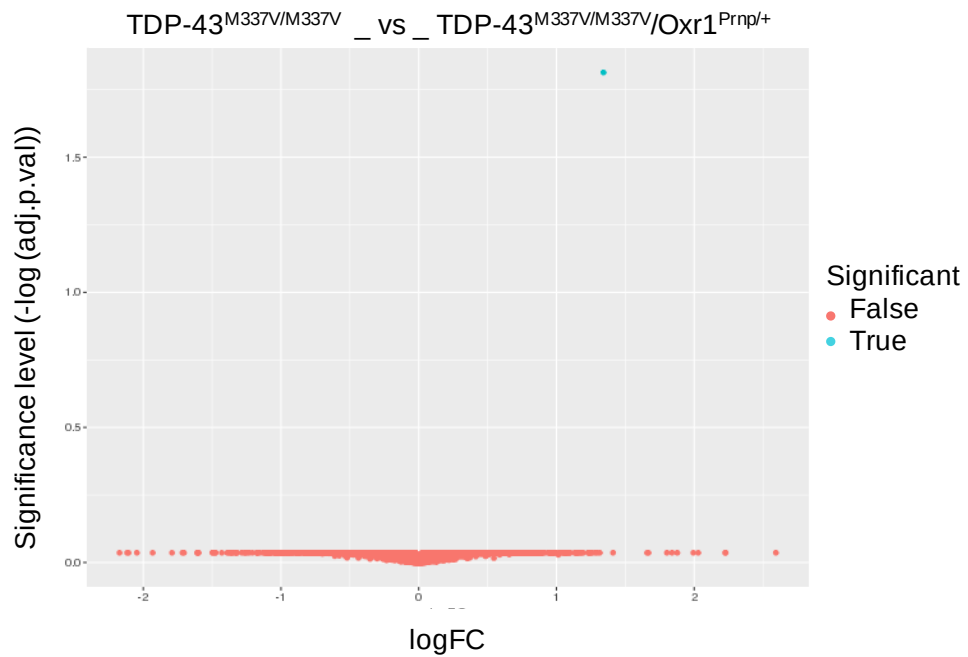
A**B**

Figure 4.10 The transcriptome is unaltered in the spinal cord of TDP-43^{M337V/M337V} mice at 18 months of age. Volcano plot of microarray data showing gene expression changes between; **(A)** NTg and TDP-43^{M337V/M337V} mice, **(B)** TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} mice. Dots represent genes whose expression was unaltered (red; false) and differentially expressed (green; true). Oxr1 is the only gene differentially expressed between TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} mice. X-axis represents the fold change (log2 scale), y-axis represents the level of significance (-log₁₀(adj.p.value)).

4.3.9 Overexpression of Oxr1 reduces aberrant TDP-43 mislocalisation in primary motor neurons from heterozygous TDP-43^{M337V/+} mice.

Given that 18-month-old TDP-43^{M337V/M337V} mice did not display cytoplasmic mislocalisation and aggregation of TDP-43 in spinal motor neurons, they were uninformative as an *in vivo* system to the effect of Oxr1 on the cellular localisation of TDP-43. Importantly, previous work in the Talbot group demonstrated that primary motor neurons which heterozygously express human TDP-43^{M337V} (TDP-43^{M337V/+}) present with aberrant TDP-43 mislocalisation, even in the absence of external stressors such as sodium arsenite (Gordon *et al.*, 2019). Therefore, as an alternative approach, I cultured spinal motor neurons under unstressed conditions from E13.5 mice. For this experiment, I used a different breeding strategy in order to generate TDP-43^{M337V/+} and TDP-43^{M337V/+}/Oxr1^{Prnp/+} embryos. To investigate the cellular localisation of TDP-43 in primary motor neurons, I compared TDP-43 fluorescence intensity between the nucleus and cytoplasm and only analysed cells positive for SMI-32, a commonly used marker for motor neurons (Carriedo, Yin and Weiss 1996).

First, using an antibody recognizing the human TDP-43^{M337V} and endogenous mouse TDP-43 protein (Figure 4.11A), I found that TDP-43^{M337V/+}/Oxr1^{Prnp/+} motor neurons display a significant ~14% decrease in cytoplasmic total TDP-43, compared to TDP-43^{M337V/+} motor neurons (Figure 4.11C). Furthermore, total TDP-43 was found predominantly in the cytoplasm in ~33% of TDP-43^{M337V/+} motor neurons, compared to only ~2% of TDP-43^{M337V/+}/Oxr1^{Prnp/+} motor neurons (Figure 4.11D). This suggests that overexpression of Oxr1 reduces the aberrant mislocalisation of total TDP-43 in TDP-43^{M337V/+} primary motor neurons. I next investigated the localisation of the human-specific pool of TDP-43^{M337V} in primary motor neurons using a GFP antibody directed against the C-terminal Ypet tag (Figure 4.11B). I observed a significant ~17% decrease in cytoplasmic GFP in TDP-

43^{M337V/+}/Oxr1^{Prnp/+} motor neurons compared to TDP-43^{M337V/+} motor neurons (Figure 4.11E). In addition, GFP was found predominantly in the cytoplasm in ~51% of TDP-43^{M337V/+} motor neurons, compared to only ~3% of TDP-43^{M337V/+}/Oxr1^{Prnp/+} motor neurons (Figure 4.11F). Together, this suggests that overexpression of Oxr1 reduces the aberrant mislocalisation of human TDP-43^{M337V} in TDP-43^{M337V/+} motor neurons. No nuclear clearing of either TDP-43^{M337V} or total TDP-43 was observed in primary motor neurons under unstressed conditions.

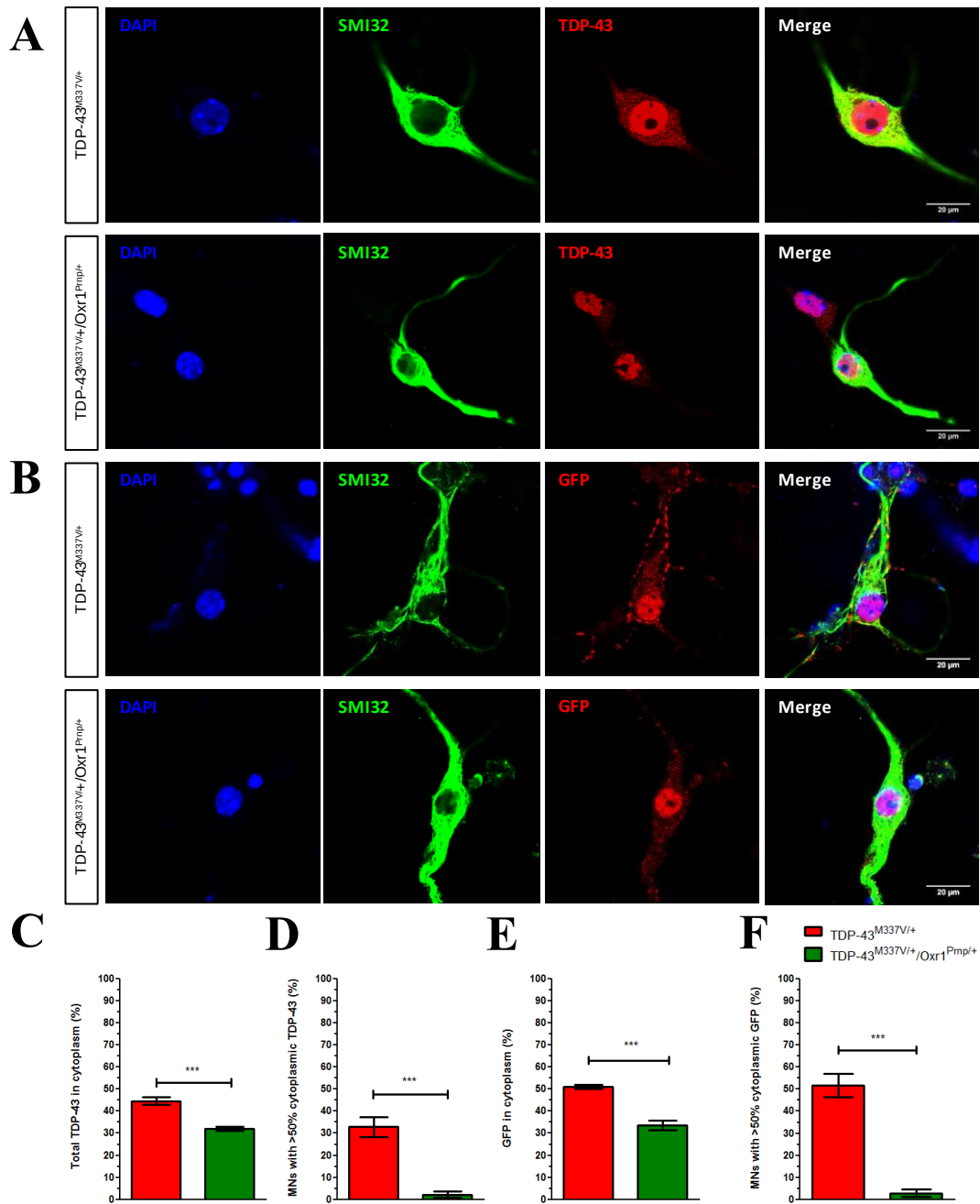


Figure 4.11 Overexpression of Oxr1 reduces mislocalisation of TDP-43 in TDP-43^{M337V/+} motor neurons under resting conditions. (A-B) Representative images of SMI32-positive primary motor neurons from TDP-43^{M337V/+} and TDP-43^{M337V/+}/Oxr1^{Pmp/+} mice stained with (A) anti-TDP-43 and (B) anti-GFP to mark the mutant YPET-tagged TDP-43. (C) Motor neurons from TDP-43^{M337V/+}/Oxr1^{Pmp/+} mice have a significant decrease in cytoplasmic TDP-43 compared to TDP-43^{M337V/+} mice. (D) TDP-43^{M337V/+}/Oxr1^{Pmp/+} mice have a significant decrease in the number of motor neurons with >50% cytoplasmic TDP-43 compared to TDP-43^{M337V/+} mice. (E) Motor neurons from TDP-43^{M337V/+}/Oxr1^{Pmp/+} mice have a significant decrease in cytoplasmic mutant YPET-tagged TDP-43 compared to TDP-43^{M337V/+} mice. (F) TDP-43^{M337V/+}/Oxr1^{Pmp/+} mice have a significant decrease in the number of motor neurons with >50% cytoplasmic mutant YPET-tagged TDP-43 compared to TDP-43^{M337V/+} mice. Values are mean $\pm$ SEM (45-60 motor neurons were analysed per embryo, 6 embryos were analysed per genotype; ***p<0.001, Unpaired student's t-test). Scale bars: 20 μ m.

4.3.10 Overexpression of Oxr1 does not affect formation or number of stress granules in TDP-43^{M337V/+} motor neurons

Formed under conditions of cellular stress, such as OS, stress granules are dynamic cytoplasmic foci that store and repress the translation of housekeeping mRNAs, allowing the prioritized translation of stress response mRNAs important for cell survival (Anderson and Kedersha 2002; Kedersha and Anderson 2007). Stress granules are generated by the reversible aggregation of RNA-binding proteins with low complexity prion-like domains, such as TDP-43 (Gilks *et al.* 2004; Colombrita *et al.* 2009; Dewey *et al.* 2011; McDonald *et al.* 2011). It has been suggested that mutant TDP-43 may alter stress granule dynamics on several levels, such as stress granule formation, size and disassembly (Liu-Yesucevitz *et al.* 2010; Dewey *et al.* 2011; McDonald *et al.* 2011; Colombrita *et al.* 2012; Aulas and Vande Velde 2015). Previous work by the Talbot group has demonstrated that the formation and area of stress granules is significantly reduced in TDP-43^{M337V/+} primary motor neurons compared to TDP-43^{WT/+} and NTg controls, following exposure to sodium arsenite (Gordon *et al.*, 2019). In addition, using motor neurons differentiated from mouse embryonic stem cells (ESCs), it was also demonstrated that the number of stress granules was significantly reduced in TDP-43^{M337V/+} motor neurons compared to TDP-43^{WT/+} controls, following exposure to sodium arsenite (Gordon *et al.*, 2019). Taken together, these data suggest that expression of TDP-43^{M337V} alters stress granule dynamics in motor neurons *in vitro*.

Therefore, I wanted to investigate whether overexpression of Oxr1 would rescue the stress granule defect observed in TDP-43^{M337V/+} motor neurons. Using a well-established stress granule marker, G3BP1, I analysed the formation and number of stress granules in primary motor neurons from our mouse cross (Figure 4.12A). Following treatment with sodium arsenite, I observed no difference in the number of motor neurons that successfully formed stress granules between TDP-43^{M337V/+} and TDP-43^{M337V/+}/Oxr1^{Pmp/+} -derived

cultures (Figure 4.12B). In addition, there was no significant difference in the number of stress granules per motor neuron between TDP-43^{M337V/+} and TDP-43^{M337V/+}/Oxr1^{Pmp/+}-derived cultures (Figure 4.12C). Taken together this suggests that overexpression of Oxr1 does not affect the formation or number of stress granules in TDP-43^{M337V/+} motor neurons. Furthermore, given that 100% of TDP-43^{M337V/+} motor neurons successfully formed stress granules after treatment with sodium arsenite, this would suggest that expression of mutant TDP-43^{M337V} does not affect the ability of motor neurons to form stress granules. However, it should be noted that in this study, and the majority of others investigating stress granule dynamics (Guil, Long and Caceres 2006; Colombrita *et al.* 2009; Liu-Yesucevitz *et al.* 2010; Molliex *et al.* 2015), cells were treated with a higher concentration of sodium arsenite (0.5mM) compared to the study by Gordon *et al.* (0.05mM; Gordon *et al.*, 2019).

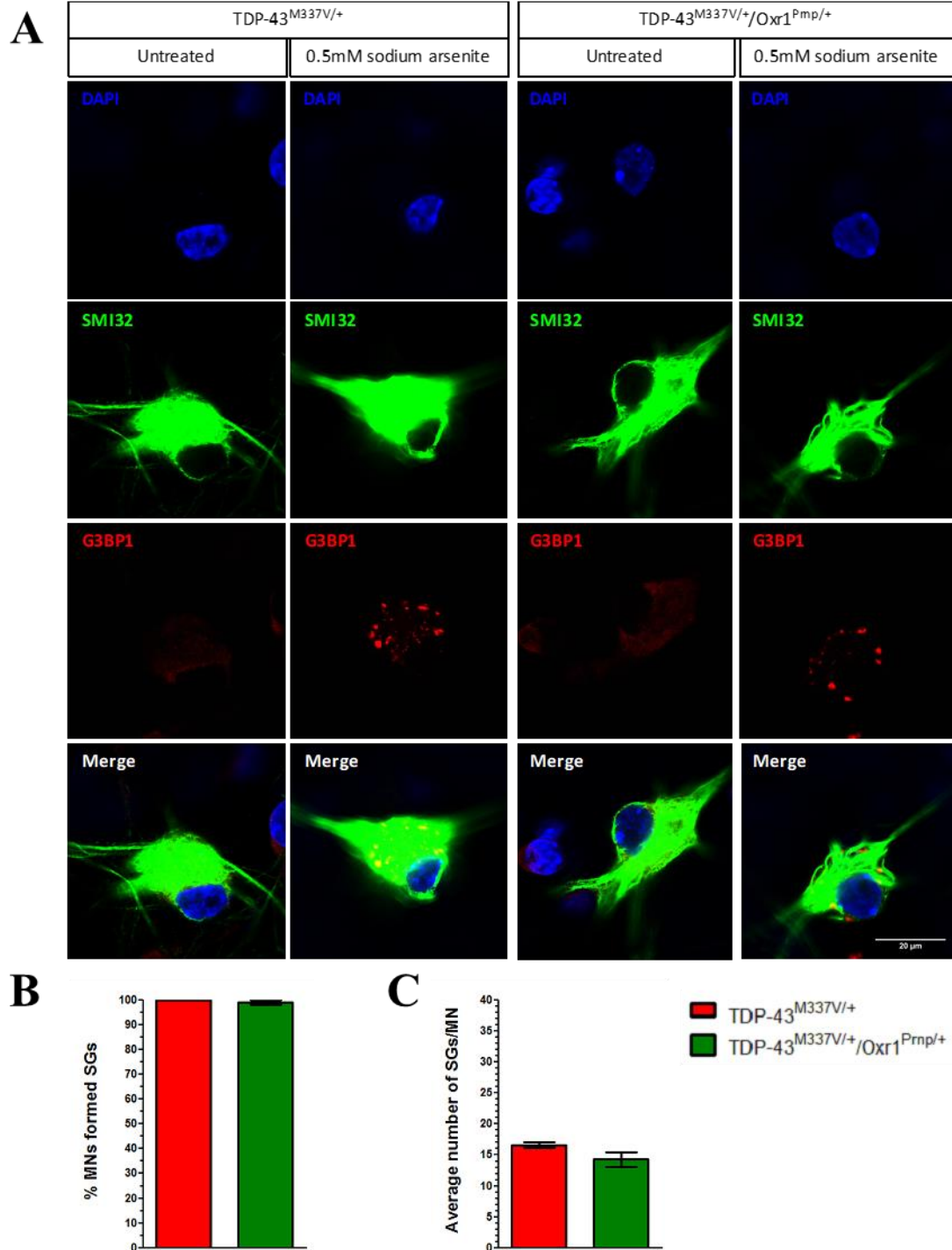


Figure 4.12 Overexpression of Oxr1 does not affect the formation or number of stress granules in TDP-43^{M337V/+} motor neurons. (A) Representative images of G3BP1-positive stress granules in SMI32-positive primary motor neurons in response to sodium arsenite treatment to induce oxidative stress. (B) No significant difference was observed in the number of motor neurons that formed stress granules between TDP-43^{M337V/+} and TDP-43^{M337V/+}/Oxr1^{Prnp/+} mice. (C) No significant difference was observed in the number of stress granules per motor neuron between TDP-43^{M337V/+} and TDP-43^{M337V/+}/Oxr1^{Prnp/+} mice. Values are mean $\pm$ SEM (n=4; Unpaired student's t-test). Scale bar: 20 μ m. SGs: stress granules.

4.3.11 Oxr1 binds to TDP-43 *in vivo* and may be associated with improved TDP-43^{M337V} solubility.

A previous proteomic study in a neuronal cell line demonstrated that Oxr1-C binds to wild-type Tdp-43 and Tdp-43^{M337V} (Finelli *et al.* 2015). Furthermore, overexpression of Oxr1-C in cells expressing TDP-43^{M337V} improves mislocalisation and aggregation of Tdp-43 under OS conditions (Finelli *et al.* 2015). Importantly, this decrease in cytoplasmic aggregation was only observed for mutant proteins with a preserved binding for Oxr1, such as Tdp-43^{M337V} (Finelli *et al.* 2015). Therefore, this suggests that the interaction between Oxr1 and Tdp-43 is important for proper cellular localization of Tdp-43. Here, I demonstrate that overexpression of Oxr1-FL improves motor dysfunction and neuromuscular phenotypes in TDP-43^{M337V/M337V} mice, and reduces TDP-43 mislocalisation in primary motor neurons expressing hTDP-43^{M337V}. Therefore, in order to investigate how overexpression of Oxr1-FL may be improving these ALS-associated phenotypes, it was important to test whether Oxr1-FL binds to TDP-43 in the disease context *in vivo*.

To this end, I performed co-immunoprecipitation from the spinal cords of 18-month-old TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice. When I pulled down TDP-43 with a specific antibody for both human and mouse TDP-43, I co-immunoprecipitated both Oxr1-FL and Oxr1-C (Figure 4.13A), demonstrating that TDP-43 binds to both Oxr1 isoforms *in vivo*. In order to confirm the interaction between TDP-43 and transgenic HA-tagged Oxr1, I also immunoblotted with anti-HA. While I found that HA-tagged Oxr1-FL co-immunoprecipitated with the IgG control, it co-immunoprecipitated with TDP-43 with a higher efficiency (Figure 4.13A). Firstly, these data support previous findings demonstrating that Oxr1-C binds to TDP-43 (Finelli *et al.* 2015). More importantly, these data also suggest that Oxr1-FL, which is overexpressed in TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice, also binds to TDP-43.

A defining biochemical feature of ALS is the accumulation of insoluble TDP-43 aggregates (Neumann *et al.* 2006). Previous reports have demonstrated that TDP-43 mutations, such as M337V, results in increased accumulation of insoluble TDP-43 protein (Johnson *et al.* 2009; Bilican *et al.* 2012; Janssens *et al.* 2013; Wobst *et al.* 2017). Therefore, I first wanted to investigate whether expression of hTDP-43^{M337V} would result in accumulation of insoluble protein in the spinal cord of TDP-43^{M337V/M337V} mice at 18 months of age, and then also determine whether overexpression of Oxr1 could improve TDP-43 solubility. To this end, I used western blot analysis on fractionated soluble and insoluble (urea soluble) spinal cord lysates from NTg, Oxr1^{Pmp/+}, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice. Using an antibody that detects both endogenous mouse TDP-43 (~43kDa) and transgenic human TDP-43^{M337V} protein (~70kDa; Figure 4.13B), I did not detect any significant genotypic differences in the level of endogenous Tdp-43 (Figure 4.13C) and total TDP-43 (Figure 4.13E) for both soluble and insoluble fractions, indicating that expression of mutant TDP-43^{M337V} does not result in increased accumulation of insoluble TDP-43 protein. I also observed a non-significant ~50% decrease in insoluble hTDP-43^{M337V} in the spinal cord of TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice compared to TDP-43^{M337V/M337V} mice.

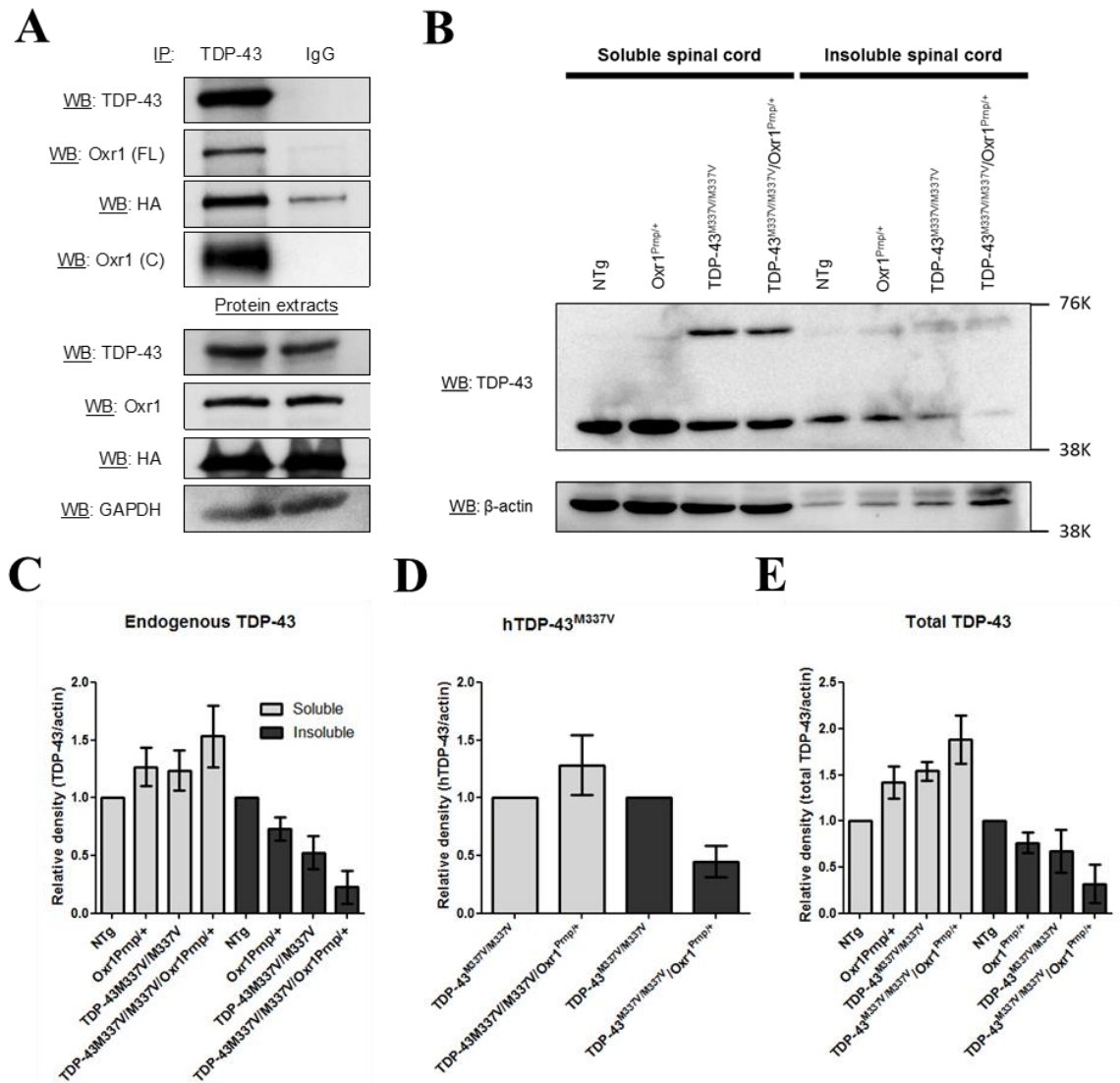


Figure 4.13 Oxr1 binds to TDP-43 *in vivo* and may be associated with improved TDP-43^{M337V} solubility. (A) Co-immunoprecipitation in spinal cord tissue from TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice reveals that TDP-43 binds to Oxr1-FL and Oxr1-C. The left lane represents proteins immunoprecipitated with anti-TDP-43 beads. The second lane represents proteins immunoprecipitated with non-specific IgG. The first band confirms successful pull-down of TDP-43. The second and third bands demonstrate that TDP-43 binds to Oxr1-FL. The fourth band reveals that TDP-43 binds to Oxr1-C. While bands five-eight represent direct protein extracts to demonstrate equal amount of proteins used (n=1). (B) Soluble and insoluble protein fractions were isolated from the spinal cords of NTg, Oxr1^{Pmp/+}, TDP-43^{M337V/M337V} and TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice at 18 months of age and analysed by immunoblotting with anti-TDP-43 and anti- β -actin. Soluble and insoluble levels of endogenous TDP-43 (C), hTDP-43^{M337V} (D) and total TDP-43 (E) was quantified by normalization to the levels of β -actin and reveals a trend towards improved TDP-43 solubility in TDP-43^{M337V/M337V}/Oxr1^{Pmp/+} mice compared to TDP-43^{M337V/M337V} littermates, however this does not reach statistical significance (n=3).

4.4 Discussion

In this chapter, I characterized a novel mouse model of ALS based on an ALS-associated human TDP-43 mutation (M337V) driven by the human promoter. Longitudinal behavioural analysis revealed that TDP-43^{M337V/M337V} mice develop motor dysfunction by 9 months of age. In addition, pathological analysis of key components of the neuromuscular system showed that TDP-43^{M337V/M337V} mice present with a neuroinflammatory response in the spinal cord, denervation at the NMJ and hind-limb muscle pathology at 18 months of age, which augments previous findings from the Talbot group that pathologically characterised TDP-43^{M337V/M337V} mice at an earlier time-point (12 months of age; Gordon *et al.*, 2019). Interestingly, I demonstrate for the first time that neuronal overexpression of Oxr1 delays adult-onset motor dysfunction, reduces denervation at the NMJ and improves muscle pathology in this novel TDP-43^{M337V/M337V} BAC mouse model of ALS. In addition, I also show that overexpression of Oxr1 in primary motor neurons expressing hTDP-43^{M337V} improves the aberrant cytoplasmic mislocalisation of TDP-43 protein, a pathological feature observed in tissue from more than 95% of all ALS cases (Neumann *et al.* 2006). Taken together, these results not only establish Oxr1 as a novel genetic modifier of TDP-43-related pathology *in vivo*, but also strengthens the argument for its use as a potential therapeutic target for the treatment of ALS.

Since TDP-43 was identified as a major component of protein aggregates found in sALS and non-SOD1 fALS cases (Neumann *et al.* 2006), a major research focus has been to generate new animal models expressing mutant forms of TDP-43 that better model the human disease. So far, none of the mutant TDP-43 driven animals have seemed to recapitulate all pathological features of ALS, as observed in humans. In addition, for the majority of models generated, even rodents expressing human wild-type TDP-43 developed ALS-like phenotypes comparable in severity to those with mutant TDP-43 (Shan *et al.* 2010;

Stallings *et al.* 2010; Wils *et al.* 2010; Xu *et al.* 2010, 2011; Swarup *et al.* 2011b). Therefore, it has been difficult to distinguish the effects of transgene overexpression from effects caused by TDP-43 mutation. To date, only three studies using rodent models have demonstrated clearly that overexpression of an ALS-linked mutant TDP-43, but not the human wild type TDP-43, is toxic *in vivo* (Zhou *et al.* 2010; Arnold *et al.* 2013; Gordon *et al.* ,2019. However, it should be noted that high levels of mutant TDP-43 overexpression in the rat model results in a very toxic and aggressive phenotype, questioning its usefulness as a model to understand ALS (Zhou *et al.* 2010).

Given that the new TDP-43^{M337V/M337V} BAC mouse model of ALS expresses hTDP-43^{M337V} at low-levels (≤ 1 -fold increase from endogenous mouse Tdp-43) and develops a phenotype unique from mice expressing wild-type hTDP-43 (Gordon *et al.*, 2019), we consider it to be a more disease-relevant mouse model than those based on overexpression to non-physiological levels. However, as evident from my findings and others previously (Arnold *et al.* 2013; Gordon *et al.* 2019), the major caveat of TDP-43 transgenic rodent models with low to moderate levels of expression is that certain ALS-associated phenotypes such as motor neuron degeneration may not develop. Also, phenotypes that do manifest tend to do so at a later age and are usually mild in severity, especially compared to those with high levels of TDP-43 expression or other models of ALS such as the SOD1^{G93A} mouse (Philips and Rothstein 2015). For example, here I found that while TDP-43^{M337V/M337V} mice develop defects in motor coordination and muscle strength at 9 months of age, they present no further exacerbation of motor impairment at later time points and statistical significance between TDP-43^{M337V/M337V} mice and NTg controls is lost. This is somewhat similar to observations made by Arnold *et al.*, who generated transgenic mice that express hTDP-43^{M337V} at moderate levels (~ 1.5 -fold increase from endogenous mouse Tdp-43), using the murine prion promoter (Arnold *et al.* 2013). They demonstrated that while TDP-43^{M337V}

mice develop a defect in motor coordination at 10 months of age compared to NTg controls, there was no significant difference in motor coordination between these two genotypes at 17 months of age (Arnold *et al.* 2013). Together, these studies highlight the subtlety of ALS-associated phenotypes presented by mutant TDP-43 mouse models with low to moderate levels of transgene expression.

Previous work by our group has demonstrated that intermediate isoforms of OXR1 are upregulated in post-mortem spinal cord samples from ALS patients and Oxr1-FL is upregulated in the spinal cord of pre-symptomatic SOD1^{G93A} mice (Oliver *et al.* 2011). This suggests that Oxr1 up-regulation may be a consequence of motor neuron degeneration in the spinal cord; but more importantly, Oxr1 may serve as an early marker of neurodegeneration before any overt phenotypic abnormalities occur. In this study, I did not observe upregulation of Oxr1 RNA or protein in the spinal cord of 18-month-old TDP-43^{M337V/M337V} mice. I hypothesize that this could be a reflection of the lack of motor neuron loss and very limited pathology found in the spinal cord of these mice at this time-point. Notably, the only ALS-associated pathological feature observed in the spinal cord of these mice was a subtle neuroinflammatory response, characterised by increased astrogliosis and microgliosis. However, given that I do not observe motor neuron degeneration in these mice, I hypothesize that these reactive glial cells are more likely exerting a neuroprotective effect on neighbouring motor neurons. Indeed, it has been demonstrated recently that reactive glial cells can selectively clear neuronal hTDP-43 thereby improving motor neuron survival in a TDP-43 mutant mouse model of ALS (Spiller *et al.* 2018). Future experiments characterizing the phenotype of these cell types may be worthwhile and could potentially shed light on why low-level ubiquitous expression of hTDP-43^{M337V} does not lead to motor neuron degeneration in mice by this time-point.

ALS is widely considered a distal axonopathy, in that many molecular changes influencing motor neuron degeneration occur at the NMJ at very early stages of the disease prior to symptom onset (Fischer *et al.* 2004; Moloney, de Winter and Verhaagen 2014). Here, I demonstrate that expression of hTDP-43^{M337V} in mice causes denervation and degeneration of the NMJ in distal lumbrical muscles. While the exact causative mechanism for this has not been determined, it is well established that TDP-43 is known to process and transport key presynaptic mRNAs that encode presynaptic proteins involved in the organization and functional elements of an efficient NMJ (Narayanan *et al.* 2013; Alami *et al.* 2014). In addition, TDP-43 is also a component of macromolecular complexes that generate miRNAs important for NMJ maintenance (Williams *et al.* 2009; Lagier-Tourenne, Polymenidou and Cleveland 2010). Therefore, whether by gain or loss of function, it is likely that mutations in TDP-43 leads to impaired NMJ function. This is supported by multiple studies characterizing other mutant TDP-43 rodent models that also present with NMJ degeneration (Wegorzewska *et al.* 2009; Zhou *et al.* 2010; Swarup *et al.* 2011b; Arnold *et al.* 2013).

Remarkably, I show that overexpression of Oxr1 can reduce lumbrical muscle denervation and NMJ degeneration in TDP-43^{M337V/M337V} mice. Given that lumbrical muscles in the paw are directly involved in grip strength and locomotion, the protection Oxr1 provides against NMJ degeneration and muscle denervation could potentially explain why I observe significantly increased motor function in TDP-43^{M337V/M337V}/Oxr1^{Prnp/+} mice compared to TDP-43^{M337V/M337V} littermates. Due to its antioxidant-like properties (Finelli and Oliver 2017), one way in which Oxr1 could be reducing NMJ degeneration in this model is by reducing the levels of OS in pre-synaptic nerve terminals. Indeed, presynaptic nerve terminals at the NMJ are sensitive to ROS, and under OS conditions, neurotransmitter releasing machinery can be compromised (Pollari *et al.* 2014). Therefore, additional

experiments that confirm the localisation of Oxr1 to pre-synaptic terminals and investigate the redox state of the NMJ are needed. In addition, one physiological aspect that is receiving much attention in terms of early signs of NMJ degeneration is altered synaptic transmission (Rocha *et al.* 2013). Interestingly, recent evidence has shown that pre-symptomatic (3-month-old) TDP-43^{Q331K} mice display impaired neuromuscular transmission several months prior to any defects in motor function and motor neuron loss (Chand *et al.* 2018). Furthermore, this defect was characterised by impaired fusion and release of synaptic vesicles, which is consistent with the possible presynaptic role for TDP-43 as described above (Williams *et al.* 2009; Lagier-Tourenne, Polymenidou and Cleveland 2010; Narayanan *et al.* 2013; Alami *et al.* 2014; Chand *et al.* 2018). Therefore, future studies that explore neuromuscular transmission in these mice could be worthwhile.

Despite clear evidence that protein aggregation is a hallmark feature of ALS, many questions about the functional significance of aggregation remain unanswered. For example, which cellular mechanisms drive or are affected by protein aggregation? Are aggregates toxic and damaging to cells? Are they neuroprotective by sequestering misfolded protein? Or are they inert and have no direct link to disease pathogenesis? Here, I demonstrate that TDP-43^{M337V/M337V} mice develop neuromuscular defects in the absence of TDP-43 aggregation in lower motor neurons. Additionally, analysis of these mice demonstrated a lack of significant alterations in the biochemical solubility of TDP-43, contrary to what has been reported in human patients (Inukai *et al.* 2008; Tsuji *et al.* 2012; Nonaka *et al.* 2013). These observations are supported by other studies investigating TDP-43 solubility and aggregation in rodent models of ALS. For example, mice expressing hTDP-43^{M337V} at moderate levels developed motor dysfunction at 10 months of age without the presence of insoluble TDP-43-positive aggregates in lower motor neurons (Arnold *et al.* 2013). Additionally, in 2-month-old rats, inducing motor neuron-specific expression of TDP-

43^{M337V} resulted in motor dysfunction, rapid motor neuron loss, axonal and NMJ degeneration, neuroinflammation and premature death by 3 months of age without the presence of TDP-43-positive aggregates in lower motor neurons (Huang *et al.* 2012). Therefore, my findings add to the growing body of evidence suggesting TDP-43 aggregation itself may not be a necessary condition for the development of ALS.

Following this, considerable evidence has accumulated over the years suggesting that earlier stages of the protein mislocalisation process are more directly tied to neurodegeneration than the formation of protein aggregates themselves, and that protein aggregation might even represent a beneficial defence mechanism that operates to eliminate irreversibly aggregated proteins (Hung and Link 2011; Pastore and Temussi 2012; Blokhuis *et al.* 2013; Carija *et al.* 2017). Indeed, TDP43-mediated neurotoxicity has been associated with an increased cytoplasmic mislocalisation of this protein, whereas the formation of aggregates was not a necessary process (Barmada *et al.* 2010; Igaz *et al.* 2011). Previous work by the Talbot group has demonstrated that under basal conditions, primary motor neurons heterozygously expressing hTDP-43^{M337V} at low levels resulted in increased cytoplasmic mislocalisation of both exogenous and endogenous TDP-43 protein, compared to NTg motor neurons or those heterozygously expressing similar levels of wild type hTDP-43 protein (Gordon *et al.*, 2019). Furthermore, this cytoplasmic redistribution of TDP-43 in primary motor neurons was associated with impaired stress granule formation, resulting in fewer stress granules in mutant expressing cells under OS (Gordon *et al.*, 2019). Given the apparent disconnect between the TDP-43 mislocalisation phenotype *in vitro* and *in vivo*, it was hypothesized that primary motor neurons cultured under artificial conditions could be subjected to enough stress that induces hTDP-43^{M337V} mislocalisation, despite no additional stressors, such as sodium arsenite, being used. Nonetheless, it is clear that primary motor neurons from this novel mouse model provide a valuable *in vitro* tool that can be used to

further explore the role of TDP-43 mislocalisation and altered stress granule dynamics in ALS.

In this chapter, I show that overexpression of Oxr1 significantly reduces the mislocalisation of mutant TDP-43^{M337V} protein in these primary motor neurons under unstressed conditions. In addition, I show that overexpression of Oxr1 does not rescue the defect in stress granule formation, previously observed in these cells (Gordon *et al.*, 2019). Therefore, my work suggests that Oxr1 can reduce the mislocalisation of mutant TDP-43 protein in the absence of environmental stress, such as OS, in a manner that is likely independent from the stress granule response. This is consistent with previous work demonstrating that overexpression of Oxr1-C in cells expressing hTDP-43^{M337V} reduced the mislocalisation and aggregation of TDP-43 protein, without having any effect on the formation of stress granules (Finelli *et al.* 2015).

The subcellular localisation of ALS-associated proteins, such as TDP-43, can be influenced by a number of factors including genetic mutation/protein misfolding (Johnson *et al.* 2009; Barmada *et al.* 2010; Bilican *et al.* 2012), impaired protein degradation (van Eersel *et al.* 2011), prolonged environmental stress (Colombrita *et al.* 2009; Dewey *et al.* 2011), and post-translational modifications such as phosphorylation, oxidation, acetylation, ubiquitination and proteolytic processing (Cohen *et al.* 2012, 2015; Nonaka *et al.* 2016; Carra *et al.* 2017). Interestingly, previous work from the Oliver group demonstrated that Oxr1 reduces the cytoplasmic mislocalisation and aggregation of mutant TDP-43 through a pathway independent of proteasome- and autophagy-mediated degradation (Finelli *et al.* 2015). Therefore, given that I and others previously (Finelli *et al.* 2015) demonstrate an interaction between TDP-43 and Oxr1 at the protein level, I hypothesize that Oxr1 modulates TDP-43 localisation either by directly/indirectly modifying TDP-43 and/or by restoring correct protein folding. Indeed, recent data from the Oliver group suggests that Oxr1

possesses chaperone activity similar to that of peroxiredoxin 2 (Prdx2; Svistunova et al. unpublished work), and can modify other proteins thereby modulating their structure and/or function. For example, Oxr1 can alter the post-translational modifications, such as oxidation and S-nitrosylation, of Prdx2 thereby changing Prdx2 oligomerization and function (Svistunova et al. unpublished work). Therefore, future studies investigating the role Oxr1 as a potential chaperone that promotes proper folding of TDP-43 are needed, and experiments that test whether the interaction between Oxr1 and TDP-43 alters post-translational modifications of TDP-43 will be important.

In conclusion, I demonstrate that overexpression of Oxr1 protects against motor dysfunction and neuromuscular defects in a novel TDP-43 mutant mouse model of ALS. Therefore, Oxr1 is the first antioxidant protein that, when genetically overexpressed, can delay or mitigate pathological features associated with TDP-43-related ALS *in vivo*. The cytoplasmic mislocalisation of TDP-43 protein is a characteristic feature of ALS and other TDP-43 proteinopathies, therefore the development of therapies that aim to reduce TDP-43 mislocalisation has been widely investigated. Excitingly, I show that genetic overexpression of Oxr1 can decrease the cytoplasmic mislocalisation of TDP-43 in primary motor neurons expressing hTDP-43^{M337V}. Therefore, I propose that Oxr1 has potential as a novel therapeutic target for the treatment of these devastating diseases.

Chapter 5

Transcriptional control of Oxr1 as a potential therapeutic strategy in ALS

5.1 Introduction

ALS is a devastating condition which results in muscle wasting, paralysis and death usually within 2–5 years of diagnosis (Andersen and Al-Chalabi 2011). With an estimated mortality of 30,000 patients a year worldwide, ALS has a significant impact on society and the public health sector (Petrov *et al.* 2017). Despite almost half a century of studies investigating the therapeutic potential of numerous chemical compounds and cell-based therapies, treatment is largely limited to palliative care (Miller *et al.* 2009; Andersen *et al.* 2012). Over 60 drugs with different mechanisms of action have been trialled, and only two compounds, riluzole and edaravone, have come to market (Mitsumoto, Brooks and Silani 2014; Petrov *et al.* 2017). Riluzole, an anti-glutamatergic therapy, has only a modest effect on survival with no significant effect on muscle strength or function (Bensimon, Lacomblez and Meininger 1994; Lacomblez *et al.* 1996). Edaravone, an anti-oxidative therapy (Watanabe *et al.* 1994), was shown to slow disease progression in a group of patients with early onset and rapidly progressing disease (Yoshino and Kimura 2006). However, due to limitations in phase III trials the clinical efficacy of edaravone is still undetermined (Abe *et al.* 2017; Hardiman and van den Berg 2017). Therefore, finding an effective therapeutic strategy for ALS patients is crucial.

An extensive pipeline of new potential therapeutics for ALS is now available, and some of these drugs aim to target known pathogenic mechanisms, such as OS (Bucchia *et al.* 2015; Petrov *et al.* 2017). With the exception of edaravone, all antioxidants tested in ALS

clinical trials have essentially failed (Yoshino and Kimura 2006; Orrell, Lane and Ross 2008). Several stipulations have been put forward over the years to account for this failure. Firstly, most antioxidant compounds reduce only one subtype of ROS (Gilgun-Sherki, Melamed and Offen 2001; Phaniendra, Jestadi and Periyasamy 2015). Secondly, there are difficulties with the specificity of the antioxidants in scavenging the particular ROS that drives disease pathogenesis (Chan and Chan 2015). In addition, free radical scavenging alone is unlikely to reverse damage to DNA, proteins and lipids that would have accumulated by the time of treatment (Uttara *et al.* 2009). Thirdly, like the majority of therapeutic compounds, antioxidants struggle to cross the blood brain barrier, and therefore fail to penetrate the CNS at a pharmacologically relevant dose (Gilgun-Sherki, Melamed and Offen 2001). Given that exogenously administered antioxidants have failed to accomplish their therapeutic goal, a reasonable alternative approach is to activate endogenous antioxidant defence systems in the CNS (Joshi and Johnson 2012; Chan and Chan 2015).

OXR1 is found in all eukaryotic genomes and is expressed in the human CNS as many different isoforms (Volkert *et al.* 2000; Durand *et al.* 2007). The protection OXR1 provides against OS was originally demonstrated in bacteria (Volkert, Elliott and Housman 2000). Since then, its antioxidant properties have also been investigated in a wide range of other cell types and organisms (Elliott and Volkert 2004; Jaramillo-Gutierrez *et al.* 2010; Murray *et al.* 2013; Kobayashi *et al.* 2014; Sanada *et al.* 2014; Yang *et al.* 2014; Liu *et al.* 2015). Importantly though, OXR1 is a critical regulator of neuronal survival in response to OS, and is upregulated in the spinal cord of ALS patients, suggesting it plays an important role in ALS pathogenesis (Oliver *et al.* 2011). More recently, overexpression of Oxr1 in the SOD1^{G93A} mouse model of ALS not only delayed the expression of OS and inflammatory markers in the spinal cord, but also improved motor neuron survival, motor coordination and extended life-span, suggesting that Oxr1 can modulate SOD1-related ALS pathology

and has the potential to serve as a new therapeutic target for ALS patients (Liu *et al.* 2015). Importantly, mice only overexpressing Oxr1 in their CNS are phenotypically indistinguishable from wild-type littermates, which demonstrates that increasing Oxr1 levels would not be detrimental if used as a therapeutic strategy (Liu *et al.* 2015).

Restoring protein homeostasis has also emerged as an attractive therapeutic target for the treatment of ALS (Webster *et al.* 2017). In chapter 4, I demonstrated that overexpression of Oxr1 in a novel TDP-43^{M337V} mouse model of ALS improved motor coordination, muscle strength and neuromuscular defects, such as NMJ degeneration. This suggests that Oxr1 can also modulate TDP-43-related ALS pathology as well. While we do not know the exact mechanisms by which Oxr1 protects against such pathology, I showed that Oxr1 can bind to TDP-43 *in vivo*, and that overexpression of Oxr1 in TDP-43^{M337V/+} primary motor neurons can reduce the cytoplasmic mislocalisation of mutant TDP-43^{M337V} protein. These observations are consistent with previous findings from the Oliver group (Finelli *et al.* 2015). Together, these data highlight potentially novel neuroprotective functions of Oxr1 in regulating protein homeostasis. Whether Oxr1 achieves this by acting as a chaperone, altering post-translational modifications (such as those caused by OS), or by other mechanisms remains to be determined. Nonetheless, it is increasingly clear that up-regulation of OXR1 is proving to be a valuable approach to reduce OS, inflammation and protein dyshomeostasis, which are common pathogenic mechanisms associated with ALS and other neurodegenerative diseases (Finelli *et al.*, 2015; Liu *et al.*, 2015; Oliver *et al.*, 2011).

5.2 Aims of Chapter 5

Overexpression of *Oxr1* shows considerable promise as a therapeutic strategy for the treatment of ALS. Therefore, the aim of this chapter is to investigate the transcriptional regulation of *Oxr1* and determine whether endogenous *Oxr1* expression can be manipulated as a protective strategy. In order to achieve this, I characterised promoter regions of *Oxr1* and developed a new cell-based luciferase reporter assay that can be used to screen a library of compounds and identify those that are able to activate the *Oxr1* promoter.

5.3 Results

5.3.1 *In silico* analysis of human and mouse *Oxr1* promoters

Before considering strategies that could be used to manipulate endogenous *Oxr1* expression, it is crucial to understand how the gene is transcriptionally regulated. As yet, no promoter has been functionally characterised for any of the *Oxr1* isoforms (Figure 5.1). Therefore, human and mouse genomic sequences 2000 bp upstream of the initial ATG start codon of *Oxr1-FL* and *Oxr1-C* were first tested for alignment using the Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov>). Interestingly, I identified a ~320 bp sequence immediately upstream of the translation start site of *Oxr1-C* that was highly conserved between human and mouse genomes (Figure 5.2). This suggests that this small core promoter region is especially important in regulating the expression of *Oxr1-C*. I found no significant conservation between human and mouse genomes for promoter sequences of *Oxr1-FL*. Therefore, I decided to focus subsequent analysis on the conserved *Oxr1-C* promoter region. Importantly, *Oxr1-C* is as potent an antioxidant and confers the same level of neuroprotection against OS-induced cell death as *Oxr1-FL* (Oliver et al. 2011), so can therefore be used as a target for up-regulation. Furthermore, given that *Oxr1-FL* contains additional domains with unknown functions, targeting *Oxr1-C* alone could have

fewer undesirable side-effects. Subsequent analysis of the conserved *Oxr1-C* promoter (*Oxr1-Cp*) using the bioinformatic transcription factor binding sites (TFBS)-prediction tool Patch 1.0 connected to the TRANSFAC® database (<http://gene-regulation.com>; (Wingender *et al.* 1996) revealed a number of highly conserved predicted TFBSs (Figure 5.2). This suggests that transcription factors such as Forkhead box L1 (FoxL1) and NK2 homeobox 5 (NKX2.5) are likely involved in the regulation of human and mouse *Oxr1-C* by binding to their corresponding *cis*-regulatory elements.

UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9) Assembly

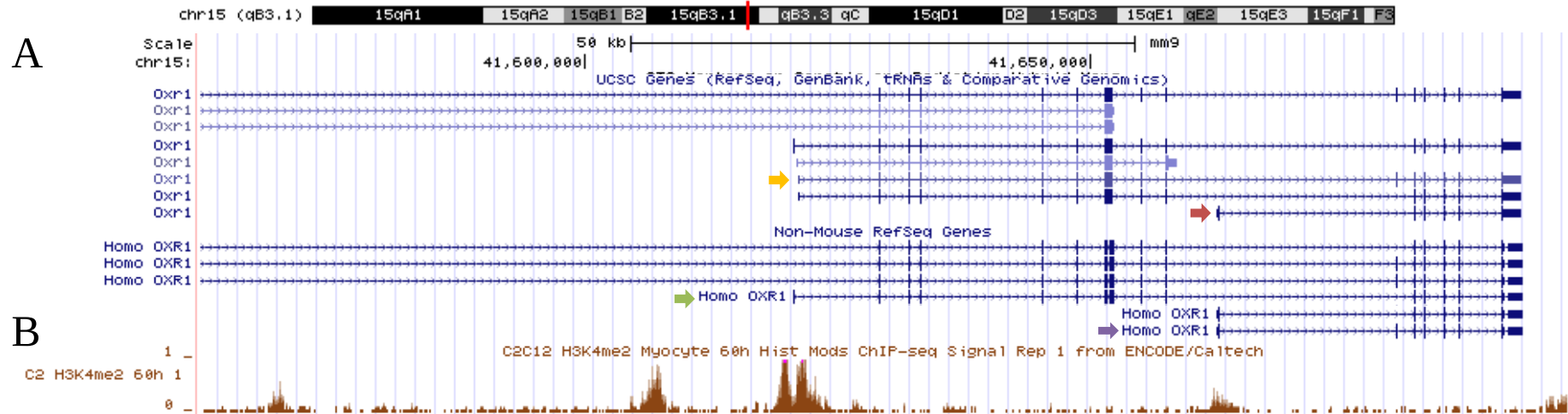


Figure 5.1 *Oxr1* isoforms and potential promoter regions. (A) Schematic diagram taken from the UCSC Genome Browser showing mouse *Oxr1-FL* (yellow arrow; GenBank accession number - NM_130885), mouse *Oxr1-C* (red arrow; NM_001130164), human *OXR1-FL* (green arrow; NM_181354) and human *OXR1-C* (purple arrow; NM_001198534). (B) Chemical modifications, such as methylation, to histone proteins present in chromatin influence gene expression by changing how accessible the chromatin is to transcription. The brown density graphs represent signal enrichment of histone H3(di-methyl K4) (H3K4me2), a marker associated with promoters and enhancers, across the genome as determined by ChIP-seq assays in C2C12 myoblast cells (*performed by California Institute of Technology as part of the ENCODE project*). Additional histone marks and other chromatin associated ChIP-seq data is available at <https://genome.ucsc.edu>.

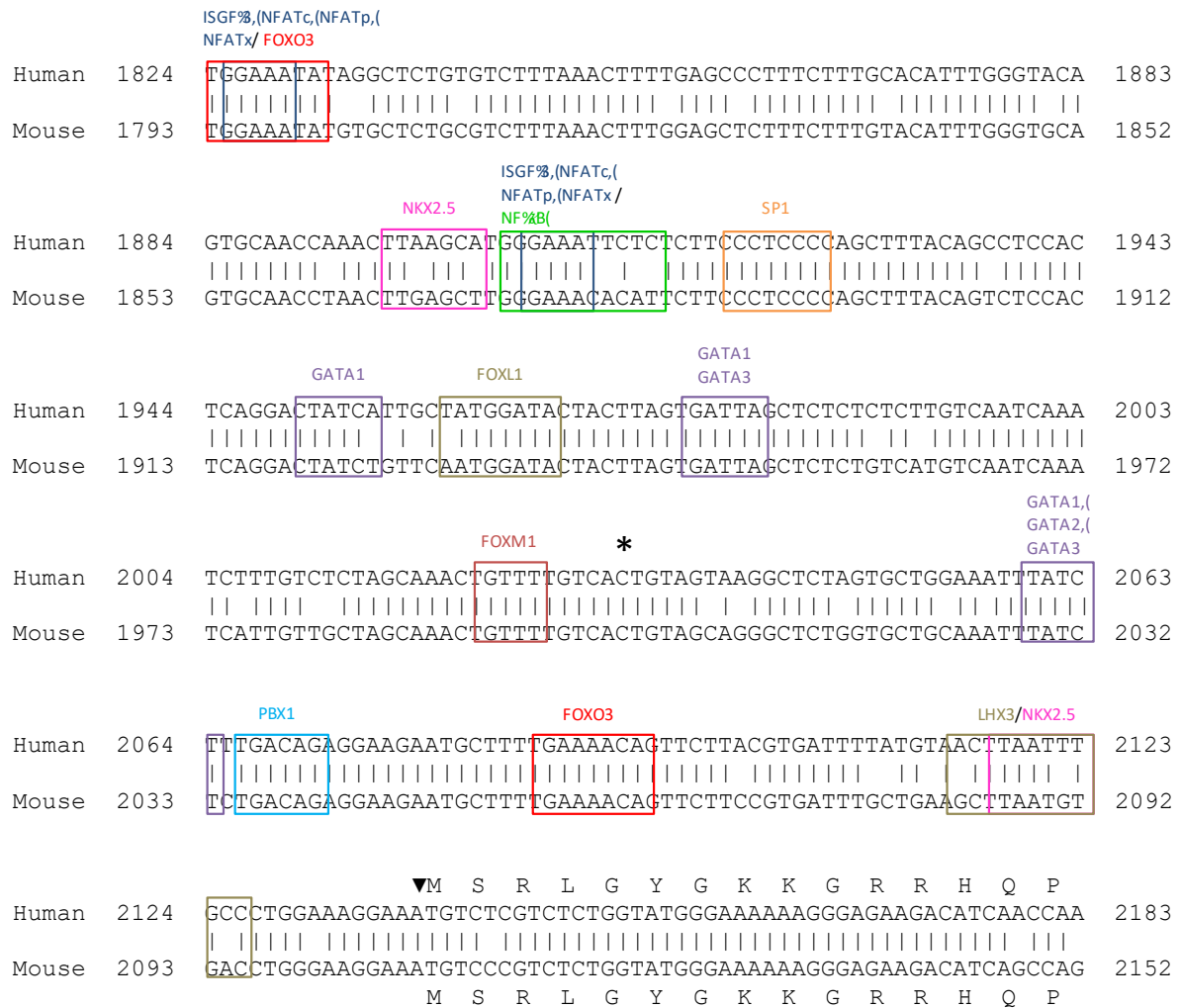


Figure 5.2 Sequence alignment of human and mouse promoter regions of *Oxr1-C*. Sequence conservation between the human (top: NM_001198534) and mouse (bottom: NM_001130164) promoter region of *Oxr1-C*. The asterisk represents the transcription start site of the 5' untranslated region. The triangle depicts the translation start site and the translated exon is shown with corresponding amino acids. All putative transcription factor binding sites are shown in boxes with the predicted transcription factor shown above. FOXL1: Forkhead Box L1, FOXM1: Forkhead Box M1, FOXO3: Forkhead Box O3, GATA1/2/3: GATA binding protein 1/2/3, ISGF-3: Interferon-Stimulated Gene Factor 3 Gamma, LHX3: LIM Homeobox 3, NF-κB: Nuclear Factor Kappa B, NFATc/p/x: Nuclear Factor of Activated T-cells cytoplasmic 1/2/3, NKX2.5: NK2 Homeobox 5, PBX1: Pre-B-Cell Leukemia Transcription Factor 1, SP1: Specificity Protein 1.

5.3.2 The conserved promoter region of *Oxr1-C* shows significant promoter activity

A common practice for functionally characterizing a promoter is to measure its activity using a cell-based assay with a reporter gene driven by the promoter of interest. A popularly applied reporter gene is the firefly luciferase (*FLuc*) derived from *Photinuspyralis*, due to its high sensitivity and cost efficiency (de Wet, Wood, DeLuca 1987; Fan and Wood 2007). Therefore, the conserved promoter region of human *OXR1-C* (hOXR1-Cp) and mouse *Oxr1-C* (mOxr1-Cp) were cloned in to *FLuc* reporter vectors (pGL4.10). In addition, in order to investigate potential regulatory elements further upstream that could be critical for promoter activity, a larger promoter region (~1 Kb) upstream of mouse *Oxr1-C* was also cloned in to a pGL4.10 vector. Following transient transfection of the promoter constructs in to N2a cells, a luciferase reporter assay was used to measure the activity of the cloned promoter region. A Renilla luciferase (*RLuc*) construct (*pRL-TK*), derived from the sea pansy *Renillareniformis* (Lorenz *et al.* 1991), was also co-transfected and used as an internal control to which expression of *FLuc* was normalised. Interestingly, I observed an increase in luminescence from cells transfected with either pGL4.10/hOXR1-Cp (25-fold) or pGL4.10/mOxr1-Cp (17-fold), compared to those transfected with the control pGL4.10/Empty construct (Figure 5.3). This suggests that the cloned conserved promoter regions of *Oxr1-C* are functional and can drive the expression of the *FLuc* reporter. In addition, given that the human promoter region was active in cells derived from mouse tissue highlights the conservation between promoters. I also observed that there was a 42-fold increase in luminescence from cells transfected with pGL4.10/mOxr1-Cp 1Kb compared to those transfected with pGL4.10/Empty (Figure 5.3), suggesting that there are additional regulatory elements upstream of the conserved region in the mouse *Oxr1-C* promoter that can increase its activity in this assay system.

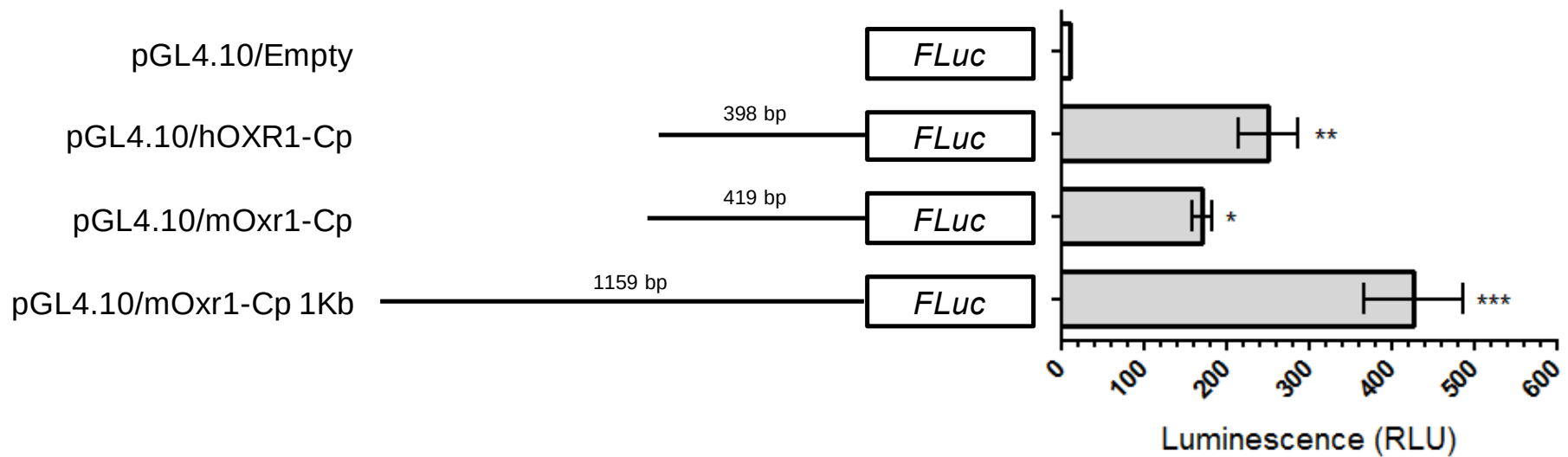


Figure 5.3 The conserved promoter region of *Oxr1-C* shows significant activity. Schematic illustrates the length (bp) of the *Oxr1-C* promoter regions cloned in to the pGL4.10 vector. Luciferase assays in N2a cells demonstrate that the conserved promoter region of *Oxr1-C* can drive the expression of *firefly* luciferase (*FLuc*). A larger promoter region (~1Kb) of mOxr1-C has greater promoter activity than the shorter conserved region of mOxr1-Cp. Values are the mean $\pm$ SEM (n=4, One-way ANOVA followed by Dunnett's Multiple Comparison Test comparing to pGL4.10/Empty (*p<0.05, **p<0.01, ***p<0.001)).

5.3.3 Mutating specific TFBSs alters Oxr1-C promoter activity

Given the functional activity of the conserved Oxr1-C promoter, I then determined whether there are any key TFBSs that could be critical for Oxr1-C expression. Therefore, a selection of predicted TFBSs (Figure 5.2) in the conserved Oxr1-C promoter region were mutated by site-directed mutagenesis in order to disrupt the binding of corresponding TFs. Following transient transfection either the wild-type Oxr1-C promoter or the mutant promoter constructs into N2a cells, a luciferase reporter assay was used to measure promoter activity. Interestingly, I observed a similar pattern of promoter activity for the different mutant constructs between the human (Figure 5.4A) and the mouse (Figure 5.4B) promoters. More specifically, I found that mutating the Nkx2.5 binding site (human – TTGCGCA; mouse – TTCCGCT; underlined bases represent mutation) caused a significant increase in Oxr1-C promoter activity, whereas mutating Pbx1 (TGGTAG) and Sp1 (AACTCCC) binding sites caused a reduction in activity compared to the wildtype promoter (not significant for mOxr1-Cp; Figure 5.4A & B). In addition, I also observed that mutating the NFκB (mouse - GTTAAACACAT) and FoxM1 (mouse - TGGGT) binding site in mOxr1-Cp caused a significant increase in promoter activity, compared to the wild-type promoter (figure 5.4B). Taken together, these results suggest that there are key regulatory elements found in this conserved Oxr1-C promoter region that could be critical for the normal expression of both human and mouse Oxr1-C.

Even though mutating predicted TFBSs was an attempt to disrupt the binding of corresponding TFs, it should be noted that introducing a mutated sequence could unintentionally give rise to alternative TFBSs. This could potentially affect the activity of the Oxr1-C promoter region and should therefore be considered when interpreting data. In this study, I found that mutating the human Nkx2.5 binding site generated a predicted CCAAT/Enhancer Binding Protein Beta (C/ebpβ) binding site (TTGCGCA), and mutating

the mouse Nkx2.5 binding site produced a predicted Ets Proto-Oncogene 1 (Ets-1) and Ets-related transcription factor (Elf-1) binding site (ACTTCC; Patch 1.0, <http://gene-regulation.com>). In addition, mutating the mouse Nfkb binding site generated a predicted Pou Class 1 Homeobox 1 (Pou1f1) binding site (TA AACAC), and mutating the conserved Sp1 binding site produced a Retinoid X Receptor Alpha (Rxra) binding site (TAACT; Patch 1.0, <http://gene-regulation.com>). Therefore, it is entirely possible that the changes in promoter activity I observed in my assay could be due to the action of other transcription factors capable of binding to the mutated sequences, however additional studies would be needed to confirm this.

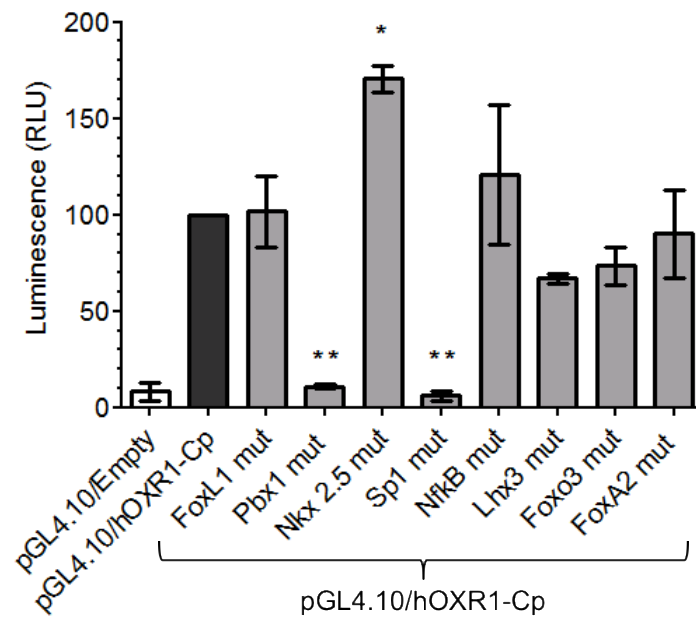
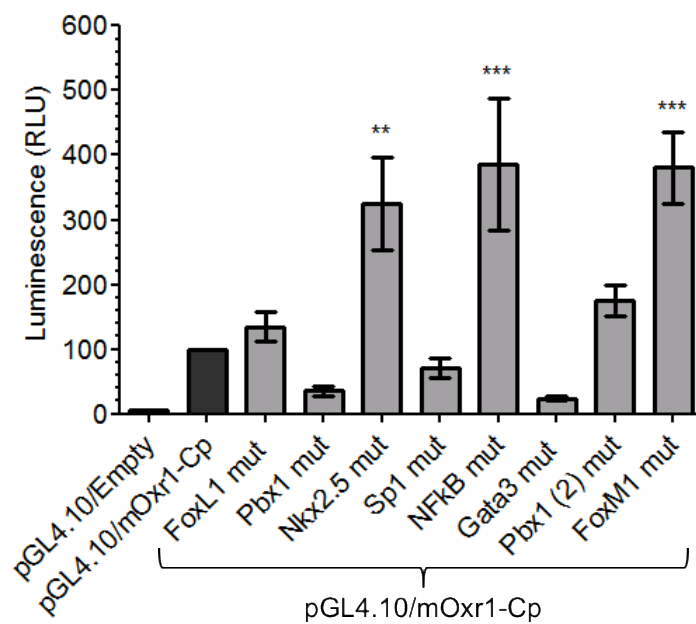
A**B**

Figure 5.4 Mutating specific TFBSs alters *Oxr1-C* promoter activity. Luciferase Assays demonstrate that human (A) and mouse (B) *Oxr1-Cp* activity can be altered by mutating predicted TFBSs. Values are the mean $\pm$ SEM (n=4, One-way ANOVA followed by Dunnett's Multiple Comparison Test compared to pGL4.10/hOxr1-Cp or pGL4.10/mOxr1-Cp (*p<0.05, **p<0.01, ***0.001)).

5.3.4 Establishment of a cell-based screening system for the identification of small compounds capable of Oxr1 upregulation.

5.3.4.1 Generation of stably transfected N2a lines

In general, the first step to establishing a cell-based high throughput screening system is to generate a biological detection system. Transiently transfected cell lines not only express luciferase constructs for brief periods of time, but also display relatively large variances in expression due to transfection inefficiencies (Zhang *et al.* 2012). By contrast, stably transfected cell lines involve integrating the luciferase constructs in to the host genome resulting in reduced assay variation because luciferase constructs are consistently expressed over long periods of time (Johnston and Johnston 2002; Zhang *et al.* 2012). In addition, there is no need for an internal control such as *Renilla* luciferase. Therefore, I generated stable N2a lines using an *FLuc* construct (pGL4.22) that contains a mammalian selectable marker. Given that a standard drug development procedure moves from cell assay to animal model to clinical trial, an ideal compound would need to be capable of inducing the expression of endogenous Oxr1-C in both human and mouse neurons. Therefore, I cloned the conserved promoter region of mouse Oxr1-C in to the pGL4.22 vector. Importantly, I demonstrated previously that the activity of the conserved promoter region of Oxr1-C was comparable between human and mouse (Figures 5.2 and 5.3).

Following stable transfection of either pGL4.22/Empty (control line) or pGL4.22/mOxr1-Cp, cells were grown under selection with the antibiotic puromycin before surviving colonies were picked and re-plated. After several weeks, I identified lines positive for the luciferase construct that displayed appropriate levels of *FLuc* expression (Figure 5.5). More specifically, I generated three individual pGL4.22/mOxr1-Cp cell lines that expressed *FLuc* at varying levels (Figure 5.5B), likely due to chromatin structure or regulatory elements at different genome integration sites. In addition, I also generated three control cell

lines that express *FLuc* at basal levels. Therefore, these stable cells lines can potentially be used to establish a sensitive and robust high-throughput screening system to identify small compounds that upregulate *Oxr1-C*.

A

Cell lines (N2a)	Colonies picked	Lines positive for construct	Lines expressing luciferase
pGL4.22/Empty (control)	96	12	3
pGL4.22/mOxr1-Cp	96	14	3

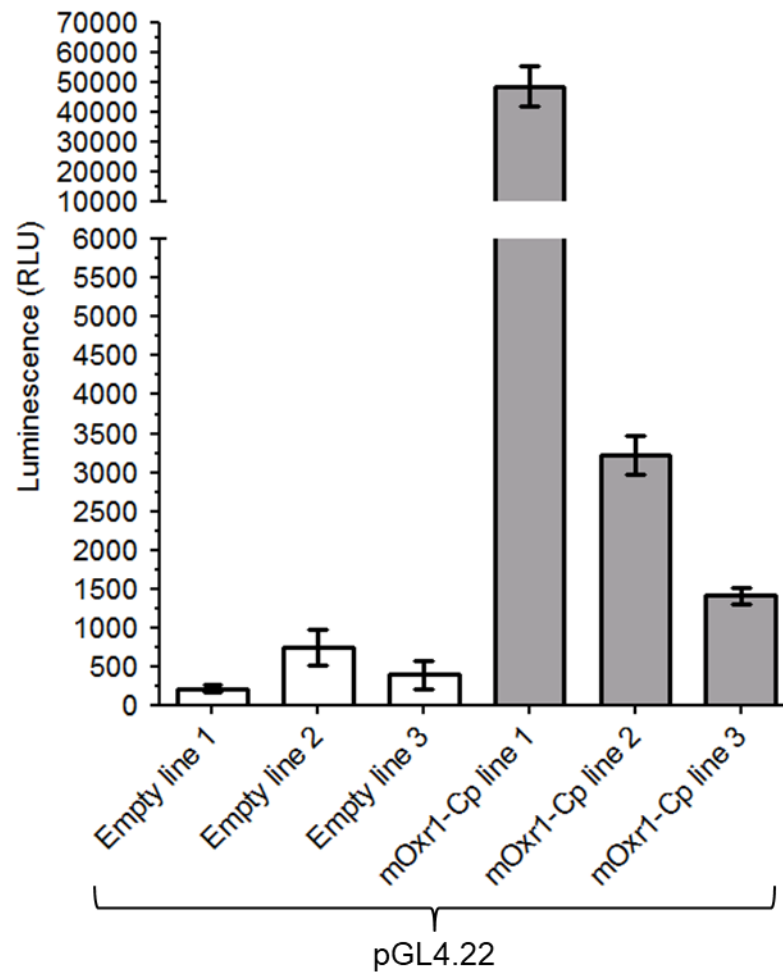
B

Figure 5.5 Generation of stable N2a lines that express *FLuc* driven by *mOxr1-Cp*. (A)

A table showing the number of N2a colonies analysed for stable integration of the pGL4.22/Empty or pGL4.22/mOxr1-Cp construct that also express *FLuc*. **(B)** Luciferase assay demonstrates appropriate levels of *FLuc* expression in either pGL4.22/Empty control lines or the pGL4.22/mOxr1-Cp lines. Values are the mean $\pm$ SEM.

5.3.4.2 Assay optimization and pilot compound screen

Once an assay has been created, optimization is crucial to find ideal conditions for compound screening (Zhang *et al.* 2012). Cell density, compound concentration and duration of treatment are all parameters that were considered before carrying out a pilot screen. In order to treat cells that were actively dividing and under 80% confluent, I decided to seed 0.5×10^5 cells/well in a 96-well plate format 24 hours before treatment (data not shown). In addition, 24 hours was chosen as the duration of treatment in order to avoid cell detachment and death (due to over confluency) by the time of luciferase assays (data not shown). Compound concentration should be sufficiently potent to achieve bioactivities, while avoiding concentrations that could pose significant cytotoxicity. Therefore, cells were treated with compounds at a final concentration of 10 μ M (as advised by the Department of Chemistry, University of Oxford).

Due to its ability to dissolve molecules with a wide range of chemical properties, compounds are usually stored in dimethyl sulfoxide (DMSO). However, DMSO is harmful to living cells, and its toxicity can be cell line specific (Waybright, Britt and McCloud 2009). Therefore, it was important to determine the optimal concentration of DMSO to be used in my assay. Using my stable N2a cell lines, cell viability assays revealed that treatment with 0.25 to 1.0% DMSO did not cause a significant difference in cytotoxicity, compared to untreated cells (Figure 5.6A). However, I did observe a small but significant increase in cytotoxicity when cells were treated with 2% DMSO (Figure 5.6A), suggesting that this concentration would be unsuitable to use in subsequent screens. It is also extremely important that the concentration of DMSO is compatible with other biological reagents (such as enzymes) in the assay to ensure the screen is not adversely affected. Therefore, I also tested the effect of increasing DMSO concentration on *FLuc* activity in the three pGL4.22/mOxr1-Cp cell lines. In general, I observed a progressive decrease in luminescence

as the concentration of DMSO increased (Figure 5.6B), suggesting that higher concentrations of DMSO can affect *FLuc* activity. Importantly, there was minimal difference (<2%) in luminescence from cell lines treated with 0.25% DMSO, compared to untreated cells (Figure 5.6B). Therefore, I decided to solubilize compounds at a final concentration of 0.3% DMSO for the pilot screen.

Pilot screens are usually carried out in small scale using a minor batch of known compounds to determine whether the assay is sufficiently feasible and reliable for high throughput screening. All three N2a pGL4.22/mOxr1-Cp lines were used to screen 80 compounds from a kinase inhibitor library (Figure 5.7), and 80 compounds from an epigenetic modulator library (Figure 5.8). All compound data was normalized to the 0.3% DMSO control and positive hits were defined as being greater than the mean of all compounds + (2 × standard deviations (STDEV) of all compounds). While, I found numerous compounds from both libraries that caused an increase in *FLuc* activity in the N2a pGL4.22/mOxr1-Cp lines, only nine were identified as positive hits: kinase inhibitor compound 1 (K1), K7, K8, K23, K25, K36, K47, epigenetic modulator compound 29 (E29) and E54. Interestingly, E54 caused the largest increase in *FLuc* activity and was active in all three pGL4.22/mOxr1-Cp cell lines (Figure 5.8), suggesting it could be an excellent candidate compound for Oxr1 upregulation (Figure 5.9).

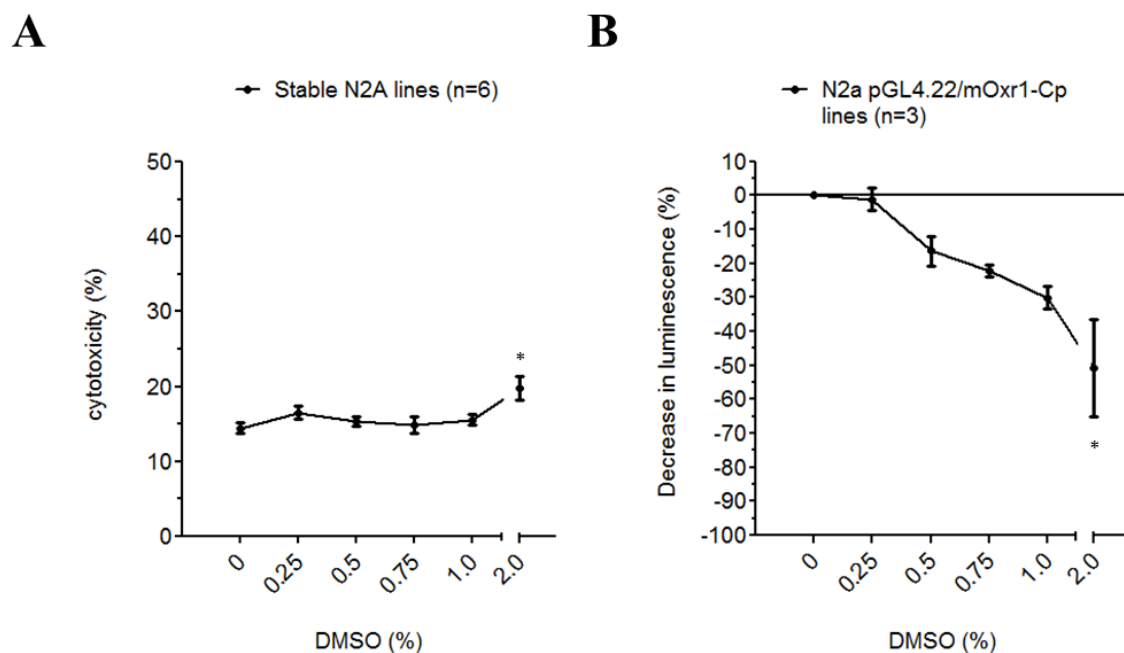
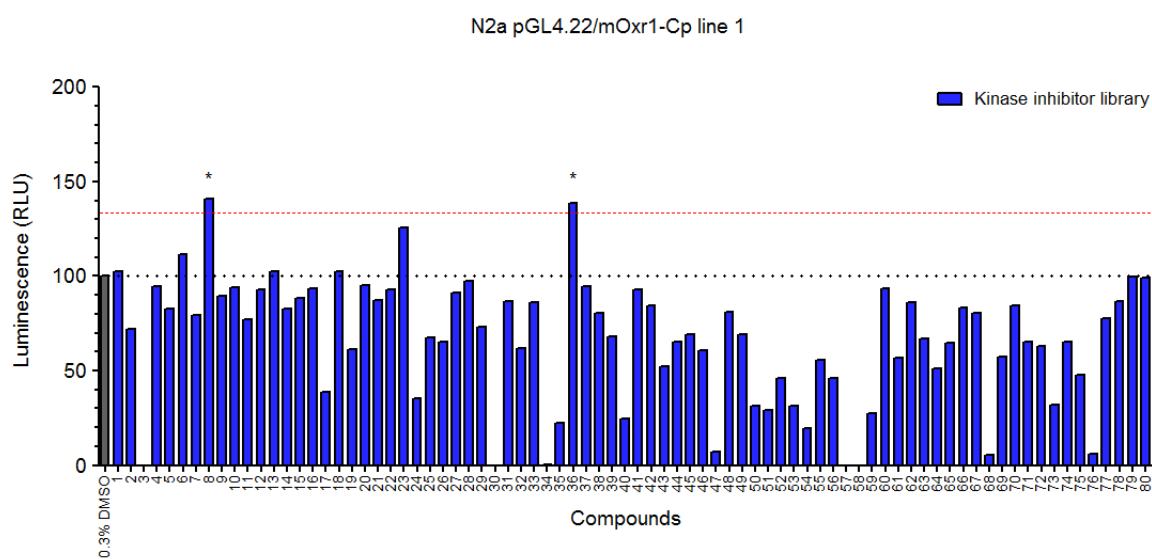
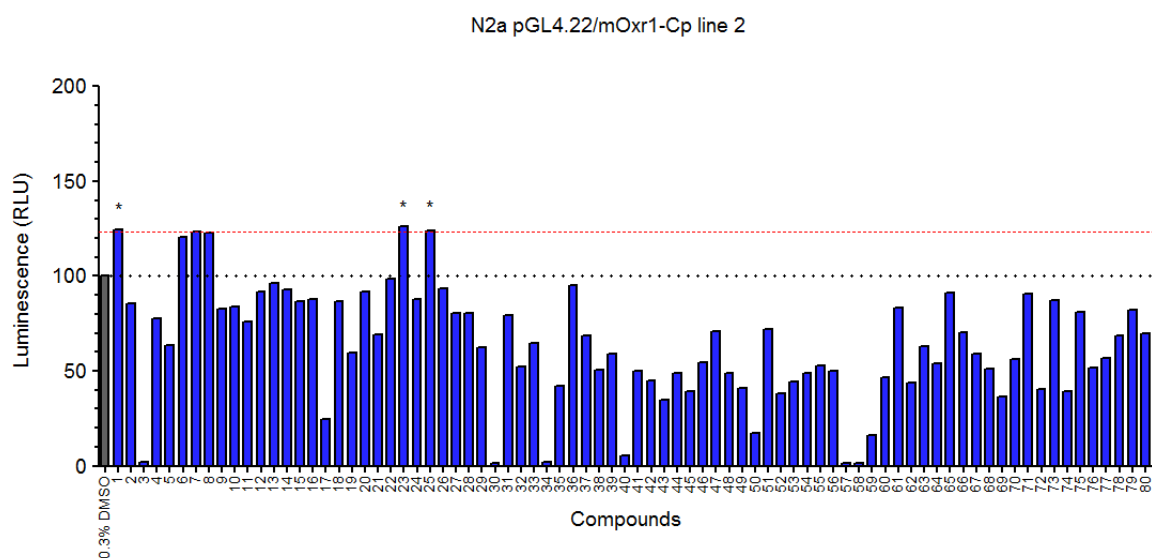


Figure 5.6 Optimization of DMSO concentration for pilot compound screen. (A) cell viability assays demonstrate that stable N2a lines treated with a range of 0.25 and 1.0% DMSO display no significant increase in cytotoxicity, compared to untreated cells. N2a lines treated with 2% DMSO display a significant increase in cytotoxicity, compared to untreated cells (B) luciferase assays demonstrate that *FLuc* activity gradually decreases as the concentration of DMSO increases. Values are the mean $\pm$ SEM (n=1, one-way ANOVA followed by Bonferroni's multiple comparison test, *p<0.05, **p<0.01).

A



B



C

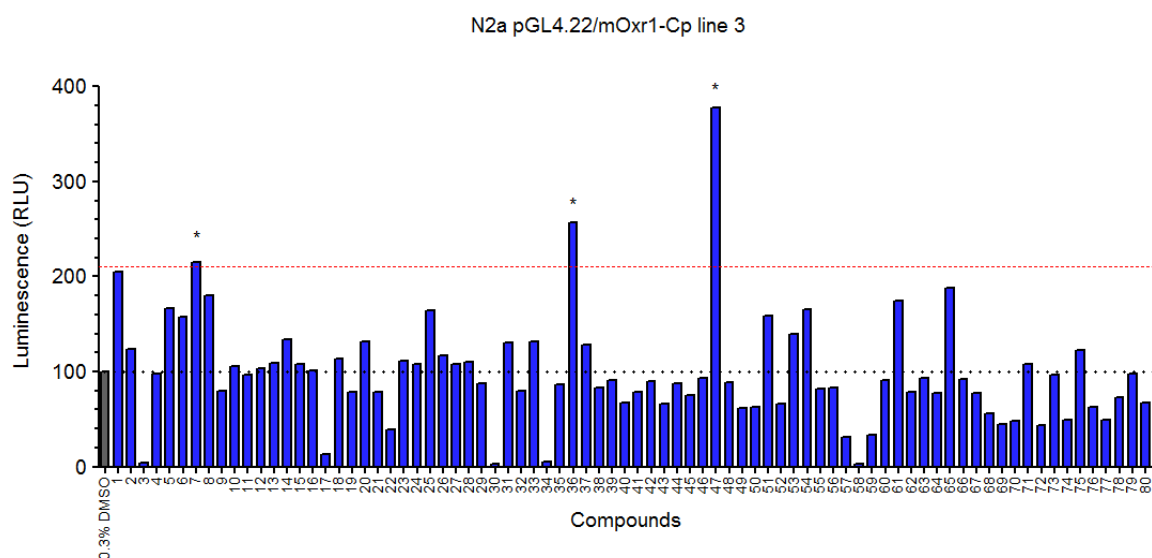
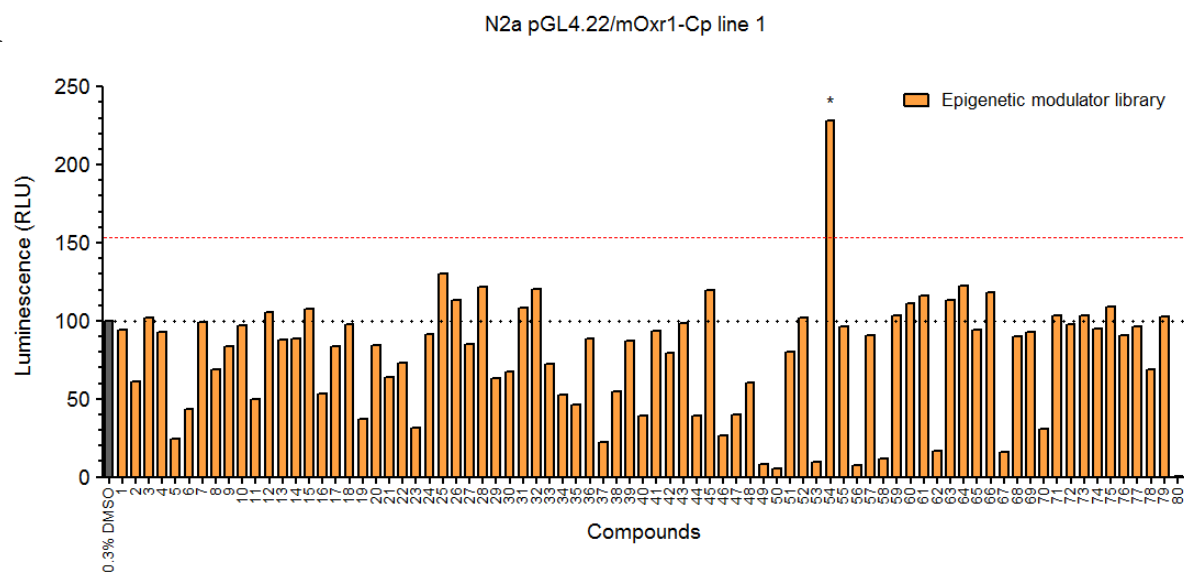
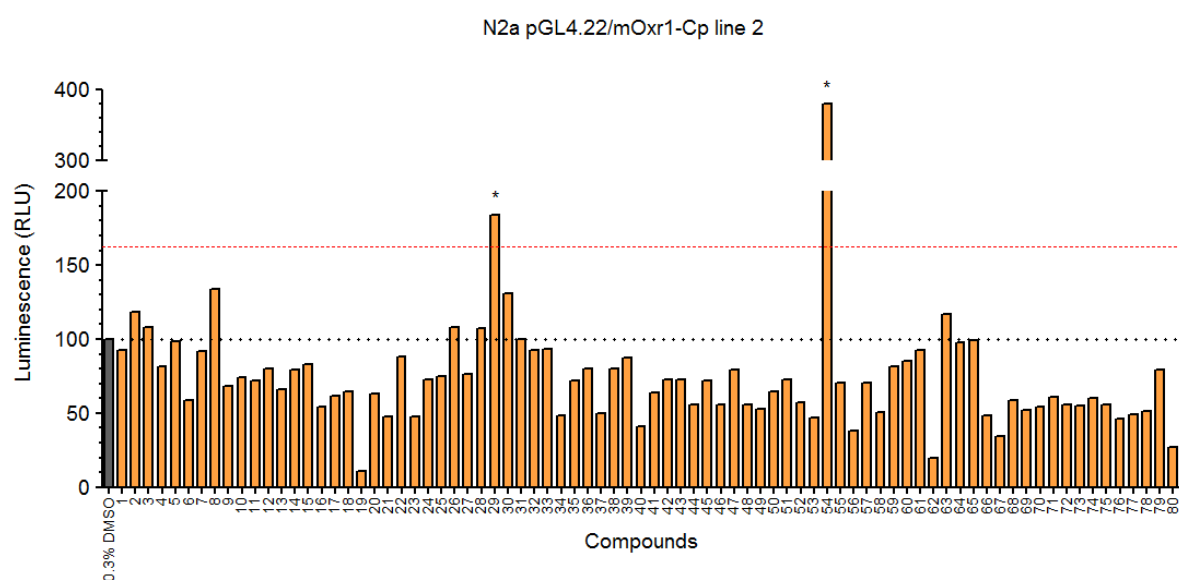


Figure 5.7 Pilot compound screen with kinase inhibitor library. N2a pGL4.22/mOxr1-Cp line 1 **(A)** line 2 **(B)** and line 3 **(C)** were treated with 80 compounds from a kinase inhibitor library at 10 μ M for 24 hours. Luciferase assays demonstrate that *FLuc* activity is significantly increased when treated with certain compounds that may activate the Oxr1-C promoter region. Values are the mean of triplicate readings normalized to 0.3% DMSO control. * * * : represents the level of FLuc activity in cells treated with 0.3% DMSO. -----: represents the threshold used to identify positive hits, which was set at $2 \times \text{STDEV}$ from the mean of all compounds. *: represents the compounds identified as positive hits.

A



B



C

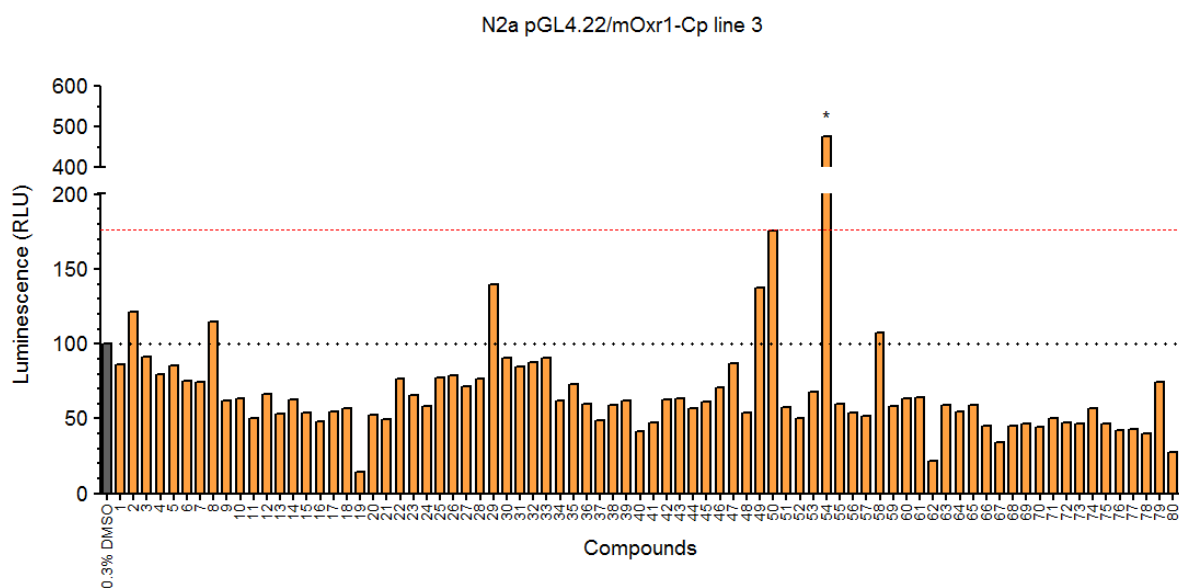


Figure 5.8 Pilot compound screen with epigenetic modulator library. N2a pGL4.22/mOxr1-Cp line 1 **(A)** line 2 **(B)** and line 3 **(C)** were treated with 80 compounds from an epigenetic modulator library at 10 μ M for 24 hours. Luciferase assays demonstrate that *FLuc* activity is significantly increased when treated with certain compounds that may activate the Oxr1-C promoter region. Values are the mean of triplicate readings normalized to 0.3% DMSO control. * * * : represents the level of *FLuc* activity in cells treated with 0.3% DMSO. -----: represents the threshold used to identify positive hits, which was set at $2 \times$ STDEV from the mean of all compounds). *: represents the compounds identified as positive hits.

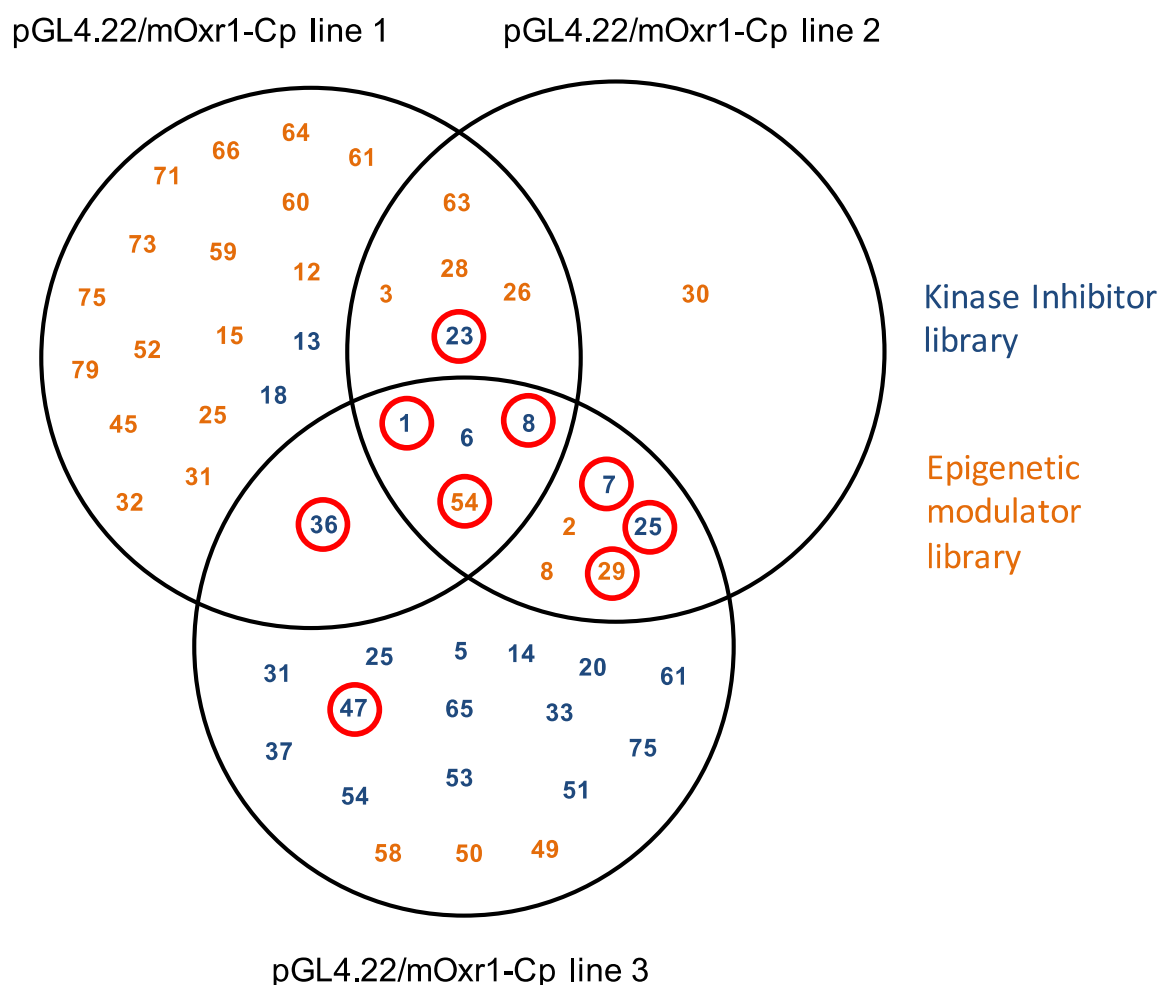


Figure 5.9 Summary of compounds that increase *FLuc* activity in the three pGL4.22/mOxr1-Cp cell lines. Venn diagram showing the compounds from a kinase inhibitor library (blue) and an epigenetic modulator library (orange) that caused an increase in *FLuc* activity in the three pGL4.22/mOxr1-Cp cell lines. Compounds that were identified as above the significance threshold in at least one of the cell lines are circled in red.

5.3.4.3 Counter screens used to identify false positive compounds

In order to determine whether positive hits are real and not ‘off-target effects’, I also screened all compounds using one of the N2a pGL4.22/Empty control lines (Figure 5.10). Importantly, I identified a number of compounds (K1, K5, K47 and E54) as positive hits in the promoter-less FLuc control line (Figure 5.10), suggesting that these compounds likely have a non-specific off-target effect in my assay. For luciferase reporter-based assays, one major concern is identifying false active compounds that increase the luminescent signal not through activation of the target, but through posttranslational stabilization of the luciferase enzyme (Auld *et al.* 2008b). Therefore, an additional counter screen was performed to rule out such false positive hits. Eight positive hits from the pilot screen were selected and tested for their ability to inhibit purified FLuc using a biochemical enzyme assay. PTC-124 is a known potent reversible inhibitor of purified FLuc (Auld *et al.* 2009), and was therefore used as a positive control in the counter screen. In agreement with previous findings, I observed that the activity of purified FLuc displayed a concentration-dependent inhibition with PTC-124 treatment ($IC_{50} = 0.00179 \mu M \pm 8.46e-05$; Figure 5.11). However, I found that none of the eight compounds tested were capable of inhibiting FLuc activity to a comparable level (Figure 5.11). Furthermore, for all compounds tested, data points did not fit to a clearly defined sigmoidal curve and it was therefore not possible to calculate accurate relative IC_{50} values within this concentration range (Figure 5.11). This suggests that these compounds are not potent stabilizers of FLuc.

Another concern with this type of assay is to identify false positive compounds that increase the luminescent signal by increasing the rate of cell proliferation, thereby increasing the number of cells expressing FLuc. Therefore, I tested cell proliferation in all three N2a pGL4.22/mOxr1-Cp cell lines in response to treatment with the selected compounds. Interestingly, K25 was the only compound to cause a significant increase in cell proliferation

in these cell lines (Figure 5.12). This suggests that K25 could have caused a non-specific increase in FLuc activity in the pilot screen by increasing proliferation of reporter cell lines. Interestingly, K36 was found to have a negative correlation on cell proliferation (Figure 5.12). Given that K36 increased FLuc activity in two of the three pGL4.22/mOxr1-Cp lines in the pilot screen, did not increase FLuc activity in the pGL4.22/Empty control line, and did not cause concentration-dependent inhibition of purified FLuc in the counter screen, these data suggest that K36 could be a good candidate for compounds that activate the conserved promoter region of mOxr1-C. A full summary of data obtained from the pilot compound screen and counter screens is provided in Table 5.1.

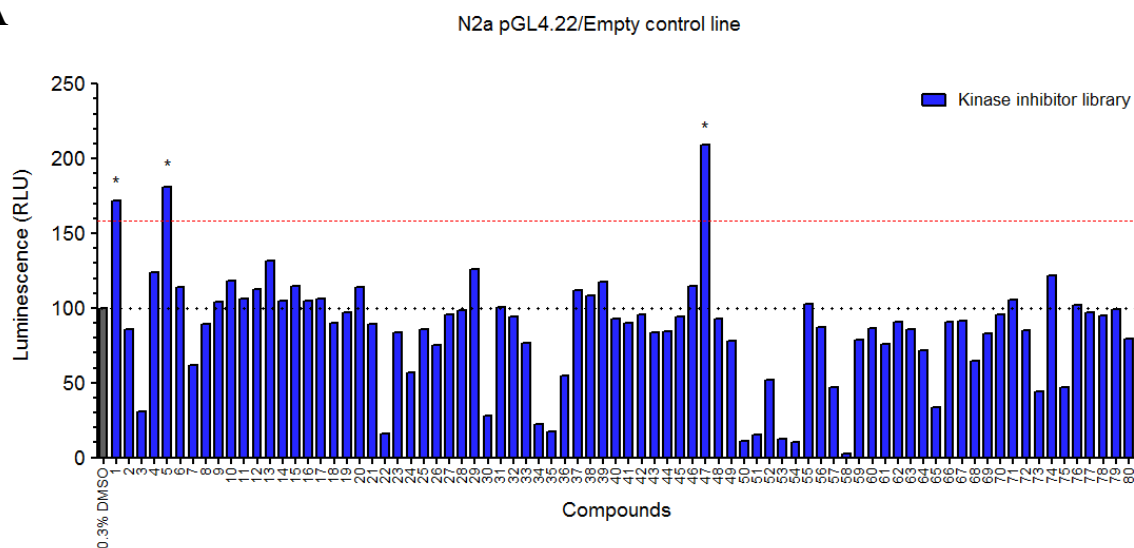
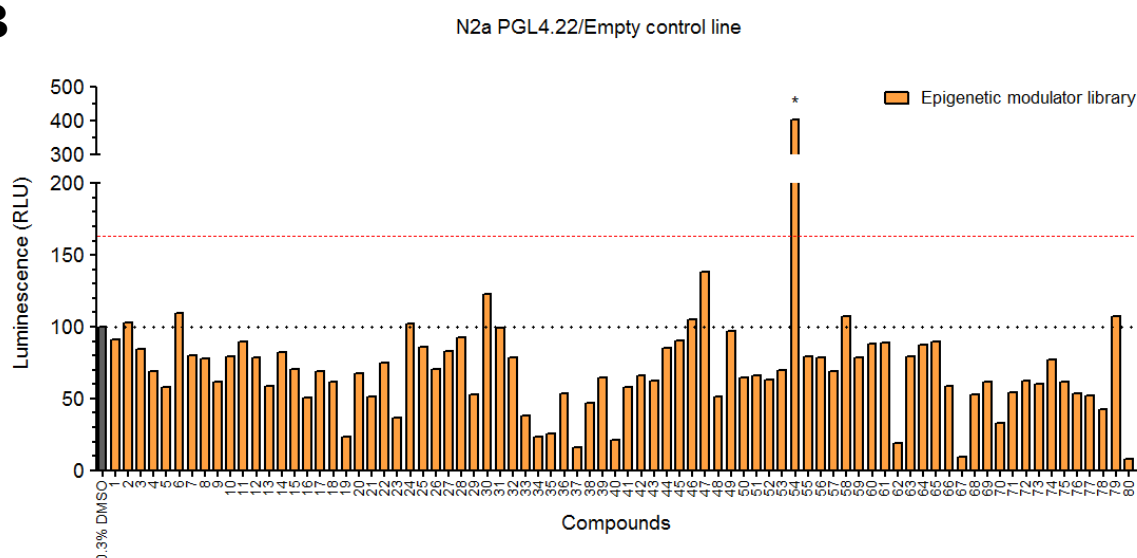
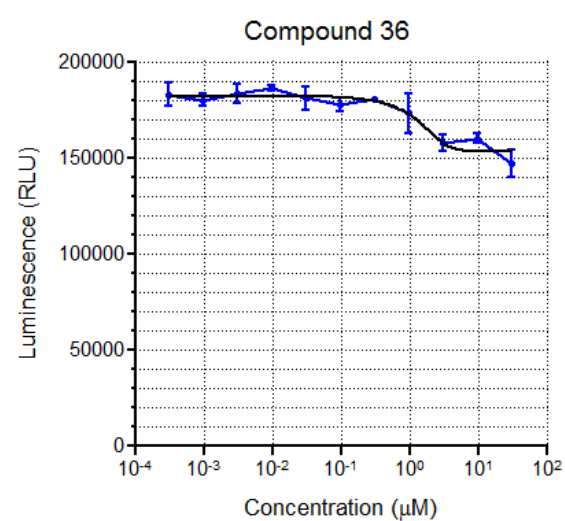
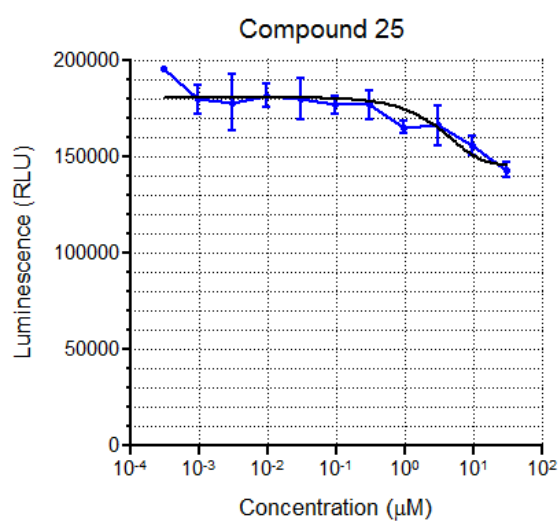
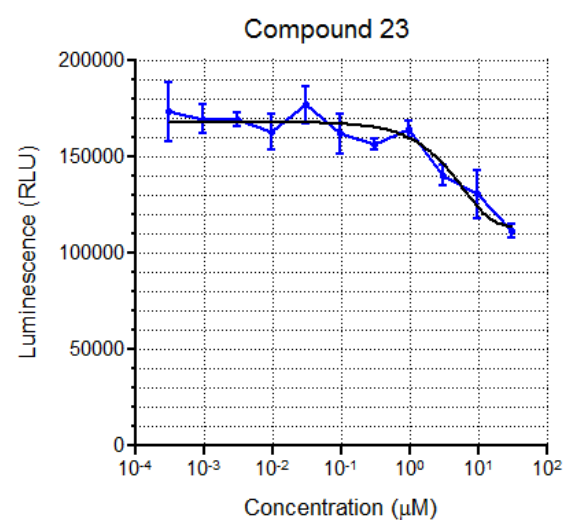
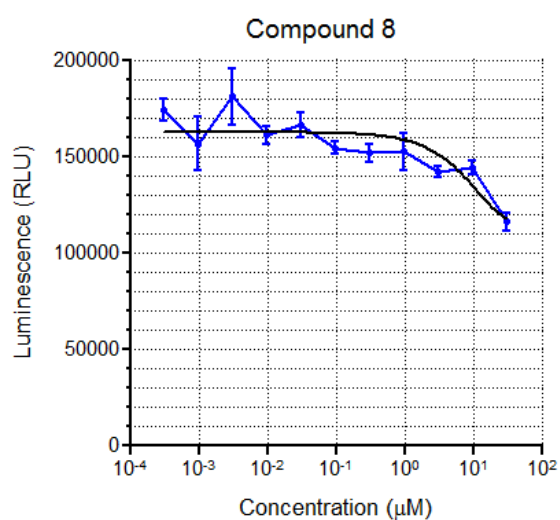
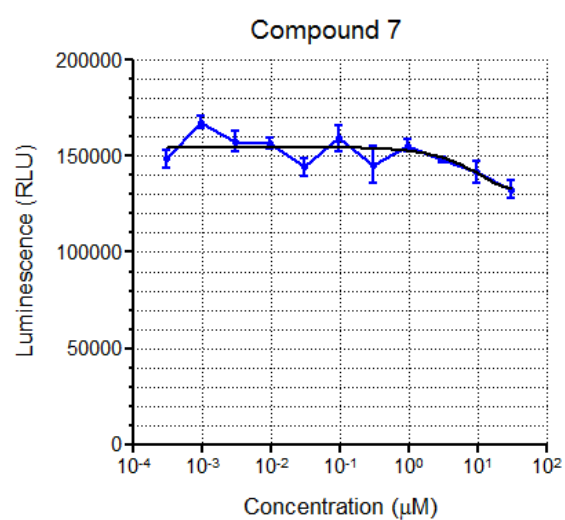
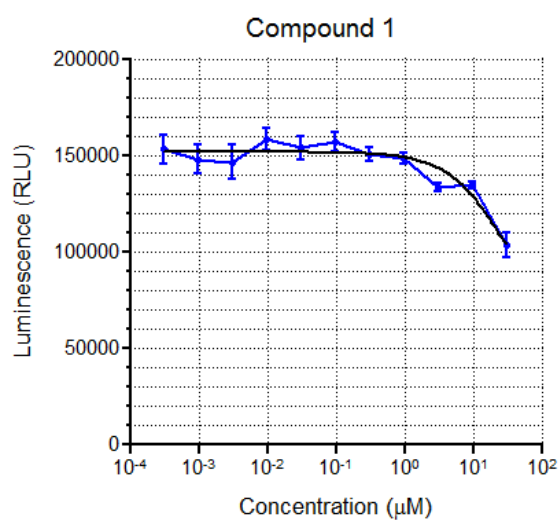
A**B**

Figure 5.10 Pilot screens using a pGL4.22/Empty control cell line. N2a pGL4.22/Empty control line was treated with 80 compounds from a kinase inhibitor library **(A)** and 80 compounds from an epigenetic modulator library **(B)** at 10 μ M for 24 hours. Luciferase assays demonstrate that certain compounds may have non-specific off-target effect on *FLuc* activity. Values are the mean of triplicate readings normalized to 0.3% DMSO control. * * * : represents the level of *FLuc* activity in cells treated with 0.3% DMSO. -----: represents the threshold used to identify positive hits, which was set at $2 \times \text{STDEV}$ from the mean of all compounds). *: represents the compounds identified as positive hits.



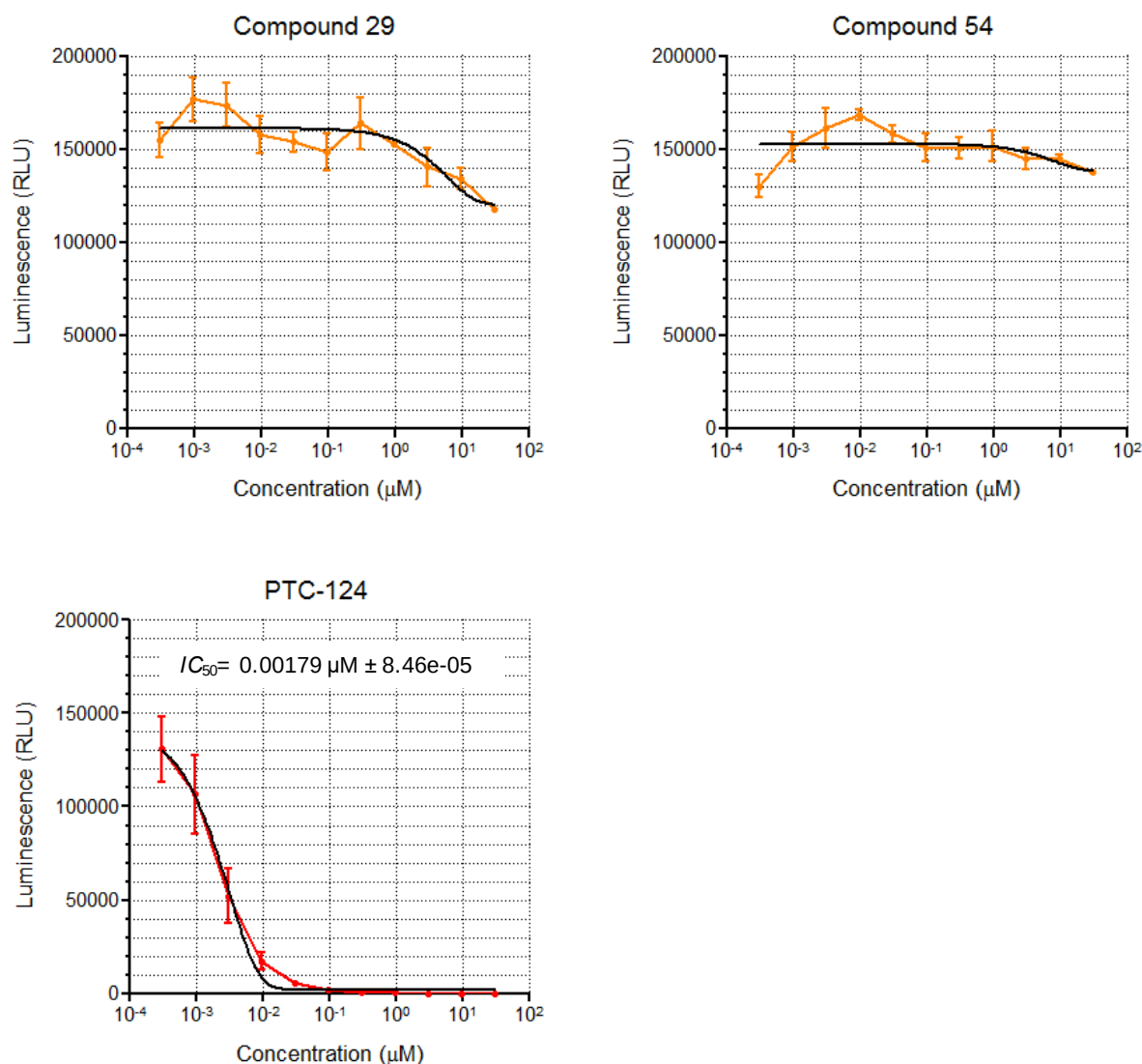


Figure 5.11 Positive hits are not potent FLuc stabilizers. Luciferase assays demonstrate the activity of purified FLuc enzyme, using substrates (luciferin and ATP) at K_m concentrations (10 μM) and increasing concentrations of compound (0.3 nM - 30 μM). Activity of FLuc shows concentration-dependent inhibition with PTC-124 treatment, a known FLuc stabilizer. Each compound concentration was tested in triplicate and values are the mean $\pm$ SEM. Data was fitted to a four-parameter logistic function optimized with least squares regression in order to calculate relative IC_{50} values. Data was obtained from one independent experiment.

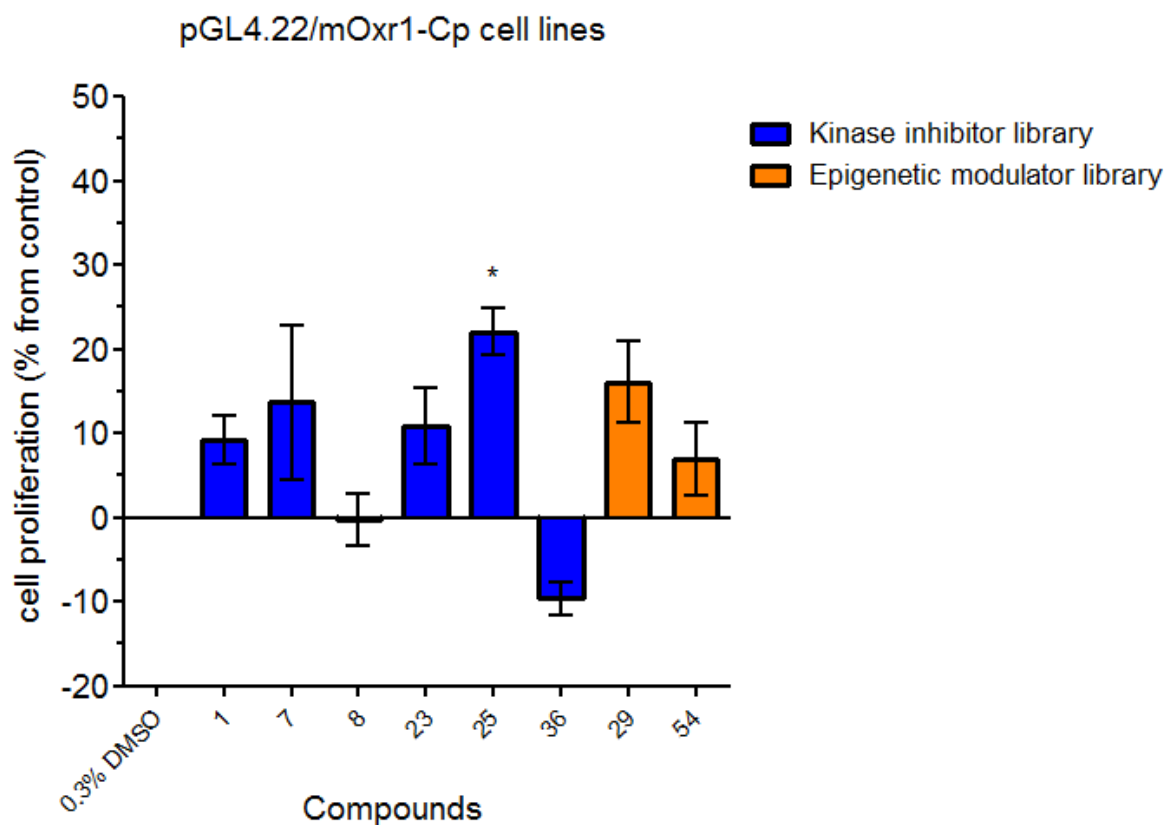


Figure 5.12 Counter screen to determine the effect of compound treatment on cell proliferation. Proliferation assays demonstrate the effect of compound treatment on cell proliferation in all three N2a pGL4.22/mOxr1-Cp cell lines. Cells were treated with compounds in triplicate at 10 μ M for 24 hours. Values are the mean $\pm$ SEM, normalized to 0.3% DMSO control (n=3, one-way ANOVA followed by Dunnett's multiple comparison test compared to 0.3% DMSO, *p<0.05).

Table 5.1 Summary of hits from pilot compound screen and counter screens

Library	Compound	pGL4.22/mOxr1-Cp								pGL4.22/ Empty control line		<i>FLuc</i> Inhibition (<i>IC</i> ₅₀ values; μM)
		Line 1		Line 2		Line 3		Average				
		Increase in luminescence (%)	Increase in cell proliferation (%)	Increase in luminescence (%)	Increase in cell proliferation (%)	Increase in luminescence (%)	Increase in cell proliferation (%)	Increase in luminescence (%)	Increase in cell proliferation (%)	Increase in luminescence (%)	Increase in cell proliferation (%)	
Kinase Inhibitor	1	3	15	24	6	105	7	44	9	72	9	N/A
	7	-21	-4	23	28	115	17	39	14	-38	12	N/A
	8	41	5	23	-6	80	0	48	0	-11	-11	N/A
	23	26	18	26	13	11	2	21	11	-16	7	N/A
	25	-33	17	24	27	65	23	19	22	-14	1	N/A
	36	38	-11	-5	-5	157	-12	63	-9	-45	-38	N/A
Epigenetic modulator	29	-36	16	84	24	39	8	29	16	-47	-17	N/A
	54	128	6	280	15	377	0	262	7	303	-2	N/A

5.4 Discussion

In this chapter, I have shown that there is a highly conserved region immediately upstream of the translation start site of Oxr1-C, which exhibits significant promoter activity. Furthermore, I demonstrated that this promoter region has a number of conserved TFBSs that are critical for its activity. In addition, I have successfully established multiple neuronal cell lines that stably express FLuc under the control of the mouse Oxr1-C promoter region. These cell lines were then used in setting up and optimizing a cell-based luciferase reporter assay in order to carry out a pilot bench-top screen. While my screen originally identified nine compounds suggested to activate the Oxr1-C promoter region and increase FLuc expression, follow-up counter screens were able to identify compounds that may have non-specific off-target effects. Nonetheless, I have established a novel assay that can be further validated and optimized in the future in order to carry out high-throughput screening of compounds that upregulate Oxr1.

Previous work by the Oliver group and work presented in Chapter 4 suggests that upregulation of Oxr1 could be a valuable therapeutic strategy for the treatment of ALS (Finelli *et al.* 2015; Liu *et al.* 2015). However, before considering such strategies, it is important to characterize the regulatory elements of *OXR1*. Here, I conducted a screen demonstrating mutations in Pbx1, Sp1 and Nkx2.5 binding sites significantly alters the activity of the OXR1-C conserved promoter region. This suggests that Pbx1, Sp1 and Nkx2.5 could be essential elements for the regulation of *OXR1-C*, however complimentary chromatin immunoprecipitation sequencing (ChIP-seq) experiments will be important in validating these findings. For example, a ChIP-seq experiment identified hOXR1-FL as a target gene of Pbx1 in ovarian cancer cells by global mapping of Pbx1 binding sites (Thiaville *et al.* 2012). In addition, ChIP-seq data from a cardiac muscle cell line demonstrated that Nkx2.5 likely binds to the promoter of the longest isoform of mouse Oxr1

(NM_001130166; He *et al.*, 2011). More recently, ChIP-seq was used to identify genome-wide binding sites of the transcription factor orthodenticle homeobox 2 (Otx2) in juvenile mouse cortex (Sakai *et al.* 2017). It was not only demonstrated that Otx2 bound to the promoter region of multiple transcripts of Oxr1 (including Oxr1-C), but also that cortical neurons from Otx2-deficient mice displayed a significant downregulation of Oxr1 expression, indicating direct transcriptional regulation (Sakai *et al.* 2017). This also suggests that Otx2 may contribute to the protection of cells against OS-induced damage through transcriptional regulation of Oxr1. Future studies that elucidate these upstream regulatory elements of Oxr1 will provide crucial information for the development of pharmacological therapies that upregulate Oxr1.

Current drug discovery relies heavily on high-throughput screening of chemical libraries against molecular targets of interest in order to find compounds with the desired mechanism of action. Generally, high-throughput screening incorporates two steps: (1) Establishment of a biological detection system; (2) Screening and validation of effective compounds. In this chapter, I established a new luciferase reporter cell-based screening system that I used to screen 160 compounds for their ability to increase Oxr1-C promoter activity. While this was only a pilot screen, I identified a small number of compounds that appeared to increase Oxr1-C promoter activity. The candidate with the most potential, K36, was identified as a positive hit in two of the three reporter lines and passed all of the proposed counter screens, suggesting that the effect I observed was real and not off-target interference. More importantly, these results also confirm that I have a functional assay that is sensitive enough to detect changes in Oxr1-C promoter activity, across multiple cell lines.

Indeed, similar screening approaches have been used previously with great success. One example is within the field of Duchenne muscular dystrophy (DMD) research. DMD is a lethal, X-linked muscle-wasting disease caused by lack of the cytoskeletal protein dystrophin

(Cohn and Campbell 2000). Recently, Guiraud *et al.* studied the ability of a small compound called SMT022357 to increase the transcription of the endogenous mouse utrophin gene (Guiraud *et al.* 2015), which is a structural and functional autosomal paralogue of dystrophin (Love *et al.* 1989). Specifically, they used murine myoblast cells containing a stably integrated 8.4 kb of the human utrophin promoter linked to an FLuc reporter gene and found that treatment with SMT022357 caused a 3-fold increase of in FLuc activity compared to treatment with a vehicle (Guiraud *et al.* 2015). This suggests SMT022357 is able to increase the activity of the utrophin promoter. Another example is within the field of Friedreich's ataxia (FRDA), which is a disease caused by large GAA expansions in intron 1 of the frataxin gene (FXN), resulting in silenced FXN expression (Campuzano *et al.* 1996). Interestingly, Lufino *et al.*, modified BAC vectors carrying the whole FXN genomic DNA Locus by inserting the luciferase gene in exon 5a of the FXN gene and replaced six GAA repeats present in the vector with a ~310 GAA repeat expansion. Luciferase reporter assays were then successfully used in screening a library of novel small compounds, one of which was identified and also found to upregulate FXN expression in FRDA patient primary cells (Lufino *et al.* 2013).

Endogenous gene promoters are positioned in a unique chromatin landscape and contain binding sites for enhancers, repressors and other cofactors, which may be located far away from the promoter (Bonora, Plath and Denholtz 2014). In the two examples of screening approaches provided above (Lufino *et al.* 2013; Guiraud *et al.* 2015), full promoter regions or whole endogenous loci were cloned to ensure a more realistic control of the target gene. Therefore, it may be worthwhile for future studies to also consider testing larger Oxr1 promoter regions to use in luciferase-reporter based compound screens. On the other hand, these conventional transcriptional reporter systems still critically lack the chromatin context and other potential regulatory components of the transcriptional machinery (Kain and

Ganguly 2001). Therefore, a better approach would be to integrate a luciferase reporter cassette directly downstream of the endogenous Oxr1-C promoter using CRISPR/Cas9 genome engineering technology (Sander and Joung 2014; Rojas-Fernandez *et al.* 2015; Liu *et al.* 2017). Alternatively, cell lines can be engineered at the endogenous Oxr1 locus to generate an in-frame luciferase or luminescent peptide protein fusion (Schwinn *et al.* 2018). Only in these reporter systems would all components of endogenous gene regulation, including the native chromatin architecture, be engaged.

Because high-throughput screening requires the ability to test compounds within the range of 10,000 to 100,000 per day (Mayr and Fuerst 2008), I acknowledge additional assay optimization and validation will be needed to upscale from the bench top screen. Assay miniaturization is often employed to create a more high-throughput screening system (Mayr and Bojanic 2009). In this study, I used 96-well plates to handle compounds and perform screens, however the majority of pharmaceutical, biotech and screening centres use 384- or 1536-well plate formats (Mayr and Bojanic 2009). Miniaturization increases throughput and improves variation between wells. Therefore, it may be worthwhile in the future to expand this assay to a 384-well plate format, which can accommodate four times more samples than a 96-well plate format. The goal at this stage would then be to maintain a robust signal detection and achieve acceptable signal-to-background ratios despite the considerable reduction in reagent volume and cell number.

Assay automation is another step that makes high-throughput screening possible (Mayr and Fuerst 2008). The core of the automated system resides in one or more automatic multi-channel liquid handling instruments, which are essential to the production of high-quality data (Zhang *et al.* 2012). In this study, all reagents required for the assay (including compounds) were manually pipetted, which increases assay variation. Therefore, another goal for future screens would be to assess the liquid handling compatibility with the assay.

While most current liquid handling equipment handles the 384-well plate format very well, accuracy and variation of dispensing low volumes of liquid (1–5µl) can also be a concern and should be tested (An and Tolliday 2010). Furthermore, once the new assay has passed the liquid handling validation step, a plate uniformity assessment would be needed in order to test for drift and edge effects, which are more likely to occur in assays with smaller reaction volumes (Zhang *et al.* 2012).

A final factor that will need to be considered when developing this assay further is the quality of the process and reproducibility of data. The most widely accepted measurement of assay quality is the Z' factor, which essentially measures the separation of a positive activity and background control in the absence of intervention of test compounds (Zhang, Chung and Oldenburg 1999; Malo *et al.* 2006). Calculating this measurement requires the use of both positive and negative controls, which are also typically required to assure uniformity from plate to plate, and from screening batch to batch. However, similar to my assay, not all screens have a positive control to accurately measure the phenotypic change a positive hit might produce. In fact, finding those hits is the reason for running a novel screen and if there was a positive control, there would be no reason to run the screen. In these situations, different processing methods are employed in order to normalize raw data. For example, the Z Score, Enhanced Z Score and B Score methods exclude the positive and negative control measurements and instead use the samples themselves as controls under the assumption that the majority of samples are inactive and the data is normally distributed (Malo *et al.* 2006). Such measurements should be calculated in future pilot high-throughput screens in order to fully validate the use of this assay to find up-regulators of Oxr1-C.

While luciferase-based reporter assays are well suited for high-throughput screens due to their high sensitivity, accuracy and robustness, there are a number of limitations, such

as off-target activity, that need to be considered when analysing data. One type of off-target activity occurs because of a direct interaction of a compound with FLuc enzyme. This can inhibit FLuc activity and produce false positive or false negative results in a cell based assay depending on whether the compound is a reversible or irreversible competitive inhibitor (Auld *et al.* 2009). In this chapter, I performed a biochemical enzyme assay to test the ability of selected compounds to inhibit purified FLuc. I found that this screen worked well, evident by the concentration-dependent inhibition of FLuc by the positive control, PTC-124. However, I did not find that any of the selected compounds had similar inhibitory activity against FLuc, which could be a result of the small number of compounds screened. In a titration-based screen of the PubChem library against FLuc, ~360,800 IC₅₀ values were determined from a similar enzymatic assay using Km concentrations of D-luciferin and ATP (Thorne *et al.* 2012). Interestingly, more than 10,000 compounds showed potent concentration-response curves and 168 compounds had potencies <100 nM (Thorne *et al.* 2012). Similar hit rates (~4%) were observed when screening the Novartis compound file at 10µM (Ho *et al.* 2013). These hit rates are considered significant as many HTS assays using FLuc reporters are found to have hit lists which are enriched (up to 98%) with FLuc inhibitors (Auld *et al.* 2008a, 2008b; Thorne, Auld and Inglese 2010). This highlights the importance of counter screening all potential positive hits in future studies using the biochemical assay performed in this study.

With that in mind, the major disadvantage of using the counter screen described above is that it can only deselect interfering compounds without being able to determine if these also have interesting activity. Therefore, an alternative valuable approach would be to develop an orthogonal assay using the same Oxr1-C promoter but a different luciferase reporter. A newer luciferase isolated from the deep-sea shrimp *Oplophorus gracilirostris*, optimized for stability and brightness by Promega, has also been used in compound screens

(Hall *et al.* 2012). The optimized luciferase is called NanoLuc™ due to its small molecular weight (19kDa) and superior brightness. NanoLuc™ inhibitors have been previously identified and there is minimal (~6%) overlap with known FLuc inhibitors (Ho *et al.* 2013). By screening with both reporter systems, one would seek to find compounds that are active in both reporter cell lines as these would likely be true positive hits that increase Oxr1-C promoter activity. At the same time, truly active compounds that were not identified in one reporter line (due to luciferase inhibition) would likely be identified in the other reporter line, which would reduce the number of false negatives. In support of this idea, second-generation coincidence reporter vector systems such as the pNLCoI Vectors by Promega allow expression of both FLuc and NanoLuc™ fused to a PEST destabilization domain (*NlucP*) from the same mRNA transcript. The stoichiometric expression of both luciferases is achieved by use of the P2A sequence from porcine teschovirus-1, which promotes a ribosomal skip and expression of the two unfused enzymes with distinct compound interference profiles. When used in high-throughput compound screening, false hits caused by direct interference with one or the other luciferases can be distinguished from true hits that show a similar response for both, reducing workload associated with follow-up screens.

In conclusion, I have identified and characterised a functional promoter region of Oxr1-C and used this promoter in establishing a new luciferase reporter cell-based assay that can be used to identify compounds that upregulate Oxr1. With additional optimization and validation this assay can be used in a more high-throughput screen in the future. I have also proposed certain counter screens that are necessary in reducing the number of false positive hits thereby increasing the chance of identifying a truly active compound.

Chapter 6

General discussion

6.1 Summary

The main aim of this thesis was to investigate the neuroprotective function of Oxr1 in motor neurons, particularly focusing on its role in modulating disease-relevant phenotypes observed in ALS. In Chapter 3, I found that deletion of *Oxr1* specifically in ChAT-expressing motor neurons did not increase their vulnerability to degeneration *in vivo*, but did however cause a local neuroinflammatory response in the spinal cord of aged mice. In Chapter 4, I demonstrated that Oxr1 binds to TDP-43 *in vivo* and reduces the cytoplasmic mislocalisation of transgenic mutant TDP-43^{M337V} protein in mouse primary motor neurons. In addition, I found that neuronal overexpression of Oxr1 improved motor dysfunction and neuromuscular defects in a novel BAC transgenic TDP-43^{M337V} mutant mouse model of ALS. In Chapter 5, I investigated the transcriptional regulation of Oxr1-C and identified a number of conserved TFBSs in a highly conserved promoter region that were critical for its activity. Furthermore, I established a new cell-based luciferase reporter assay and carried out a pilot ‘bench-top’ screen to identify compounds capable of activating the conserved promoter region of Oxr1-C. Together, this work established Oxr1 as a novel genetic modifier of TDP-43-related pathology *in vivo*, demonstrating the potential for OXR1 to serve as a therapeutic target in ALS and possibly other neurodegenerative diseases. Moreover, this work has also laid the foundations upon which a screening strategy can be further developed and implemented in order to identify compounds that upregulate Oxr1 expression.

6.2 Is Oxr1 essential for the function or survival of other neuronal populations outside of the cerebellum?

Because OS plays a central role in neurodegenerative disorders such as ALS (Lin and Beal 2006), understanding the function of antioxidants and complex antioxidant defence networks will be important for the development of novel therapies targeting these diseases. Highly conserved among eukaryotes, Oxr1 has antioxidant-like properties and can protect cells against OS and OS-induced cell death in a variety of organisms ranging from yeast and nematodes to flies and mice (Volkert, Elliott and Housman 2000; Oliver *et al.* 2011; Kobayashi *et al.* 2014; Sanada *et al.* 2014). Conversely, losing Oxr1 expression can render cells more vulnerable to OS-induced cell death (Oliver *et al.* 2011). This is exemplified in the *bella* mouse mutant, which harbours a spontaneous deletion of the *Oxr1* gene (Oliver *et al.* 2011). These mice display increased levels of oxidative DNA damage and degeneration specifically in cerebellar GCs (Oliver *et al.* 2011). Given that Oxr1 is expressed not only in cerebellar GCs, but throughout the entire CNS, this suggests that it plays an important role in additional neuronal populations as well (Volkert, Elliott and Housman 2000; Durand *et al.* 2007; Finelli *et al.* 2016).

In Chapter 3, I reported that deletion of *Oxr1* specifically in ChAT-expressing motor neurons did not increase their vulnerability to degeneration *in vivo*, suggesting that Oxr1 may be necessary for the function and survival of some neuronal populations but not others. This is supported by additional studies carried out by the Oliver group, which investigated the importance of Oxr1 to the survival of other neuronal populations commonly affected in neurodegenerative disorders. Dopaminergic neurons in the substantia nigra pars compacta are the neuronal population vulnerable in Parkinson's disease (PD) (Braak and Braak 2000). Given that OS is highly implicated in PD pathology (Lin and Beal 2006), it was decided that conditional deletion of *Oxr1* in dopaminergic neurons would be a new and valuable model

for assessing the selective vulnerability of neurons in the midbrain. To specifically delete *Oxr1* in dopaminergic neurons, the conditional-ready *Oxr1* knockout mouse line (*Oxr1^{tm1c/tm1c}*) was crossed to a transgenic line in which the expression of Cre recombinase is controlled by the dopamine transporter (*DAT*) promoter (Zhuang *et al.* 2005). Characterization of *Oxr1^{tm1c/tm1c}/DAT-Cre* mice revealed that these mice displayed no difference in survival and motor function up to 12 months of age, compared to age-matched controls (Wu *et al.*, unpublished work). Furthermore, there was no significant decrease in the survival of dopaminergic neurons within the substantia nigra pars compacta in *Oxr1^{tm1c/tm1c}/DAT-Cre* mice at 18 months of age, compared to age-matched controls (Wu *et al.*, unpublished work). This suggests that specific deletion of *Oxr1* does not lead to the selective vulnerability of dopaminergic neurons to degeneration *in vivo*.

OS is also known to play a key role in the pathology of Alzheimer's disease (AD; Lin & Beal 2006), which is characterized by the degeneration of neurons in the neocortex and hippocampus (Saxena and Caroni 2011). Entorhinal cortex and hippocampal CA1 projection neurons are particularly vulnerable to OS-related insults and degeneration in AD (Saxena and Caroni 2011). However, *Oxr1* expression is highest in the CA3 region of the mouse hippocampus (Finelli *et al.* 2016), suggesting it could have a more important protective function against OS in these neurons. Therefore, the Oliver group also decided to generate a conditional knockout mouse model in which *Oxr1* is specifically deleted in hippocampal neurons in the CA3 region. To do this, *Oxr1^{tm1c/tm1c}* mice were crossed to a transgenic line in which Cre recombinase is conditionally expressed in CA3 hippocampal neurons under the control of the endogenous promoter for glutamate receptor ionotropic kainate 4 (*Grik4*; Nakazawa *et al.* 2011). It was reported that 6-month-old *Oxr1^{tm1c/tm1c}/Grik4-Cre* mice displayed a subtle deficit in short term novelty-preference memory when assessed using the Y-maze test, but did not show any defect in general motor

function or activity (Eastwood et al., unpublished work). Furthermore, there was no significant difference in the survival of CA3 neurons in *Oxr1^{tm1c/tm1c}/Grik4-Cre* mice at 6-months of age, compared to age-matched controls (Eastwood et al., unpublished work). This suggests that specific deletion of *Oxr1* does not lead to the selective vulnerability of CA3 hippocampal neurons to degeneration *in vivo*.

Taken together, these studies suggest that *Oxr1*-depleted cerebellar GCs are more vulnerable to degeneration than *Oxr1*-depleted motor neurons, dopaminergic neurons or CA3 hippocampal neurons. There are a number of possible explanations for this observation, which are described in more detail in Chapter 3.4. However, in summary, it could simply be that: (1) cerebellar GCs have an inherent increased vulnerability to OS, compared to these other neuronal populations; (2) *Oxr1* plays a more important role in cerebellar GCs than in motor neurons, dopaminergic neurons or hippocampal neurons; (3) *Oxr1* is constitutively deleted in *bella* mutant mice, whereas *Oxr1* is specifically deleted in neurons and not in surrounding glial cells in conditional knockout models. Therefore, normal glial function such as trophic support is likely preserved and could contribute to the health and survival of neurons in conditional knockout mice. However, another interesting hypothesis that could potentially explain the differential vulnerability of these *Oxr1*-depleted neuronal subtypes, is that other family members of the TLDc domain-containing proteins could be compensating for the loss of *Oxr1*. It has been demonstrated previously that all TLDc domain-containing proteins are able to confer protection against OS-induced cell death (Finelli et al. 2016). According to the alignment of TLDc domain containing proteins, NCOA7 is the closest family member to OXR1 (Shkolnik et al. 2008). Furthermore, similar to OXR1, NCOA7 exists as a full-length isoform (NCOA7-FL) and a shorter isoform (NCOA7-B; Figure 1.3A; Shkolnik et al. 2008; Finelli et al. 2016). Despite confirming that

Ncoa7 is protective against OS *in vitro*, its precise mechanisms and *in vivo* functions remain unknown.

A recent study investigated the potential role of Ncoa7-FL in distal renal tubular acidosis (dRTA; Merkulova et al. 2018), which is a disorder characterised by metabolic acidosis arising from the inability of intercalated cells in the collecting duct of the kidney to secrete protons or reabsorb bicarbonate from urine (Rodriguez Soriano 2002). This is necessary to maintain systemic acid-base homeostasis at the whole organism level (Rodriguez Soriano 2002). dRTA can be caused by insufficient activity of the vacuolar proton pumping ATPase (V-ATPase; Breton & Brown 2013), which was previously demonstrated to interact with Ncoa7-FL (Merkulova et al. 2015). Therefore, Merkulova et al. hypothesized that loss of Ncoa7-FL function would affect V-ATPase activity in the kidneys *in vivo*, possibly causing dRTA (Merkulova et al. 2018). To test this, *Ncoa7-FL* knockout mice that had been generated as part of the Knockout Mouse Project (<https://www.komp.org/>) were characterised (Merkulova et al. 2018). Interestingly, in contrast to *bella* mice, homozygous *Ncoa7-FL* knockout mice are viable and fertile with normal weight and without any overt behavioural, anatomical or physiological abnormalities (Merkulova et al. 2018), which is consistent with the data obtained during systematic broad-based standardized phenotyping of Ncoa7-FL KO mice performed by the International Mouse Phenotyping Consortium (<http://www.mouse-phenotype.org/>). Furthermore, Ncoa7-FL knockout mice did not develop severe dRTA, despite showing reduced abundance of several V-ATPase subunits (Merkulova et al. 2018). Given that Oxr1-FL is also expressed in the kidney and was found to interact with V-ATPase (Merkulova et al. 2015; Finelli et al. 2016), Merkulova et al. hypothesized that Oxr1 could have been partially compensating for loss of Ncoa7 function (Merkulova et al. 2018).

Importantly, *Ncoa7* is also expressed in spinal motor neurons, CA3 hippocampal neurons and in neurons from the ventral tegmental area and substantia nigra pars compacta, but not in cerebellar GCs in adult mice (Allen Mouse Brain atlas, Entrez ID: 211329, <http://mouse.brain-map.org/gene/show/84287>; Figure 1.3C; Finelli et al. 2016). Therefore, it is entirely possible that *Ncoa7* could be compensating for loss of *Oxr1* in *Oxr1*-depleted neurons in *Oxr1^{tm1c/tm1c}/ChAT-Cre*, *Oxr1^{tm1c/tm1c}/DAT-Cre* and *Oxr1^{tm1c/tm1c}/Grik4-Cre* mice, but not in *Oxr1*-depleted GCs in *bella* mice. This could be another explanation as to why we observe neurodegeneration in *bella* mice but not in any of the *Oxr1* conditional knockout models. In order to investigate this hypothesis, mouse models in which both *Oxr1* and *Ncoa7* are specifically deleted in certain neuronal populations could be generated and characterized to determine whether neuronal vulnerability to OS-induced cell death is increased. Studies such as this could also hopefully shed light on whether loss of function in one TLDC protein could be functionally compensated by another.

6.3 What are the molecular functions of OXR1 and the TLDC domain?

Despite numerous studies consistently demonstrating that overexpression of OXR1 has antioxidant and neuroprotective properties, its molecular functions remain unclear (Oliver *et al.* 2011; Finelli *et al.* 2015, 2016; Liu *et al.* 2015; Finelli and Oliver 2017). However, it is now evident that these protective properties seem to rely on a fully-functional TLDC domain (Finelli *et al.* 2016). This is exemplified by the fact that: (1) An insertional mouse mutant that removes the last 101 amino acids of the TLDC domain of *Oxr1* presented a phenotype that is identical to the recessive *Oxr1* deletion *bella* mouse (Finelli *et al.* 2016); (2) The *bella* phenotype is rescued *in vitro* and *in vivo* by expression of *Oxr1-C*, which mainly consists of the TLDC domain only (Oliver *et al.* 2011). While the three-dimensional structure of the

TLDC domain has been solved (Blaise *et al.* 2012), it lacks structural similarity to known protein folds, giving little indication to how it may function.

It was originally hypothesised that OXR1 might function as an antioxidant protein by reacting directly with ROS, similar to antioxidant enzymes such as SOD1 or glutathione reductase (Massey and Williams Jr 1965; McCord and Fridovich 1969; Oliver *et al.* 2011). Indeed, overexpression of OXR1 can reduce superoxide and hydrogen peroxide levels *in vitro* (Yang *et al.* 2014; Finelli *et al.* 2016). However, it was later demonstrated that OXR1 and the TLDC domain does not possess any enzymatic activity (Oliver *et al.* 2011; Sanada *et al.* 2014). Therefore, it was also hypothesized that OXR1 may influence the expression of key oxygen free-radical scavengers that, in turn, reduce the levels of ROS in the cell. Interestingly, it has been demonstrated that Oxr1 can modulate the expression of several antioxidant genes, such as catalase, heme oxygenase, glutathione peroxidase and p21 (Jaramillo-Gutierrez *et al.* 2010; Oliver *et al.* 2011; Yang *et al.* 2014). How OXR1 can regulate the expression of antioxidant genes remains unclear. There is some evidence for nucleolar and nuclear expression for OXR1 and the *Drosophila* OXR1 homologue mustard (mtd) *in vivo* that could suggest a potential role in transcription (Fischer *et al.* 2001; Natoli *et al.* 2008; Wang, Berkey and Watnick 2012). This is somewhat supported by data demonstrating Oxr1 interacts with several transcriptional regulators such as Hnrnpa1, TDP-43, Fus, cyclin-dependent kinase inhibitor 2A (Cdkn2a) and paired box 6 (Pax6; Finelli *et al.* 2015). Interestingly, it has been demonstrated previously that NCOA7 also has nuclear localisation and is implicated in transcriptional regulation (Durand *et al.* 2007; Arai *et al.* 2008; Paramanik and Thakur 2010). Therefore, given its structural similarity, future studies investigating how OXR1 may influence transcription and whether this occurs by either a direct or indirect mechanism are warranted.

Another functional hypothesis could be that OXR1 can influence OS response pathways indirectly by modulating the activity of effector proteins. This is supported by data collected from an unbiased protein interaction study, which identified that Oxr1 binds to proteins involved in mTOR and EIF2 pathways (Finelli *et al.* 2015), both of which have key regulatory roles in the OS response (Ron and Harding 2007; Teske *et al.* 2011). In addition, as previously mentioned, Oxr1 has also been shown to interact with V-ATPase (Merkulova *et al.* 2015). Interestingly, yeast mutants lacking V-ATPase subunits display increased sensitivity to hydrogen peroxide compared to wild-type cells (Thorpe *et al.* 2004; Milgrom *et al.* 2007). Furthermore, these V-ATPase mutant cells exhibit chronic OS, including elevated ROS levels and protein modifications characteristic of accumulated oxidative damage, even in the absence of exogenous stressors (Milgrom *et al.* 2007). Together, this suggests that V-ATPase activity can help to protect cells from endogenous OS. Therefore, another potential mechanism of Oxr1 in the OS response could be through modulation of V-ATPase activity or stability of the V-ATPase complex. However, it will be important to investigate the relationship between Oxr1 and V-ATPase activity in neuronal cells and determine whether this is a key feature of its neuroprotective properties.

More recently, a novel molecular function of Oxr1 in regulating glucose metabolism in neurons was identified (Finelli *et al.* 2018). Glucose can be processed by oxidative catabolism (glycolysis and the pentose phosphate pathway (PPP)) as well as oxidative phosphorylation in mitochondria (Dashty 2013). Glucose metabolism is essential for the brain as it provides the energy required for cellular function and survival (Mergenthaler *et al.* 2013), and when defective can contribute to neurodegenerative disease (Kapogiannis and Mattson 2011; Cistaro *et al.* 2012; Yamashita *et al.* 2017). Cerebellar GCs are predicted to consume most of the glucose in the cerebellum, suggesting that regulation of energy metabolism in these cells is particularly important (Howarth, Gleeson and Attwell 2012).

Therefore, given that cerebellar GCs are specifically affected in *Oxr1* knockout mice (*bella* and *Oxr1^{d/d}* (mice with constitutive deletion of two exons of the TLDc domain leading to truncation of the protein), Finelli et al. hypothesised that dysregulation of glucose metabolism could be a mechanism driving neurodegeneration in these models (Finelli et al. 2018). Following metabolic profiling from the cerebella of pre-symptomatic P18 *Oxr1^{d/d}* and wild-type littermates, it was revealed that 52 metabolites, especially those in the glycolytic and PPP pathways, were significantly deregulated in *Oxr1^{d/d}* cerebellum (Finelli et al. 2018). This suggests that loss of *Oxr1* causes deregulation of glucose metabolism in the cerebellum.

Interestingly, OS and glucose metabolism are two pathways that are functionally linked. It is well established that some neuronal populations preferentially metabolise glucose via the PPP in order to: (1) reduce the level of ROS produced by oxidative phosphorylation; (2) generate reduced nicotinamide adenine dinucleotide phosphate (NADPH), which provides reducing power in the antioxidant system (Mullarky and Cantley 2015). Therefore, given that *Oxr1* plays a well-established role in protecting against OS (Finelli and Oliver 2017), Finelli et al. measured glycolysis under OS conditions and discovered that cerebellar GCs lacking *Oxr1* do not reduce their glycolytic rate (Finelli et al. 2018), as would be expected in wild-type cells to reduce the overproduction of ROS (Mullarky and Cantley 2015). This further supports that loss of *Oxr1* leads to deregulation of glycolytic metabolism, which is exacerbated under OS.

Importantly, glucose-6-phosphate isomerase (GPI in human/Gpi1 in mouse) is an enzyme that functions at the crossroads of the glycolytic and PPP pathways (Cordeiro et al. 2003), and can also act as a cytokine that stimulates cell proliferation and migration (Watanabe et al. 1996; Zong et al. 2015). GPI can be present as: (1) a monomer, which functions primarily as a cytokine without enzymatic activity (Mizrachi 1989; Tsutsumi et al. 2003; Yanagawa et al. 2005); (2) a dimer, which contains a sugar binding site responsible

for its enzymatic activity (Sun *et al.* 1999; Tanaka *et al.* 2002); (3) a tetramer, which has an unknown function (Yanagawa *et al.* 2005). Interestingly, using co-immunoprecipitation in a neuronal cell line, it was demonstrated that both Oxr1-FL and Oxr1-C bind to Gpi1, highlighting the importance of the TLDC domain for this interaction (Finelli *et al.* 2018). Furthermore, it was also demonstrated that Oxr1-FL can regulate the function of Gpi1 by modulating the ratio of monomeric to oligomeric forms, favouring Gpi1 as a monomer and therefore its activity as a cytokine over its glycolytic role (Finelli *et al.* 2018). This effect was not observed for Oxr1-C, suggesting that the association between Oxr1 isoforms and Gpi1 is complex and may serve different functions (Finelli *et al.* 2018). Nonetheless, together this data proposes another hypothesis in which Oxr1 may carry out its neuroprotective function: by modulating Gpi1 activity in order to inhibit the glycolytic pathway and dampen ROS production. Intriguingly, Ncoa7 and Tbc1d24 were also identified as novel binding partners for Gpi1, suggesting they too may be able to regulate OS and glucose metabolism through modulation of Gpi1 function. As mentioned in section 6.2, there is reciprocal expression of Oxr1 and Ncoa7 in the cerebellum, which is in contrast to their concordant expression in other regions of the CNS (Shao, Halachmi and Brown 2002; Finelli *et al.* 2016). Therefore, in line with the hypothesis stated in section 6.2, this study provides molecular evidence that may explain, at least in part, the specific pattern of cell death in the Oxr1^{d/d} cerebellum and lack of cell death in the Oxr1 conditional knockout models.

In addition to its antioxidant function, previous work by the Oliver group suggests that Oxr1 may also function in other processes such as protein homeostasis (Finelli *et al.* 2015). Impaired protein homeostasis is undoubtedly a critical factor in ALS pathology, with particular focus on the cytoplasmic mislocalisation of TDP-43 protein (Medinas, Valenzuela and Hetz 2017; Gao *et al.* 2018). How mislocalised TDP-43 confers toxicity is still under debate, however evidence suggests that both loss-of-nuclear-function and gain-of-

cytoplasmic-function mechanisms may be involved, likely acting synergistically (Ratti and Buratti 2016). Furthermore, increasing evidence suggests that protein aggregation does not seem to be a necessary step to induce toxicity and drive neurodegeneration (Pastore and Temussi 2012; Arnold *et al.* 2013; Carija *et al.* 2017). In Chapter 4, I demonstrated that Oxr1-FL and Oxr1-C bind to TDP-43 *in vivo*, and overexpression of Oxr1-FL can reduce the cytoplasmic mislocalisation of mutant TDP-43^{M337V} protein in mouse primary motor neurons. The exact molecular mechanisms by which Oxr1 reduces TDP-43^{M337V} mislocalisation is unknown and requires further investigation. Given that TDP-43 and Oxr1 interact *in vivo*, I speculate that Oxr1 modulates TDP-43 localisation either by directly/indirectly altering post-translational modifications found on TDP-43 and/or by restoring correct protein folding. Indeed, previous work by the Oliver group demonstrated that Oxr1 binds to chaperonin containing TCP1 subunit 2 (CCT2), CCT6A, CCT7, DnaJ homolog, subfamily C, member 19 ((DNAJC19/HSP40; Finelli *et al.* 2015), all of which are proteins known to be involved in protein folding (Frydman 2001; Qiu *et al.* 2006; Lopez, Dalton and Frydman 2015). In addition, more recent data from the Oliver group suggests that Oxr1 possesses chaperone activity (Svistunova *et al.* unpublished work). Therefore, future work investigating the role Oxr1 as a potential chaperone that promotes the correct folding of TDP-43 will be important. In addition, experiments that identify post-translational modifications on TDP-43, such as oxidation or acetylation, in the absence/presence of Oxr1 could be useful.

6.4 Role of OXR1 in neuroinflammation and the immune response

In this thesis, I focused on the protective properties of Oxr1 in neuronal cells. However, it is increasingly apparent that Oxr1 may also play an important role in infection

and regulating the immune response (Finelli and Oliver 2017). For example, a gain of function mutation in *mtd* (ortholog of *OXR1* in *Drosophila*) resulted in increased tolerance to oral *Vibrio cholera* infection mediated by the innate immune response (Wang, Berkey and Watnick 2012). Importantly, the mutant *mtd* phenotype could be mimicked by overexpressing a short *mtd* transcript consisting of the TLDc domain only, suggesting that this region was critical for sensitivity to infection (Wang, Berkey and Watnick 2012). Consistent with this finding in *Drosophila*, and supporting a potential role for OXR1 in regulating the immune response in mammals, mouse strains resistant to inflammation of the kidneys express significantly higher levels of Oxr1 than more sensitive strains (Li *et al.* 2014). As a result, overexpression of OXR1 was subsequently tested as a therapeutic strategy in a mouse model of lupus nephritis (Li *et al.* 2014), which is inflammation of the kidneys caused by an autoimmune disease called systemic lupus erythematosus (Mortensen, Fenton and Rekvig 2008). To do this, mesenchymal stem cells (MSCs) expressing OXR1 were delivered intravenously leading to implantation in the mouse kidneys (Li *et al.* 2014). Remarkably, OXR1-MSCs transplantation significantly decreased blood urea nitrogen and ameliorated renal pathology. Furthermore, macrophage and T-lymphocyte infiltration was significantly reduced by inhibiting the expression of inflammatory factors such as Interleukin-1 β , Interleukin 6 and NF κ B (Li *et al.* 2014). Together, these data indicate that Oxr1 is capable of reducing inflammation by regulating the immune response.

Somewhat similar findings have been demonstrated in the SOD1^{G93A} mouse model of ALS. It was shown previously that neuronal overexpression of Oxr1 in SOD1^{G93A} mice reduced the expression of several genes involved in neuroinflammation, including those found in the complement and STAT3 immune pathways (Liu *et al.* 2015). Moreover, this in turn led to decreased astrogliosis and microgliosis in the spinal cord of SOD1^{G93A} mice (Liu *et al.* 2015). In Chapter 4, I demonstrated that TDP-43^{M337V/M337V} mice display a subtle

neuroinflammatory response, characterised by increased staining for astrocytes and microglia in the spinal cord. While this observation was not supported by complimentary transcriptomic data from the spinal cord at the same time point, I also demonstrated that this inflammatory response was not significantly reduced by overexpression of Oxr1. This suggests that the protective effect of Oxr1 overexpression in this model was not likely through the regulation of inflammatory pathways. By contrast, in Chapter 3, I found that Oxr1^{tm1c/tm1c}/ChAT-Cre mice display increased microgliosis in the spinal cord compared to littermate controls, demonstrating that loss of Oxr1 can lead to neuroinflammation. This is supported by observations in Oxr1^{tm1c/tm1c}/Grik4-Cre mice, showing that loss of Oxr1 specifically in the CA3 region of the hippocampus resulted in increased astrogliosis and microgliosis (Eastwood et al. unpublished work). Similar results were also demonstrated in cerebellum and spinal cord of *bella* mice (Liu *et al.* 2015), and transcriptomic data from the cerebella of pre-symptomatic P18 Oxr1^{d/d} mice displayed dysregulation of genes involved in inflammatory pathways (Svistunova et al. unpublished work). Together, these data suggest that expression of Oxr1 is important for the regulation of neuroinflammation and the immune response. Given that neuroinflammatory pathways and neurodegeneration are functionally linked (Cappellano *et al.* 2013), it will be important in the future to investigate in more detail how Oxr1 can modulate the immune response and determine whether this is a critical factor for its neuroprotective function.

6.5 Could Oxr1 be neuroprotective against other genetic causes of ALS?

As new genes have been discovered in recent years, the ALS picture has grown more complex suggesting that there are multiple primary causes of the disease. This has implications when designing therapeutic strategies that are effective for the majority of patients. Up to 70% of all fALS cases are accounted for by mutations in four genes, C9orf72,

SOD1, TARDBP and FUS (Chiò *et al.* 2012b; Millecamps *et al.* 2012). Previous work by the Oliver group and work presented in this thesis has demonstrated the therapeutic potential of Oxr1 for SOD1 and TDP-43-linked ALS (Liu *et al.* 2015; Finelli *et al.* 2015; Chapter 4). Similar to TDP-43, FUS is a ubiquitously expressed, predominantly nuclear DNA/RNA binding protein that binds to thousands of RNA targets and regulates many aspects of RNA biology, such as splicing, transport and translation (Colombrita *et al.* 2011; Brinegar and Cooper 2016). Mutations in FUS account for 5% of fALS and <1% of sALS cases, and are associated with a broad range of clinical phenotypes including some of the most aggressive, juvenile-onset forms of the disease (Kwiatkowski *et al.* 2009; Vance *et al.* 2009; Rademakers *et al.* 2010). Interestingly, of the 50 mutations known in this 526-amino acid multi-domain protein, most cluster near or within the final exon, which disrupts the nuclear localization signal (NLS) resulting in cytoplasmic mislocalisation of FUS (Dormann and Haass 2013; Deng, Gao and Jankovic 2014; Nolan, Talbot and Ansorge 2016). Indeed, post-mortem analysis of brain and spinal cord samples from ALS patients with FUS mutations contain abnormal FUS cytoplasmic inclusions in neurons and glial cells (Kwiatkowski *et al.* 2009; Vance *et al.* 2009; Deng *et al.* 2010; Tateishi *et al.* 2010). Furthermore, these inclusions were reported to be immunoreactive for FUS, HSP70, ubiquitin and ubiquitin-binding protein P62, but strikingly not for TDP-43, implying that neurodegenerative processes driven by FUS mutations are independent of TDP-43 mislocalisation (Vance *et al.* 2009; Suzuki *et al.* 2010; Tateishi *et al.* 2010).

Importantly, previous work by the Oliver group demonstrated that both Oxr1-FL and Oxr1-C bind to wild-type Fus, ALS-associated mutant Fus^{R513C}, and to a lesser extent ALS-associated mutant Fus^{P517L} (Finelli *et al.* 2015). Furthermore, it was also shown that overexpression of Oxr1-C significantly reduced the aggregation of wild-type Fus and Fus^{R513C} in Hela cells under OS. Interestingly, cytoplasmic aggregation of Fus^{P517L} was not

altered by Oxr1-C overexpression (Finelli *et al.* 2015). Taken together, these data suggest that the binding between Oxr1 and Fus is critical for the proper cellular localization of Fus protein. Furthermore, these findings also suggest that Oxr1 could have therapeutic potential for ALS and other neurodegenerative diseases characterized by FUS pathology. Therefore, it would be worthwhile in the future to investigate whether overexpression of Oxr1 could improve pathology and disease-relevant phenotypes in certain mutant FUS mouse models of ALS.

Fortunately, given its significance in the pathophysiology of ALS/ FTD, modelling FUS pathology *in vivo* has been a major research focus. However, reproducing both motor dysfunction and the distinct neuropathological features of FUS-linked ALS has proven challenging in rodents (Nolan, Talbot and Ansorge 2016). Several groups have attempted to drive pathology by inducing pathogenic mutations such as R521C/G in the NLS (Qiu *et al.* 2014; Sephton *et al.* 2014; Sharma *et al.* 2016). For example, a study by Sharma *et al.* used Cre-LoxP to generate mice expressing a single copy of human wild-type FUS, FUS^{R521C} or FUS^{P525L} at the MAPT locus, and an additional knock-out model. As in a previous overexpression model (Mitchell *et al.* 2013), they demonstrated that overexpression of wild-type FUS alone was sufficient to induce neurodegeneration, but that mutant FUS is more pathogenic (Sharma *et al.* 2016). Pathogenicity in these models was also mutation dependent, with FUS^{P525L} mice showing motor neuron specific degeneration at a younger age and a greater degree of cytoplasmic FUS mislocalisation compared to FUS^{R521C} mice (Sharma *et al.* 2016), reflecting the mutation-dependent phenotype of human cases bearing these mutations (Bäumer *et al.* 2010; Mochizuki *et al.* 2012). Furthermore, they also reported significant denervation at the NMJ in both mutant models preceding neurodegeneration, supporting the idea that neuronal loss is a downstream consequence of NMJ denervation caused by such mutations (Sharma *et al.* 2016).

However, given that models such as this have shown that neurons are exquisitely sensitive to FUS gene/protein dose (Mitchell *et al.* 2013; Qiu *et al.* 2014; Sharma *et al.* 2016), it is unclear which aspects of neurodegeneration arise from ectopic overexpression or the effects of FUS mutation. To address this key issue, a more recent study knocked into the mouse *Fus* locus, the human 'FUSDelta14' truncation mutation (Devoy *et al.* 2017), which is associated with early-onset ALS (20 years of age) with a disease course of 22 months to death (DeJesus-Hernandez *et al.* 2010). Interestingly, heterozygous FUSDelta14 mice express mutant FUS at physiological levels, develop progressive motor dysfunction, exhibit motor neuron death and NMJ degeneration and present with diffuse cytoplasmic mislocalisation of mutant FUS protein (Devoy *et al.* 2017). Because models such as these show cytoplasmic mislocalisation of mutant FUS protein, motor neuron degeneration, NMJ denervation and motor dysfunction, they could be used as a tool to determine whether Oxr1 is similarly protective in FUS-linked ALS *in vivo*. However, additional *in vitro* studies investigating possible interactions between Oxr1 and FUS Delta14, FUS^{R521C/G} or FUS^{P525L} would be required before proceeding to genetic rescue experiments using *in vivo* models such as these.

Although the identification of ALS-causing mutations in SOD1, TDP-43 and FUS highlighted pathogenic pathways through which the disease might arise, most of the genetic burden of ALS remained unaccounted for. Then in 2011, a GGGGCC (G4C2) hexanucleotide repeat expansion in intron 1 of the C9orf72 gene was identified as the most frequent genetic abnormality in fALS (>40%), sALS (~20%) and also in inherited FTD (12%; DeJesus-Hernandez *et al.* 2011; Renton *et al.* 2011; Chiò, Borghero, *et al.* 2012; Smith *et al.* 2013). Three mechanisms have been proposed to explain how C9orf72 hexanucleotide expansions confers toxicity: (1) Loss-of-function - The expansion leads to reduced transcription of C9orf72, leading to haploinsufficiency (DeJesus-Hernandez *et al.* 2011); (2)

Gain-of-function - Repeat-containing sense and antisense RNA transcripts accumulate as RNA foci and sequester RNA binding proteins (DeJesus-Hernandez *et al.* 2011; Zu *et al.* 2013; Jovičić *et al.* 2015; Zhang *et al.* 2015); (3) Gain-of-function - Sense and antisense RNA are translated by repeat-associated, non-ATG initiated (RAN) translation into five, potentially toxic, repetitive dipeptide proteins (DPRs; Zu *et al.* 2011; Ash *et al.* 2013; Mori *et al.* 2013; Zu *et al.* 2013). While it seems more likely that the C9orf72 repeat expansions cause toxicity through a gain of function, it is not clear whether this is primarily mediated by expanded RNA or DPR proteins (Moens, Partridge and Isaacs 2017). It is also likely that the different potential mechanisms of toxicity act synergistically in bringing about the disease state (Moens, Partridge and Isaacs 2017).

Interestingly, post-mortem analysis of patients with C9orf72-related ALS display cytoplasmic mislocalisation and aggregation of TDP-43 (DeJesus-Hernandez *et al.* 2011). Furthermore, multiple neuropathological studies have concluded that it is the distribution of TDP-43 pathology that correlates best with the clinical phenotype in C9orf72 patients (Hsiung *et al.* 2012; MacKenzie *et al.* 2013; Mackenzie *et al.* 2015; Davidson *et al.* 2016). This is somewhat supported by studies using transgenic mouse models of C9orf72-related ALS. It was demonstrated by two groups that while BAC transgenic mice carrying an expanded form of the human C9ORF72 gene displayed widespread RNA foci and DPRs, they did not present with TDP-43 aggregation, motor dysfunction or motor neuron degeneration (O'Rourke *et al.* 2015; Peters *et al.* 2015). By contrast, a more recent BAC mouse model of C9orf72 ALS better recapitulated the molecular, behavioural and neurodegenerative features of ALS/FTD, including RNA foci, DPR accumulation, TDP-43 aggregation, motor neuron loss, muscle denervation, gait abnormalities, paralysis and decreased survival (Liu *et al.* 2016). Interestingly, OS and disturbances in microglial function and neuroinflammation have also emerged as potential contributors to ALS that is

associated with mutations in C9orf72 (Lopez-Gonzalez *et al.* 2016; Lall and Baloh 2017). Therefore, given that Oxr1 can protect against OS and regulate neuroinflammation, and given that I demonstrated in Chapter 4 that overexpression of Oxr1 can reduce TDP-43 mislocalisation *in vitro* and protect against motor dysfunction and neuromuscular defects in a TDP-43 mouse model of ALS, future studies investigating the protective potential of Oxr1 in C9orf72-related ALS could be worthwhile.

6.6 Upregulation of OXR1 as a therapeutic strategy

Despite decades of intense research to ultimately find a cure for ALS, there are still only a few treatments available to patients, none of which are effective in slowing disease progression. As the prevalence of neurodegenerative diseases such as ALS increases (Fang *et al.* 2009), novel therapeutic strategies will be increasingly important to alleviate this health burden. Protein aggregation due to impaired protein homeostasis seems to be a common feature in ALS pathology and is therefore an attractive therapeutic target. Most treatments to date have focused on either increasing protein folding to prevent protein aggregation or removal of aggregated proteins (Webster *et al.* 2017). OS is also deemed a major contributor to the pathology of ALS and can influence multiple cellular mechanisms implicated in the disease (Lin and Beal 2006). Therefore, it will be worthwhile in the future to target the causes and consequences of OS for therapeutic intervention as well. It is becoming increasingly evident that Oxr1 is a multifunctional protein that can protect against OS, inflammation and can even reduce the mislocalisation and aggregation of ALS-linked proteins (Chapter 4; Oliver *et al.* 2011; Finelli *et al.* 2015; Liu *et al.* 2015; Finelli & Oliver 2017). Therefore, upregulation of Oxr1 could be a novel and exciting therapeutic strategy for the treatment of

ALS. However, there are a number of questions that need to be answered before this becomes more of a reality.

How can Oxr1 expression be increased? One approach that can be used to manipulate gene expression is to alter the activity of its promoter by pharmacological treatment. In Chapter 5, I identified a highly conserved region immediately upstream of the translation start site of Oxr1-C, which exhibits significant promoter activity. In addition, I successfully established multiple neuronal cell lines that stably express FLuc under the control of this Oxr1-C promoter region. These cell lines were then used in a pilot cell-based luciferase reporter assay in order to identify compounds that activate the Oxr1-C promoter and increase FLuc expression. The pilot screen identified nine compounds that significantly increased Oxr1-C promoter activity, K36 being the most promising candidate. Following further development of this assay into a high-throughput screen, it will be important for future studies to validate potential active compounds found to increase Oxr1-C promoter activity as follows: (1) Determine whether changes in mOxr1-Cp reporter activity truly reflects endogenous Oxr1 mRNA or protein alterations in cultured cell lines or primary motor neurons; (2) Determine the protective effect of selected compounds against pathological phenotypes *in vitro*, previously rescued by neuronal overexpression of Oxr1; (3) Determine compound specificity using transcriptomics to identify other potential targets in neuronal cell lines or primary motor neurons treated with lead compounds; (4) Validate the ability of the selected compounds to cross the blood brain barrier and up-regulate Oxr1 in the mouse brain and spinal cord; (5) Determine the efficacy of selected compounds to improve pathological phenotypes observed in mouse models of ALS, such as the SOD1^{G93A} mouse or the novel BAC TDP-43^{M337V} mouse. In addition, lead compounds can also be tested on their ability to improve molecular and cellular pathology in iPSC-derived motor neurons from ALS patients harbouring the TDP-43^{M337V} mutation. Fortunately, these cells

are now available courtesy of Kevin Talbot's group ((Nuffield Department of Clinical Neurosciences, University of Oxford).

By how much and when should Oxr1 expression be increased? Previous work by the Oliver group and work presented in Chapter 4 used a transgenic mouse line in which Oxr1-FL is overexpressed in neurons from the brain and spinal cord by five-fold as early as E13.5 (Oxr1^{Pmp/+} mice; Liu et al. 2015). This level of overexpression was sufficient to slow disease progression in SOD1^{G93A} and TDP-43^{M337V} mice, indicating the level of Oxr1 needed to potentially have a significant protective effect in humans. However, the timing of Oxr1 overexpression in these studies could be considered problematic because it does not simulate the point at which treatment would begin for ALS patients (during the symptomatic stages of disease). This is somewhat supported by the fact that all antioxidants used in ALS clinical trials have failed to show efficacy, despite showing previous success in pre-clinical studies that treat animal models during the pre-symptomatic stages of disease (Barber and Shaw 2010; Niedzielska *et al.* 2016). Therefore, it may be important in the future to generate an inducible Oxr1 transgenic mouse line using technology such as the Tet-On/Off expression system. Crossing this mouse line with appropriate ALS mouse models will help determine whether switching on and increasing Oxr1 expression after the onset of motor symptoms and/or pathology will also slow disease progression. Furthermore, given that non-cell autonomous mechanisms of neurodegeneration have been implicated in ALS (Chen *et al.* 2018a), and considering the potential role of Oxr1 in modulating neuroinflammation (Liu et al. 2015; Finelli & Oliver 2017; Chapter 3), it may also be worth generating a transgenic mouse model in which Oxr1 is ubiquitously overexpressed in order to determine whether overexpression of Oxr1 in non-neuronal cells as well has an increased protective effect against ALS-like pathology.

Is the therapeutic modulation of Oxr1 safe? It has been demonstrated previously and in Chapter 4 that there is no significant difference in survival or motor function between Oxr1^{Prnp/+} and non-transgenic mice (Liu *et al.* 2015). Furthermore, aged Oxr1^{Prnp/+} mice do not exhibit any signs of motor neuron death, NMJ degeneration or muscle abnormalities, suggesting that neuronal overexpression of Oxr1 *in vivo* does not result in any detrimental pathology (Chapter 4; Liu *et al.* 2015). Consistent with these findings, transcriptomic data presented in Chapter 4 and by Liu *et al.* revealed that there were no significant gene expression changes between non-transgenic and Oxr1^{Prnp/+} mice apart from the expected Oxr1 overexpression from the Oxr1 transgene itself (Liu *et al.* 2015). While further work is needed to confirm whether the proteome or any pathways are dysregulated under these conditions, together these data suggest that overexpression of Oxr1 as a therapeutic strategy would be safe. Following this, it will also be important to consider and test the safety of any potential compounds found to upregulate Oxr1 expression. Therefore, pre-clinical *in vitro* and *in vivo* toxicological testing and safety pharmacology studies will be necessary to determine any potential adverse or lethal side effects of each lead compound.

6.7 Conclusion

The work presented in this thesis demonstrates the neuroprotective effect of Oxr1 against TDP-43-linked pathology, which is applicable to more than 95% of ALS cases. In addition, I have developed a novel screening system that will be used in the future to identify compounds capable of upregulating Oxr1 expression. I believe that by improving our understanding of the molecular mechanisms underlying ALS, particularly pathways that modify disease pathogenesis, it will help provide a framework from which novel therapeutic strategies, such as Oxr1 upregulation, can be developed to treat patients suffering from this debilitating disorder.

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