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The effects of clusterin on the aggregation and pathogenicity of TDP-43, a protein implicated in amyotrophic lateral sclerosis

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The effects of clusterin on the aggregation and pathogenicity of TDP-43, a protein implicated in Amyotrophic Lateral Sclerosis

By

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DECLARATION OF AUTHENTICITY

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the Degree of Doctor of Philosophy. It does not include any material published by another person except where due reference is made in the text. The experimental work described in this thesis is original and has not been submitted for a degree to any other university.

Rebecca Anne Brown

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive and fatal neurodegenerative disease with an international prevalence of approximately 3-7 people per 100,000. The disease is characterised by the selective degeneration of both upper and lower motor neurons, and the presence of insoluble protein aggregates within degenerating neurons and surrounding cells. Typically, pathological changes begin at a focal point of onset and spread radially outwards over time. The pathogenic mechanisms that mediate neurodegeneration in ALS are multifactorial and involve interactions between genetic, cellular and molecular pathways including protein aggregation and disruption to proteostasis, glutamate induced excitotoxicity and the activation of non-neuronal cells.

Transactive response DNA binding protein (TDP-43) is a major component of ubiquitinated inclusions in ALS and mutations in the aggregation prone C-terminal region are associated with disease pathogenesis. Normally a nuclear protein involved in RNA processing and exon splicing, in ALS TDP-43 is depleted from the nucleus and accumulates in the cytosol of motor neurons in insoluble aggregates. The human body has evolved complex protein quality control systems to prevent protein misfolding and limit the accumulation of misfolded or aggregated proteins. Chaperones are the key components of these systems in the extracellular and intracellular spaces, and work by binding to the exposed regions of hydrophobicity on non-native proteins to either assist in folding/refolding or target them for degradation. Studies have reported interactions between proteins involved in ALS and various intracellular chaperones including αB-crystallin, Hsp70 and Hsp40, and Hsp27/25, suggesting that chaperones may influence ALS onset and progression. However the effects of extracellular chaperone action on the disease still remain to be thoroughly elucidated.

Clusterin is a potent extracellular chaperone with broad substrate specificity and can be retrotranslocated from the endoplasmic reticulum (ER) to the cytoplasm under conditions of ER stress. It was previously unknown whether clusterin could inhibit the aggregation of TDP-43, or if it was a constituent of TDP-43 inclusions. Cell-to-cell spread is a “prion-like” (refer to section 1.1 and 1.7) mechanism of protein propagation and studies have shown that TDP-43 and Cu/Zn superoxide dismutase (SOD1) are both capable of such spread in cultured cells. Prion-like mechanisms of protein propagation could explain the outward spread of symptoms observed in ALS. SOD1 has been shown to spread from cell-to-cell *in vivo,* but no published reports describe if TDP-43 is also able to do so. Understanding whether TDP-43 is able to do so would lead to an increased understanding of the mechanisms of protein propagation in ALS, mechanisms which could be targeted in the development of new therapeutic strategies.

This study aimed to examine whether TDP-43 was capable of cell-to-cell spread in cell culture and *in vivo* using a *Drosophila* model, and if so whether the extracellular chaperone clusterin could prevent

this spread. Additionally it was determined whether the expression of TDP-43 in a subset of *Drosophila* glial cells was sufficient to induce motor neuron defects and mortality, and whether the co-expression of clusterin was able to reduce this. The ability of clusterin to inhibit the aggregation of TDP-43 *in vitro* was also tested. Finally, thoracic spinal cord sections from ALS patients were examined via immunohistochemistry to determine if clusterin was a constituent of TDP-43 inclusions, which would be consistent with the two proteins interacting during ALS progression.

Results from both cell-culture and *in vivo* experiments demonstrated that TDP-43 was able to spread from the glial cells where it was originally expressed, to surrounding cells which included motor neurons. Taken together these results suggest that like SOD1, TDP-43 may have prion-like modes of transmission. In *Drosophila* larvae the co-expression of clusterin was able to reduce this spread. Expression of TDP-43 in the glial cells of *Drosophila* caused a significant reduction of lifespan and locomotor defects. While the co-expression of clusterin was not able to extend lifespan, it did result in the preservation of locomotor ability. This effect may have resulted from clusterin inhibiting TDP-43 aggregation, as it was found that clusterin could potently inhibit the aggregation of TDP-$43_{286\text{-}331}$, a synthetic peptide corresponding to residues 286-331 of TDP-43. Finally, clusterin was identified in the cytoplasm of human motor neurons where it was found to be co-localised with aggregates containing TDP-43 in spinal cord tissue from ALS but not control patients. Collectively, the results suggest that clusterin may be involved in preventing the aggregation of TDP-43 in ALS, possibly by interacting with TDP-43 in the extracellular space during prion-like transmission between cells, or in the intracellular space following retrotranslocation of clusterin to the cytosol.

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ABBREVIATIONS

AD	Alzheimer's Disease
ALS	Amyotrophic lateral sclerosis
Ast-1	Murine astrocyte cells
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
Clu	Clusterin
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTF-TDP	C-terminal domain of TDP-43
CTF-TDP-GFP	C terminal region of TDP-43 (amino acids 216-414 only) tagged with GFP
dEAAT1	*Drosophila* glutamate trasnsporter EAAT2
Dlg	Discs large
DMEM	Dulbecco's Modified Eagle Medium
dSOD1	*Drosophila* SOD1
EAAT2	Glutamate trasnsporter EAAT2
EDTA	Ethylenediaminetetraacetic Acid
ELAV	Embryonic lethal abnormal visual system
ER	Endoplasmic reticulum
FALS	Familial amyotrophic lateral sclerosis
FCS	Foetal calf serum
FSC	Forward scatter
GAL80^{ts}	Temperature sensitive GAL80 protein
GFP	Green fluorescent protein
Hsf-1	Heat shock transcription factor

Hsp	Heat shock protein
hTDP-43	Human TDP-43
iPSCs	Induced pluripotent stem cells
JACoP	Just another colocalisation plugin
min	Minute(s)
MND	Motor neuron disease
mRNA	Messenger RNA
MT TDP-43-GFP	M337V TDP-43 tagged with GFP
MT	Mutant
N	Native
N2a	Murine Neuro-2a neuroblast cells
nClu	Nuclear clusterin
NES	Nuclear export signal
NLS	Nuclear localisation signal
NMJ	Neuromuscular junction
NN	Non-native
NSC-34	Murine motor neuron-like hybrid cells
OC	Overlap coefficient
PBS	Phosphate buffered saline
PC	Pearson's coefficient
PDD	Protein deposition disease
PI	Pre-induction
PMI	Post mortem interval
PSCs	Pluripotent stem cells
Q/N	Glutamine/asparagine

Ref #	Reference number
REPO	Glial differentiation factor *reversed polarity*
RFP	Red fluorescent protein
RING	Rapid Iterative Negative Geotaxis
ROS	Reactive oxygen species
RRM	RNA recognition motif
RT	Room temperature
SALS	Sporadic amyotrophic lateral sclerosis
sClu	Secreted clusterin
SEM	Standard error of mean
sHsp	Small heat shock protein
SOD1	Cu/Zn Superoxide dismutase
SSC	Side scatter
TDP-43	Transactive response DNA binding protein
TTR	Transthyretin
UAS	Upstream activation sequence
UPR	Unfolded protein response
WT	Wild type
α_2M	α_2-macroglobulin

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterised by the selective degeneration of both upper and lower motor neurons and the presence of intracellular protein inclusions within degenerating neurons. Approximately 90% of cases are sporadic, with no known genetic linkage, but about 10% of cases are familial. ALS is thought to be caused by a number of non-exclusive pathological mechanisms including mutations in the genes encoding for Cu/Zn superoxide dismutase (SOD1) and transactive response DNA binding protein (TDP-43), glutamate induced excitotoxicity, oxidative stress, mitochondrial dysfunction, impaired axonal transport, and activation of non-neuronal cells. Recently studies have implicated protein aggregation as an underlying cause of disease pathology. Protein aggregates may contribute to ALS pathogenesis and cause neurodegeneration by sequestering proteins vital for normal cellular functions, causing direct damage to organelles, and disrupting proteostasis (protein homeostasis). For many years it was believed that the mechanisms leading to protein misfolding were cell autonomous, with protein misfolding occurring independently in cells. Recently non-cell autonomous mechanisms have been suggested to contribute to disease pathology. Scrapie-associated prion protein (PrP^{Sc}) is an altered form of the normal prion protein (PrP^{C}) that can aggregate and impose its alternative conformation on neighbouring PrP^{C} proteins, acting as a "seed" and causing a chain reaction of misfolding and aggregation (Jucker and Walker, 2013). The term "prion-like" is used to describe mechanisms similar to the self-perpetuating spreading characteristics of the PrP^{Sc} protein (Fernández-Borges *et al.,* 2013). The prion protein will be discussed further in section 1.7. It appears that prion-like mechanisms of self-propagation occur in ALS, with mutant (MT) SOD1 being capable of inducing the misfolding of wild type (WT) natively structured SOD1 and cell to cell spread. Aggregating TDP-43 fragments are also capable of driving co-aggregation of full-length TDP-43 in cell culture.

Proteostasis refers to all the processes that act together to maintain proteins at their correct levels and in their native structures. The human body has evolved complex quality control systems to achieve this, with molecular chaperones being a key component of such systems. Molecular chaperones can fold newly synthesised proteins, re-fold misfolded proteins, and target misfolded proteins for degradation via the ubiquitin-proteasome system and autophagy. In the neurons of patients with ALS, aggregating proteins may overwhelm molecular chaperones and degradative systems, reducing their ability to ensure that proteins are properly folded and misfolded proteins are safely disposed of. Little is known about the effects of chaperones on the protein aggregation that underlies ALS pathology, although it appears that some chaperones are able to inhibit the aggregation of proteins involved in ALS, at least in the early stages of disease. This review presents an overview of ALS with a focus on

protein aggregation, and outlines the evidence supporting “prion-like” propagation of TDP-43 and SOD1. In addition interactions between TDP-43, SOD1 and chaperones are discussed, with particular focus on extracellular chaperones and the role of extracellular proteostasis in ALS.

1.2 PROTEIN FOLDING, AGGREGATION AND DISEASE

The folding of a newly synthesised polypeptide chain into a precise tertiary structure along with the shielding of exposed hydrophobic regions is essential for a protein to become active and stable in the biological environment, as proteins that have failed to fold correctly are associated with a variety of pathological conditions (Dobson, 2003; Stirling *et al.*, 2003). The first step of folding begins with the rapid formation of α-helices and β-sheets, establishing H-bonds between amino acids in the primary amino acid sequence. The protein next undergoes hydrophobic collapse, forming one or more stable intermediates known as "molten globules", with most hydrophobic regions shielded from the aqueous environment, although some remain exposed. Finally, the inner hydrophobic core is rearranged and water is expelled to allow the molecule to adopt the native conformation (Csermely *et al.*, 2003; Cheung *et al.*, 2002; Yang and Gruebele, 2003; Fink, 1999; Fersht, 2000).

1.2.1 Protein Unfolding, Misfolding and Aggregation

Despite the importance of correct protein folding it has been estimated that approximately one third of newly synthesised proteins in eukaryotes do not reach their native conformation, and even native proteins may have low stability when exposed to damaging and denaturing conditions (Schubert *et al.*, 2000; Tan *et al.*, 2009). Furthermore, the exposed hydrophobic regions of proteins in transition states are particularly prone to aggregation in the crowded cellular environment (Stirling *et al.*, 2003; Minton, 2000). Stresses that can result in protein unfolding include denaturing conditions such as extremes of pH and temperature, oxidative stress and mechanical sheer stress, which is of particular concern to extracellular proteins as they are pumped around the body in blood (Dobson, 1999; Davies *et al.*, 1987; Yerbury *et al.*, 2005b). Partially unfolded proteins are energetically unfavourable, and the exposed hydrophobic domains can bind to complimentary regions of other misfolded proteins to form insoluble aggregates that are either toxic to cells or physically disrupt normal body functions. The aggregates formed may be amorphous or have an ordered structure such as in amyloid fibrils (Yerbury *et al.*, 2005b; Yerbury *et al.*, 2009).

1.2.2 Diseases Linked With Protein Aggregation

Protein aggregates are implicated in the pathology of numerous serious human diseases termed “protein deposition diseases” (PDDs) which include age related, systemic, and neurodegenerative diseases (Table 1.1; Wyatt *et al.*, 2009; Yerbury *et al.*, 2007). Neurodegenerative diseases such as ALS, Alzheimer’s disease, Parkinson’s disease, prion disease, and Huntington’s disease all involve

the aggregation and deposition of non-native proteins (Taylor *et al.,* 2002; Chiti and Dobson, 2006). PDDs can involve different types of aggregates occurring both in the intracellular and extracellular environments. For example, Alzheimer's disease involves the amyloid β protein forming amyloid aggregates in the extracellular spaces of the brain, while intracellular inclusions in motor neurons and associated cells are a hallmark of ALS (Chiti and Dobson, 2006).

Table 1.1: Examples of protein deposition diseases. (Wyatt *et al.,* 2009)

Disease	**Protein**	**Type of aggregate**	**Location**
Amyotrophic Lateral Sclerosis	Superoxide dismutase 1	Fibrillar non-amyloid	Intracellular
Alzheimer's Disease	Amyloid-β	Amyloid	Extracellular
Parkinson's Disease	α-Synuclein	Fibrillar non-amyloid	Intracellular
Type II Diabetes	Human islet amyloid polypeptide	Amyloid	Intracellular and Extracellular
Reactive Amyloidosis	Amyloid-α	Amyloid	Extracellular
Huntington's Disease	Huntingtin	Fibrillar non-amyloid	Intracellular
Creutzfeld-Jakob Disease	Prion Protein	Amyloid	Extracellular
Primary Systemic Amyloidosis	Immunoglobulin light chain	Amyloid	Extracellular
Secondary Systemic Amyloidosis	Serum amyloid- β	Amyloid	Extracellular
Corneal dystrophy	Immunoglobulin G	Amorphous	Extracellular
Age-related Macular Degeneration	62 different proteins	Amorphous	Extracellular
Renal Disease	Tamm-Horsfall protein Osteopotin	Amyloid	Extracellular

1.3 MAINTAINING PROTEOSTASIS

1.3.1 The Role of Protein Quality Control

A healthy human body synthesises approximately 188 g of protein per day (Norton *et al.*, 1981). It is obvious then that some form of quality control system must exist to prevent misfolding, limit accumulation of unfolded and aggregated proteins, dispose of any denatured proteins or aggregates, and generally ensure the efficient synthesis and maintenance of proteins (Buchner, 1996; Kaufman, 2002). Molecular chaperones are the key component of this system in both the intra- and extracellular environments. Molecular chaperones are conserved families of proteins that can prevent irreversible aggregation and keep proteins on the correct folding pathway by selectively binding to exposed hydrophobic domains on non-native proteins. Often working in tandem, the folding of most newly synthesised proteins will involve interaction with one or more molecular chaperones (Buchner, 1996; Fink, 1999; Dobson, 2003). Chaperones act as catalysts, interacting with the substrate protein and increasing the efficiency of the folding process by reducing the chance for competing reactions. Chaperones can either assist in the folding/refolding of proteins in an active ATP-dependent process, or work in a passive ATP-independent mode, simply holding non-native proteins in a stable state competent for future refolding, or targeting them for digestion via lysosomes or proteasomes (Csermely *et al.*, 2003; Fink, 1999).

1.3.2 The Intracellular Protein Quality Control System

In the intracellular environment a non-native or aggregated protein is handled in one of three ways (Figure 1.1). They may be rescued by chaperones, degraded, or form an aggregate. Proteins destined for degradation are directed from either the ER or cytosol to the lysosome (via vesicle transport or chaperones) or proteasome (via retrotranslocation or chaperones) for degradation. Proteins that reach the Golgi apparatus and remain misfolded are recognised as abnormal and may be sent back to the endoplasmic reticulum (ER) for immediate ER-associated degradation via proteasomes or lysosomes (Hong *et al*., 1996; Shamu 1998; Brodsky and McCracken, 1999; Arvan *et al*., 2002; Kaufman, 2002).

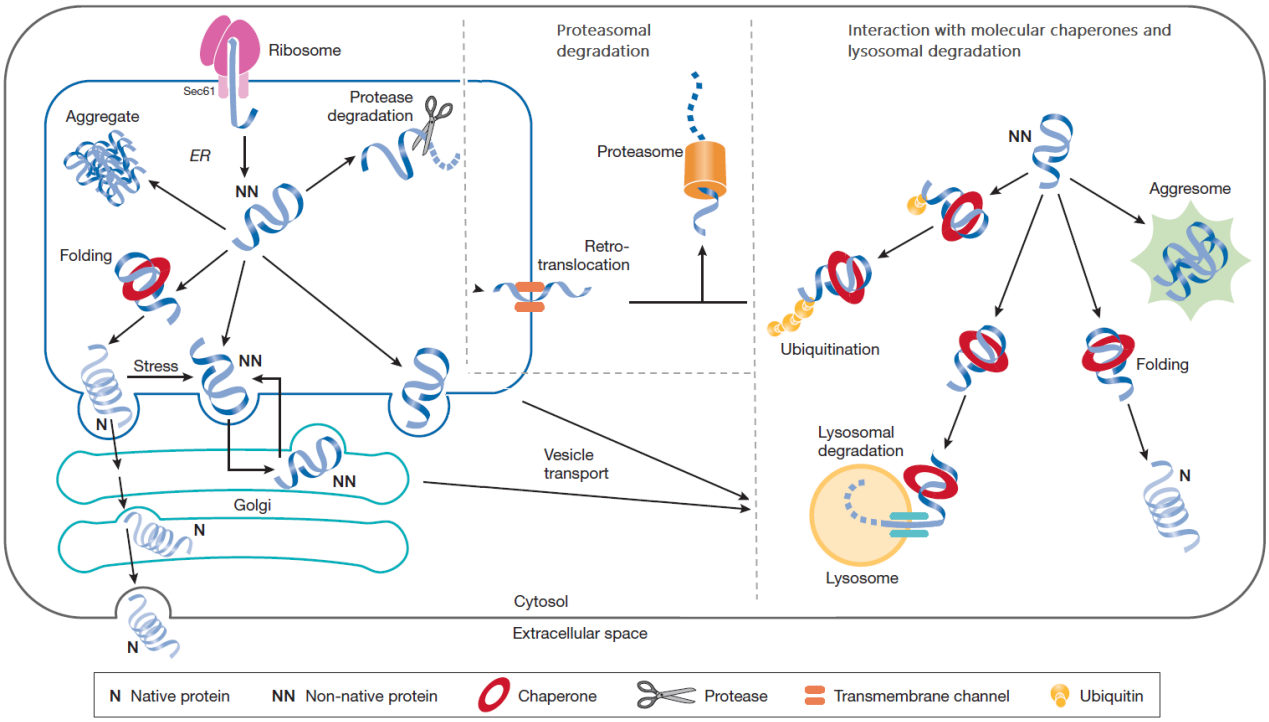

Figure 1.1: Summary of the intracellular mechanisms of protein quality control. (Modified from Yerbury *et al.*, 2005b). Non-native proteins may i) interact with molecular chaperones which either assist with refolding or direct them for proteolysis, ii) be transported from the ER to the lysosome via vesicles for degradation, or iii) be retrotranslocated from the ER to the ubiquitin-proteasome system in the cytosol. When these quality control systems fail the non-native protein may accumulate in the endoplasmic reticulum or cytosol.

The major classes of intracellular chaperones are the heat shock proteins (Hsps) and small heat shock proteins (sHsps). Many of the sHsps are upregulated during stress conditions and prevent aggregation by binding to exposed hydrophobic regions of stressed proteins in an ATP-independent process (Fink, 1999; Buchner, 1996; Hartl and Hayer-Hartl, 2002). Other heat shock proteins such as Hsp70 use ATP to facilitate folding. Hsp70 works in tandem with Hsp40, a protein which can directly interact with unfolded polypeptides, recruit Hsp70 to protein substrates, and activate the Hsp70 ATPase (Arawaka *et al.*, 2010). Molecular chaperones are the cornerstone of the protein quality control system, and when their capacity is exceeded or they can no longer perform their function, proteins can aggregate and form insoluble inclusions, resulting in disease (Kopito, 2000; Yerbury *et al.*, 2005b). Both Hsp70 and 27 have been found associated with cytosolic inclusions present in motor neurons in ALS, and over expression of both these chaperones can provide some protection against

neurodegeneration (Maatkamp *et al.,* 2004; Patel *et al.,* 2005). Chaperones and ALS will be discussed further in section 1.9 of this chapter.

1.3.3 The Extracellular Protein Quality Control System

Despite intracellular quality control, some non-native proteins are secreted and enter the extracellular space, yet under normal conditions aggregates do not accumulate and cause disease. Evidence suggesting the existence of an extracellular protein quality control system arose from research conducted in the 70's, 80's, and 90's. Margineanu and Ghetie (1981) found that *in vivo* non-native proteins were degraded more rapidly that native proteins, and later Senior *et al*. (1991) showed that liposomes with exposed hydrophobic domains on their surface were cleared from circulation more rapidly than those with a hydrophilic outer layer. While these experiments suggested the existence of an extracellular proteostasis system, the components of such a system had long remained a mystery. Extracellular proteolysis of misfolded proteins was thought unlikely, as the proteasome is 300 times less abundant in plasma than inside cells and requires ATP, which is 1000 times less abundant in the extracellular environment. Intracellular chaperones could also be excluded as their concentrations in the extracellular environment are in the ng/ml range, and their chaperone capacity would be quickly overwhelmed during times of stress (Wilson *et al.,* 2008a). The recent discovery that the abundant extracellular proteins clusterin, haptoglobin, α_2-macroglobulin (α_2M) and serum amyloid P component (Humphreys *et al.,* 1999; Yerbury *et al.,* 2005a; French *et al.,* 2008; Koistinaho *et al.,* 2004; Yang *et al.,* 1992; Zsila, 2010) all have chaperone-like activity suggests that these may play important roles in extracellular proteostasis. Extracellular chaperones such as clusterin have been shown to ATP-independently inhibit aggregation by binding to exposed hydrophobic regions of misfolded proteins to form high molecular mass complexes, in an action similar to that of the sHsps (Humphreys *et al.,* 1999; Poon *et al.,* 2000; Yerbury *et al.,* 2007; Yerbury *et al.,* 2005b; French *et al.,* 2008). In 2005(b), Yerbury *et al*. put forward a theoretical model for extracellular protein quality control (Figure 1.2). In this model, extracellular chaperones bind to exposed hydrophobic regions on non-native proteins to form stable complexes. These chaperone-client complexes are internalised via receptor-mediated endocytosis where they are directed by vesicle trafficking to the lysosome for degradation. Cell surface receptors are known that bind to clusterin, haptoglobin, and α_2M, consistent with this model (Graversen *et al.,* 2002; Sottrup-Jensen, 1989; Wilson *et al.,* 2008b).

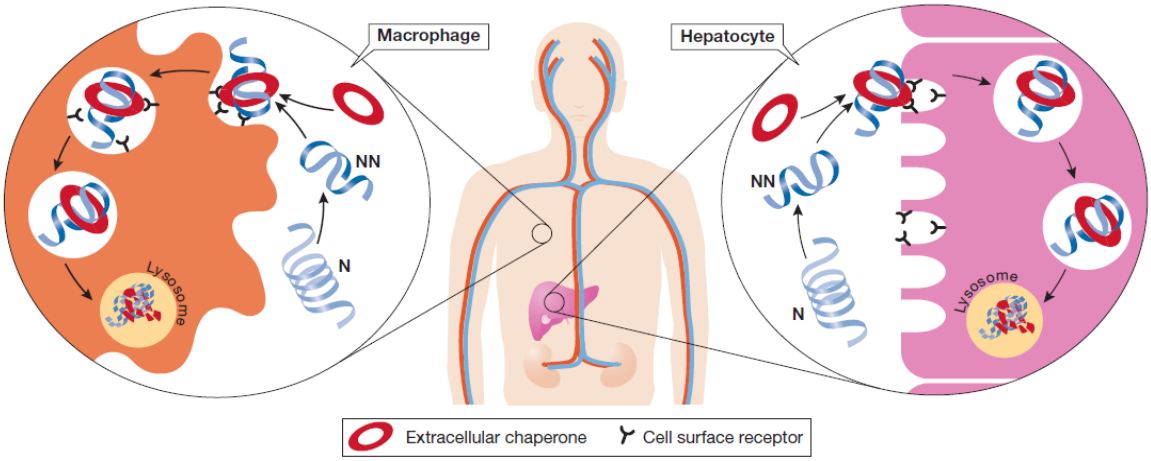

Figure 1.2: Summary of the proposed extracellular mechanisms of protein quality control. (Yerbury *et al.*, 2005b). Extracellular chaperones bind to exposed hydrophobic regions on non-native proteins to form stable complexes. These chaperone-client complexes are internalised via receptor-mediated endocytosis where they are directed by vesicle trafficking to the lysosome for degradation. This system is particularly important in the liver and macrophages. N = native protein. NN = non-native protein.

Clusterin is a conserved heterodimeric glycoprotein of 75-80 kDa that is abundant in tissue fluids such as plasma, semen, milk, urine and cerebrospinal fluid. Under normal conditions the chaperone is secreted from the ER into the extracellular space, with expression upregulated in response to various cellular stresses and diseases. Clusterin expression is generally associated with cell survival. Recently however, it has been shown that under conditions of ER stress, clusterin is retrotranslocated from the secretory system to the cytosol, where it may act as an intracellular chaperone as well as influence DNA repair, transcription and apoptosis (Nizard *et al.*, 2007; Li *et al.*, 2013; Yang *et al.*, 2000; Santilli *et al.*, 2003; Debure *et al.*, 2003). ER stress has been implicated in the pathology of ALS and ER stress signals have been observed in ALS cases and transgenic SOD1 mice (Atkin *et al.*, 2006; Tobisawa *et al.*, 2003; Kikuchi *et al.*, 2006; Saxena *et al.*, 2009). Clusterin will be discussed in more detail in the following chapters of this thesis.

1.4 AMYOTROPHIC LATERAL SCLEROSIS (ALS)

1.4.1 Overview

ALS is a progressive and fatal neurodegenerative disease first described in 1869 by the French neurologist Jean-Martin Charcot (Charcot and Joffroy, 1869). The disease is characterised by the selective degeneration of upper and lower motor neurons in the brain, brainstem and spinal cord (Figure 1.3), resulting in a progressive decline in muscle function, eventual paralysis of voluntary muscles and ultimately death within 2-5 years of symptom onset, usually due to respiratory failure (Mitchell and Borasio, 2007). There is currently no cure for ALS, and treatment is limited to palliative care aimed at improving the quality of life for patients.

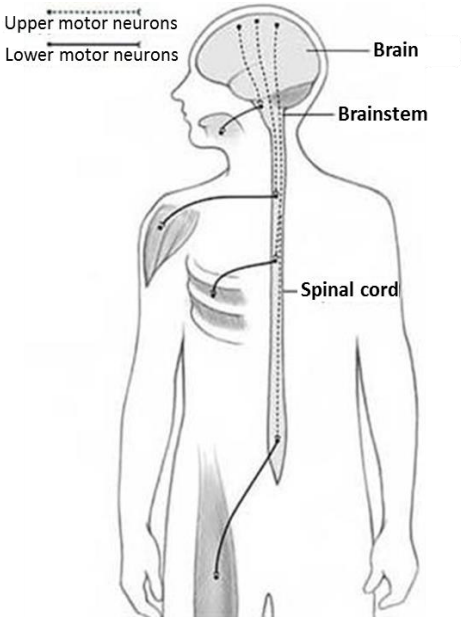

Figure 1.3: Upper and lower motor neurons. Upper motor neurons originate in the brain and connect the brain to the spinal cord. Degeneration of these neurons results in difficulty swallowing, shortness of breath and difficulty speaking. Lower motor neurons connect the brainstem and spinal cord to muscle fibres. Degeneration of these neurons results in muscle weakness and atrophy, involuntary contraction of muscle fibres, flaccidity, difficulty swallowing, and respiratory difficulties (BPCC/NSU Title III Cooperative Project, 2011).

ALS has an international prevalence of approximately 3-7 people per 100,000 (Benatar *et al.,* 2006). The incidence of ALS increases after the age of 40, peaks at ~ 55, and decreases rapidly after 80 years of age. While the disease was initially observed to be more common in males, recent studies suggest

that the male:female gender ratio is reaching equality (Swingler *et al.*, 1992; Traynor *et al.*, 1999; Ragonese *et al.*, 2012; Logroscino *et al.*, 2008). The majority of ALS cases are sporadic (90%) with no known genetic linkage, but approximately 10% of ALS cases are dominantly inherited (referred to as familial ALS). Familial (FALS) and sporadic (SALS) ALS are clinically indistinguishable (Cozzolino *et al.*, (2008). A significant step towards understanding the mechanisms underlying ALS occurred in 1993 when it was discovered that mutations in the gene encoding SOD1 were associated with ALS (Rosen *et al.*, 1993). Since then mutations causing a clinical phenotype have been identified in several other genes, including *FUS* (encodes fusion in sarcoma; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009), *OPTN* (encodes optineurin; Maruyama *et al.*, 2010), the chromosome 9 open reading frame 72 gene (*C9ORF72*; Renton *et al.*, 2011; DeJesus-Hernandez *et al*., 2011) and *TARDBP* (encodes for TAR DNA binding protein; TDP-43; Gitcho *et al.*, 2008; Sreedharan *et al.*, 2008; Van Deerlin *et al.*, 2008; Corrado *et al.*, 2009; Daoud *et al.*, 2008). While gene mutations are associated with ALS pathology, they are not the sole cause of the neurodegeneration process as there are multiple pathogenic mechanisms that may contribute.

1.4.2 Pathogenic mechanisms that may contribute to neurodegeneration

For more than a century after ALS was first described the cause of the disease remained unknown. Viruses, metals, immune dysfunction, endogenous toxins, endocrine abnormalities, trauma/injury and surgery have all been suggested to play roles in the pathogenesis of ALS, but convincing evidence supporting these hypotheses has not been demonstrated (Tandan and Bradley, 1985; Harwood *et al.*, 2009). Current understanding is that the pathogenic mechanisms mediating neurodegeneration in ALS are multifactorial and involve interactions between genetic, cellular and molecular pathways (Figure 1.5; Kiernan *et al.*, 2011). Such pathogenic mechanisms are outlined below and may include genetic factors, protein aggregation and disruption to proteostasis, glutamate induced excitotoxicity, oxidative stress, mitochondrial dysfunction, impaired axonal transport, and the activation of non-neuronal cells.

1.4.2.1 Genetic factors

SOD1 is a copper and zinc containing enzyme, abundant in motor neurones, which is located predominantly in the cytosol, lysosomes, nucleus and mitochondrial intermembrane. Its function is to catalyse the dismutation of superoxide radicals into hydrogen peroxide, providing a first line of defence for the body against damage by ROS (Dong *et al.*, 2006; Sturtz *et al.*, 2001). Rosen *et al.* identified 11 different SOD1 missense mutations in 13 different FALS families (1993). Since this discovery over 350 more SOD1 mutations have been identified at over 40 different locations scattered throughout the protein. The majority of these mutations are missense mutations, resulting in the substitution of one single amino acid by another, but a smaller number cause frame shifts, truncations, deletions or insertions (Cleveland and Rothstein, 2001). Mutations in SOD1 occur in about 20% of

FALS cases and 5% of SALS cases (Rosen *et al.,* 1993; Kiernan *et al.,* 2011). Identification of these SOD1 mutations raised the question of how they might selectively cause motor neuron degeneration; did mutations cause a loss of normal enzyme function or a toxic gain of function? Several experiments using transgenic SOD1 mice supported the toxic gain of function theory. In 1994 Gurney *et al*. showed that transgenic mice expressing MT SOD1 with normal activity levels still developed ALS. In addition mice in which SOD1 was completely depleted lived normal life spans without developing ALS (Reaume *et al.,* 1996). Mutations in SOD1 may cause a toxic gain of function by causing the protein to unfold and form intracellular aggregates which can inhibit normal cellular functions.

More recently mutations in the gene encoding the TAR-DNA binding protein TDP-43 have been linked to FALS and SALS (Gitcho *et al.,* 2008; Sreedharan *et al.,* 2008; Van Deerlin *et al.,* 2008; Corrado *et al.,* 2009; Daoud *et al.,* 2008; Yokoseki *et al.,* 2008). TDP-43 has been recognised as a major component of cytoplasmic protein aggregates in SALS, and TDP-43 mutations occur in about 3% of FALS and 1.5% of SALS cases, suggesting that TDP-43 plays a role in ALS pathogenicity (Neumann *et al.,* 2006). While TDP-43 has been implicated in ALS pathology, it is unclear how mutations cause neurotoxicity. The precise cellular functions of TDP-43 are currently unknown, therefore it is difficult to determine if mutations cause neurodegeneration via a loss of function or a toxic gain of function. The pathogenicity of TDP-43 in ALS will be discussed further in section 1.6**.** In addition to SOD1 and *TARDBP*, mutations in *FUS***,** *OPTN*, *ANG* (encodes angiogenen; Greenway *et al.,* 2006), *UBQLN2* (encodes the ubiquitin-like protein ubiquitin 2; Deng *et al.,* 2011) and *C9ORF2* (Renton *et al.,* 2011; DeJesus-Hernandez *et al*., 2011) have been found associated with ALS. The discovery of hexanucleotide repeat expansions in *C9ORF72* was a particularly important discovery, as these mutations occur 1-2 fold more frequently than SOD1 mutations, and account for ~ 34.2% of FALS cases and 5.9% of SALS cases, making them the major genetic cause of ALS worldwide (Cruts *et al.,* 2013; van Blitterswijk *et al.,* 2012).

1.4.2.2 Protein aggregation and disruption to proteostasis

In recent years evidence has accumulated to suggest that protein aggregation and disruption to proteostasis is an underlying cause of ALS pathogenesis. Insoluble cytoplasmic inclusions in the brain and spinal cord are a hallmark of FALS and SALS, and many different proteins have been identified as associated with these inclusions including TDP-43, chaperones, ubiquitin, proteasome components and SOD1 (Watanabe *et al.,* 2001; Bergemalm *et al.,* 2010; Neumann *et al.,* 2006). There are four non-exclusive theories of how aggregating proteins may contribute to ALS pathogenesis and cause neurodegeneration.

1) Aggregating proteins may sequester other proteins that are vital for normal cellular functions, causing them to be depleted, inactivated, or wrongly activated. In support of this theory several proteins have been found to be associated with SOD1 aggregates including neuronal glutamate transporters, copper chaperone for SOD1 (responsible for activating SOD1 via copper insertion) and the anti-apoptotic protein B-cell lymphoma 2 (Bergemalm *et al.,* 2010; Pasinelli *et al.,* 2006, Watanabe *et al.,* 2001).

2) Aggregating proteins may overwhelm molecular chaperones, reducing their availability to ensure proteins are properly folded and misfolded proteins are safely disposed of. In transgenic SOD1 mice the small heat shock protein αB-crystallin is the most abundant protein (besides SOD1) found associated with cytoplasmic inclusions, and the spinal cord of transgenic mice have lower chaperone activity compared to healthy mice (Wang *et al.,* 2005; Bruening *et al.,* 1999).

3) Aggregating proteins could cause direct damage to organelles, for example by physically damaging the mitochondria by aggregating on or within the organelle (Cozzolino *et al.,* 2008).

4) Aggregating proteins may overwhelm the ubiquitin-proteosome and autophagy systems, preventing the degradation of misfolded proteins (Allen *et al.,* 2003; Johnston *et al.,* 1998). As previously mentioned, misfolded and unfolded proteins can impair the function of the ER and cause ER stress, and are thus targeted for immediate ER associated protein degradation.

Recent studies have implicated ER stress in the pathology of ALS. ER stress signals and up-regulation of the unfolded protein response (UPR) have been observed in ALS cases (Atkin *et al.,* 2006; Turner *et al.,* 2006; Tobisawa *et al.,* 2003; Kikuchi *et al.,* 2006) and transgenic SOD1 mice (Kikuchi *et al.,* 2006; Saxena *et al.,* 2009). In addition a study recently provided the first morphological evidence that ER stress may be involved in ALS pathogenesis (Sasaki, 2010). Using electron microscopy, accumulations of amorphous and granular material and mis/unfolded proteins were observed in the ER of neurons from ALS patients, deposits which may trigger activation of the UPR.

1.4.2.3 Glutamate induced excitotoxicity

Another potential contributor to ALS pathogenesis is glutamate induced excitotoxicity. Glutamate is a neurotransmitter in the central nervous system which is released from pre-synaptic terminals and binds to several glutamate receptors on the post-synaptic membrane, stimulating an influx of Na^+ and Ca^{2+} ions into neurons resulting in an action potential. Glutamate is normally quickly removed from the synaptic cleft by excitatory amino acid transporters (EAATs; Figure 1.4A). Dysfunction or dysregulation of these transporters can lead to excessive glutamate induced stimulation of receptors, triggering an increased concentration of Na^+ and Ca^{2+} ions within motor neurons, which alters calcium homeostasis and results in neuronal injury and glutamate induced excitotoxicity (Shaw, 2005;

Pasinelli and Brown, 2006; Figure 1.4B). Increased levels of glutamate have been found in the cerebrospinal fluid of many patients with SALS (Rothstein *et al.,* 1990). It is thought that these increased levels result from the loss of the glutamate transporter EAAT2. Under normal conditions, the EAAT2 protein is mainly expressed in astrocytes; it is the most abundant transporter in mammals and is responsible for up to 80-90% of all extracellular glutamate uptake. Rothstein *et al*. (1995) reported that approximately 60-70% of ALS patients have a 30-95% loss of the EAAT2 protein in the motor cortex and spinal cord, supporting this hypothesis. Loss of glutamate transporters has also been reported in the SOD1 mouse and rat models of ALS, where loss of the EAAT2 protein is associated with neuronal loss (Bendotti *et al.,* 2001; Howland *et al.,* 2002). Strategies aimed at restoring the levels and function of transporters have been successful in mouse models and may represent a potential future therapeutic strategy for the treatment of ALS. For example transgenic SOD1 mice that overexpressed EAAT2 showed a delayed onset of symptoms (Guo *et al.,* 2003). Additionally high throughput screening has identified compounds that can increase EAAT2 protein through translational activation, resulting in increased transporters on glial cell surfaces, and treatment of SOD1 mice with one such compound resulted in restoration of EAAT2 expression, a significantly delayed loss of motor function and prolonged overall survival (Kong *et al.,* 2014). Expression of EAAT2 could also be increased at the transcriptional level (Lin *et al.,* 2012). Studies have demonstrated that in ALS TDP-43 may be able to directly or indirectly regulate mRNA levels of glutamate transporters, as the mRNA of EAAT2 has been identified as a potential TDP-43 target (Tollervey *et al.,* 2011). Both the loss and overexpression of *Drosophila* TDP-43 can alter mRNA levels of the fly homologue of EAAT2 (Diaper *et al.,* 2013a). Riluzole is currently the only approved drug available for the treatment of ALS, and works by inhibiting the release of glutamate from neurons and inhibiting glutamate receptors. However this drug only prolongs survival by approximately 2-3 months. Due to the multiple pathogenic mechanisms that contribute to ALS pathology, it is unlikely that therapeutic strategies targeting glutamate induced excitotoxicity alone will be sufficient to successfully treat the disease. It may be that multiple mechanisms must be targeted for treatments to be beneficial to patients. Thus it is important that every mechanism contributing to ALS is thoroughly understood.

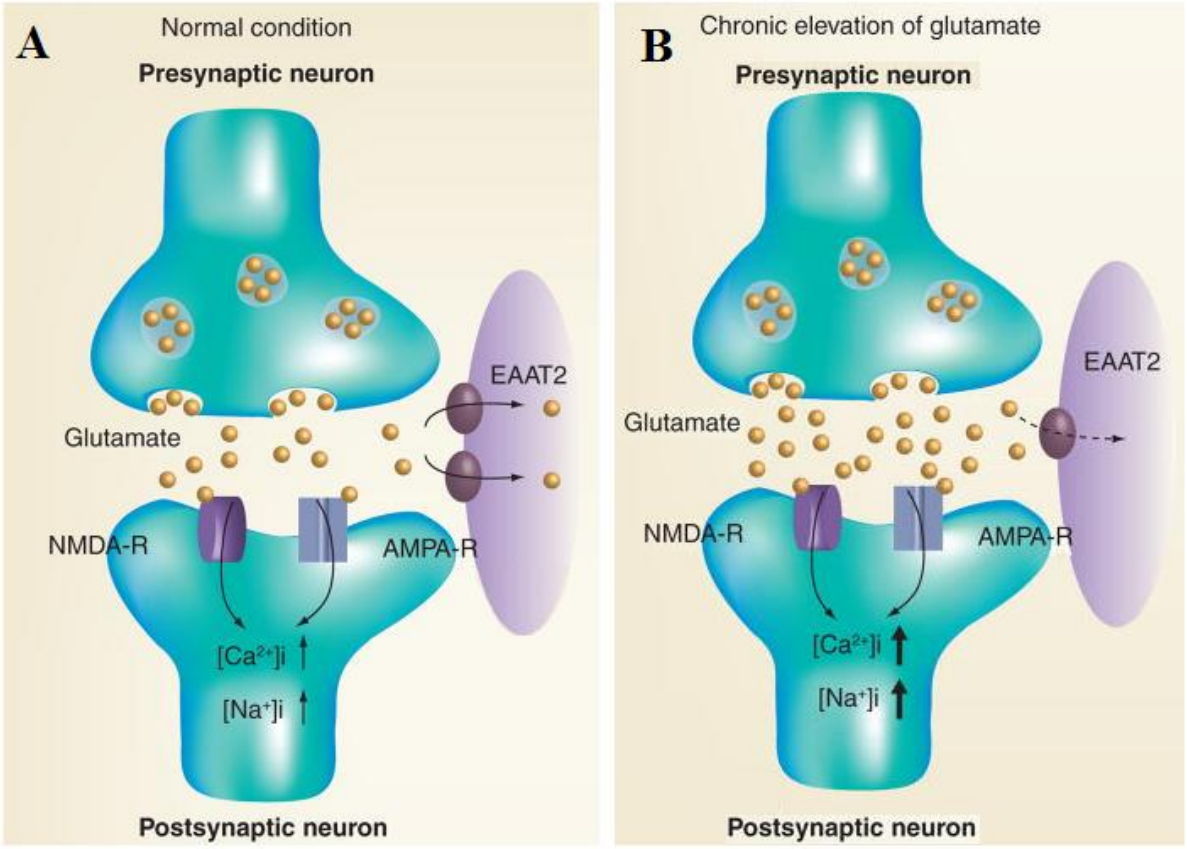

Figure 1.4: Glutamatergic neurotransmission and excitotoxicity. A) Under normal conditions glutamate is released from the presynaptic neurons. It then binds to and activates glutamate receptors (eg NMDA-R and AMPA-R) found on the postsynaptic neuron. This results in the influx of Na^+ and Ca^{2+} ions into the cell leading to an action potential. B) Chronic elevation of glutamate can occur when the uptake of glutamate is impaired, such as in ALS via the loss of EAAT2. This leads to overstimulation of glutamate receptors and increased intracellular concentrations of Na^+ and Ca^{2+} ions, resulting in excitotoxicity (Modified from Lin *et al.,* 2012).

1.4.2.4 Oxidative stress

Oxidative stress due to the accumulation of reactive oxygen species (ROS) has been linked to age associated neurodegenerative diseases such as Parkinson's and Alzheimer's disease**.** In aerobic organisms the respiratory chain is the major source of ROS. During the production of energy, about 1-5% of cellular oxygen forms ROS. ROS can have both beneficial and deleterious effects depending mainly on their concentration. At low concentrations some ROS can be beneficial, for example nitric oxide is produced by the endothelial cells to control blood pressure, and phagocytes produce ROS which can kill pathogens. However at high concentrations ROS can cause oxidative damage to DNA

and lipids (Gadoth and Göbel, 2011). Thus in healthy organisms there is a balance between the production of ROS and defence mechanisms to protect against oxidative stress. Superoxide dismutase (SOD) is a major antioxidant enzyme which converts superoxide anions to hydrogen peroxide, which is then converted to water and molecular oxygen (Potter and Valentine, 2003). Given that mutations in the *SOD1* gene can cause ALS, and the known link between oxidative stress and other neurodegenerative diseases, it has been hypothesised that oxidative stress is a mechanism which contributes to ALS. In support of this biochemical changes that reflect oxidative damage or abnormal free radical production, such as increased protein carbonyl levels, lipid peroxidation, and protein glycoxidation, have been observed in tissue samples and cerebrospinal fluid (CSF) of ALS patients (Shaw *et al.,* 1995; Ferrante *et al.,* 1997; Smith *et al.,* 1998; Tohgi *et al.,* 1999). Additionally fibroblast cultures from FALS and SALS patients were more sensitive to oxidative damage than control cultures (Aguirre *et al.,* 1998; Wijesekera and Leigh, 2009). It has also been suggested that oxidative stress in muscle may contribute to ALS pathogenesis. Mahoney *et al*. (2006) detected markers of oxidative stress and up regulation of SOD1 in the skeletal muscle of transgenic SOD1 mice.

1.4.2.5 Mitochondrial dysfunction

Mitochondria are often referred to as the power house of the cell, due to their main function of producing ATP via oxidative reactions. In addition to providing the cell with energy, mitochondria also contribute to the production of free radicals, regulate apoptosis, and play a role in calcium signalling and regulation (Newmeyer and Ferguson-Miller, 2003; Cozzolino *et al.,* 2008). There is significant evidence for mitochondrial dysfunction in human ALS, for example in SALS patients the following have been observed: reduced electron transport chain activity in motor neurons, altered mitochondrial morphology in muscle and motor neurons, increased calcium levels in motor axon terminals, and mitochondrial DNA mutations in motor cortex, muscle, and spinal cord tissue (Siklos *et al.,* 1996; Wiedemann *et al.,* 2002). In transgenic mice models of ALS disease onset correlates with the appearance and rapid increase of vacuoles originating from degenerating mitochondria, reduced electron transport chain activity and reduced ATP synthesis (Kong and Xu, 1998). In addition it has also been hypothesised that MT SOD1, a small portion of which is localised in mitochondria, can cause direct damage to the organelle. There are three main ways in which SOD1 may cause direct damage to mitochondria; 1) aggregated SOD1 could damage the mitochondria via aberrant interactions with other normal mitochondrial proteins, 2) MT SOD1 forms aggregates on the outer mitochondrial membrane, disrupting the translocator outer membrane complex, and 3) MT SOD1 could be involved in forming pores in the mitochondrial membrane, releasing cytochrome C and other pro death molecules, initiating apoptosis and resulting in neuronal degeneration (Pasinelli and Brown, 2006; Liu *et al.,* 2004; Higgins *et al.,* 2003).

1.4.2.6 Impaired axonal transport

Axonal transport is the movement of subcellular structures and proteins (cargo) from the cell body to synapses (anterograde transport) and from synapses to the cell body (retrograde transport), and is an important cellular process (Hammond, 2008). Transport can be fast, with the transport of vesicles and mitochondria at rates of 1 μm/s, or slow with enzymes and cytoplasmic proteins transported at an overall rate of 1 mm/day (De Vos *et al.*, 2007). Motor neurons are among the largest and longest cells in the body and thus rely on efficient transport systems (Pasinelli and Brown, 2006). MT SOD1 has been shown to impair both fast and slow axonal transport in SOD1 mouse models of ALS. (Zhang *et al.*, 1997; Williamson and Celveland, 1999; De Vos *et al*., 2007). Impaired axonal transport occurs at an early stage of disease progression in SOD1 mice and worsens with age (Williamson and Cleveland, 1999; Kieran *et al.*, 2005; Bilsland *et al.*, 2010). Mitochondria and neurofilaments are two of the major cargoes transported by kinesin and the dynein/dynactin complex, and defects in these transport systems results in the accumulation of mitochondria and neurofilaments in axons (Ikenaka *et al.*, 2012). Accumulations of mitochondria have been observed in the axons of motor neurons in SOD1 mice (Magrane *et al*., 2009; Bilsland *et al.*, 2010) and both mitochondria and neurofilament inclusions have been observed in the motor neurons of SALS and FALS patients (Hirano *et al.*, 1984; Sasaki *et al.*, 1996; Sasaki and Iwata, 2007; Sasaki 2011), suggesting impaired axonal transport in ALS. More recently, studies using transgenic TDP-43 mouse models of ALS reported aggregates within the axons of motor neurons which contained abnormal accumulations of mitochondria, suggesting impaired axonal transport and mitochondrial trafficking in these mice (Xu *et al.*, 2010; Shan *et al.*, 2010).

1.4.2.7 The role of non-neuronal cells

Several post mortem and animal studies suggest that the activation of non-neuronal cells contributes to the pathogenesis of ALS, affecting the viability of motor neurons. Activation of non-neuronal cells such as microglia and astrocytes is commonly observed in human ALS patients and transgenic SOD1 mice (Kawamata *et al.*, 1992; Henkel *et al.*, 2004; Hall *et al.*, 1998). When non-neuronal cells are activated they produce various inflammatory cytokines such as interleukins and tumour necrosis factor alpha, which are upregulated in the CSF or spinal cord tissues of ALS patients (Sekizawa *et al.*, 1998; Wilms *et al.*, 2003). Microglia have been observed to release factors that can enhance glutamate excitotoxicity (Tikka *et al.*, 2002). Recent studies using transgenic SOD1 mice have focused on determining whether the activation of non-neuronal cells in ALS is beneficial or deleterious. In 2001 Pramatarova *et al*. showed that expression of MT SOD1 in motor neurons alone did not result in neurodegeneration, and suggested that non-neuronal cells might need to be involved to result in ALS pathogenesis. Further studies with transgenic mice demonstrated that motor neurons expressing MT SOD1 failed to degenerate if they were surrounded by a large number of normal “supporting cells” such as microglia and astrocytes. Conversely normal motor neurons surrounded by supporting cells

expressing MT SOD1 showed signs of degeneration including ubiquitinated intra-neuronal deposits (Clement *et al.*, 2003). These results suggest that the involvement of non-neuronal cells is required for ALS pathogenesis.

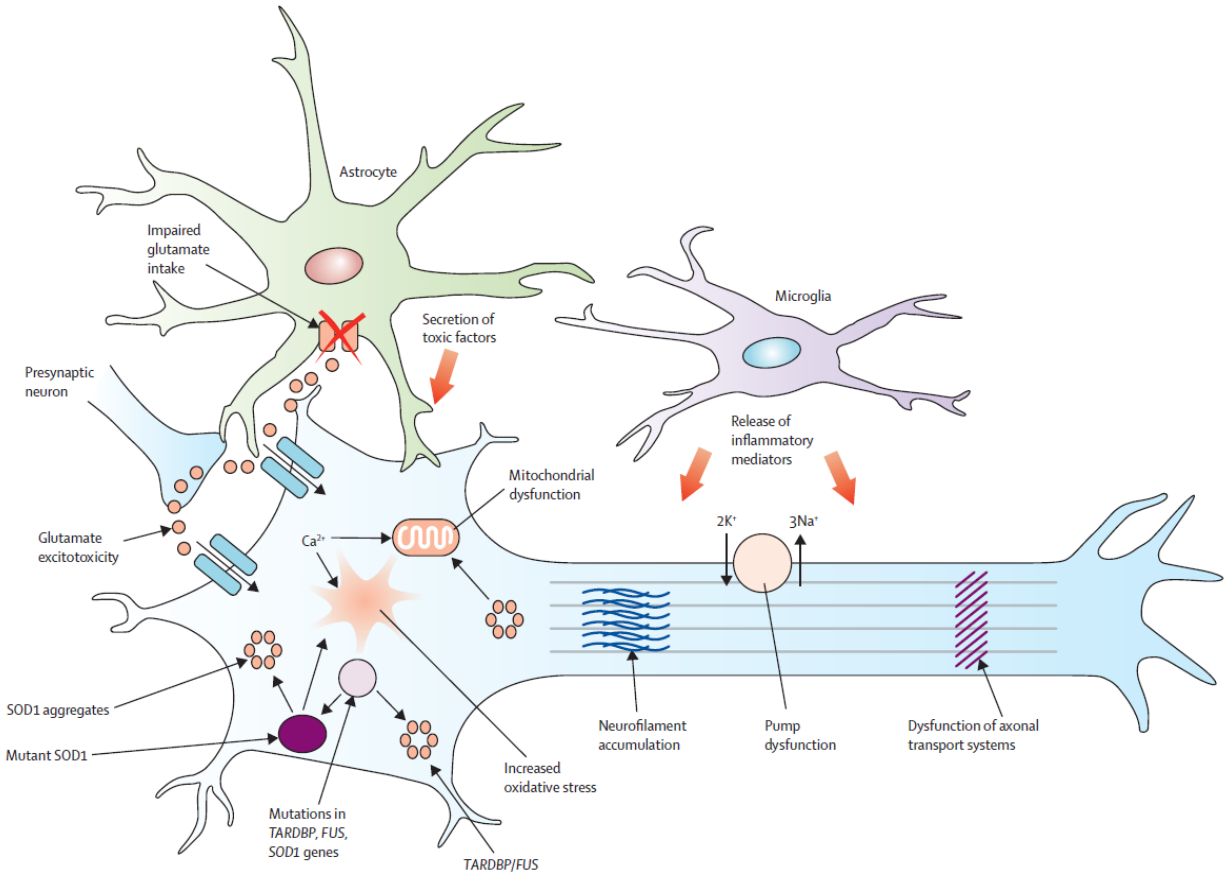

Figure 1.5: Cellular and molecular pathways implicated in neurodegeneration in ALS. ALS pathogenesis is most likely caused by the complex interactions between multiple pathogenic molecular and genetic pathways including mutations, protein aggregation and disruption to proteostasis, excitoxicity, oxidative stress, mitochondrial dysfunction, impaired axonal transport, and the activation of non-neuronal cells (Kiernan *et al.*, 2011).

1.5 SPREAD OF ALS PATHOLOGY IN THE CENTRAL NERVOUS SYSTEM (CNS)

Typically in ALS, pathological changes begin at a focal point of onset and spreads radially outwards over time, with the eventual degeneration of both upper and lower motor neurons. There are currently two proposed mechanisms for propagative progression; contiguous and non-contiguous spread.

1.5.1 Contiguous spread

Contiguous spread involves the spread of disease pathology between neighbouring neurons in either a cell autonomous (only genotypically mutant cells develop mutant phenotype) or non-cell autonomous (genotypically mutant cells can cause other cells to develop mutant phenotype) manner. Contiguous spread has been observed in several studies and has become widely accepted (Kanouchi *et al.,* 2012; Swash *et al.,* 2013). In one study retrospective analyses of ALS patient records showed that in 98% of cases early symptoms had a focal onset that spreads towards contiguous body regions, while evaluation of post-mortem nervous systems revealed that lower motor neuron loss was usually radial – greatest at the focal point of onset and decreasing outwards (Ravits *et al.,* 2007; Korner *et al.,* 2011). Other studies have observed contiguous spread that predominantly has horizontal and rostral to caudal (top to bottom) directionality, suggesting that motor neuron degeneration may have preferential directions of spread rather than simple radial spreading (Ravits *et al.,* 2007a; Gargiulo-Monachelli *et al.,* 2012; Fujimura-Kiyono *et al.,* 2011). This directionality may be due to variability in motor neuron susceptibility or the microenvironment of the neurons (Ravits and La Spada, 2009). Contiguous spread requires cell-to-cell transmission between neurons near each other. Proposed mechanisms of spread include "prion-like" propagation of abnormal cytosolic proteins in ALS, or phagocytosis or endocytosis of extracellular proteinaceous material (Swash, 2013). These mechanisms will be discussed in further detail in section 1.7.

1.5.2 Non-contiguous spread

In 2012 Gargiulo-Monachelli *et al.,* reviewed the medical records of over 300 ALS patients and observed spread to distant non-contiguous sites in 14% of the patients analysed, suggesting that not all spreading in ALS in contiguous. Non-contiguous spread may involve two phenomena of motor neuron degeneration ; 1) anterograde trans-synaptic spread ("dying forward"), where insult to the cell body causes dysfunction, or retrograde degeneration ("dying back") where insult to the distal axons causes axonal dysfunction prior to cell dysfunctions; and 2) non-synaptic spread, for example through the blood or CSF. It appears likely that contiguous and non-contiguous spreading mechanisms are not independent, but can co-exist with each other, contributing to the complex clinical presentation of ALS (Kanouchi *et al.,* 2012; Blizzard et al., 2015).

1.5.3 Histopathology

The pathological hallmark of ALS is the selective degeneration of both upper and lower motor neurons, along with activation and proliferation of astrocytes and microglia (termed astrogliosis). The cell death process of these neurons remains unclear, as they do not show the morphological features of either apoptosis or necrosis. Some motor neuron groups are less vulnerable to degeneration, including those in the upper brain stem nuclei that control eye movements, and those within the sacral spinal

cord that control the bladder (Mannen *et al.,* 1977; Cozzolino *et al.,* 2008). In addition to motor neuron degeneration, histopathology shows intracellular inclusions within degenerating neurons and glia. The most common inclusions in FALS are ubiquitinated. Ubiquitinated inclusions are found in 100% of SALS cases, suggesting that protein misfolding and disruption to proteostasis strongly contributes to the pathogenesis of ALS (Wood *et al.,* 2003; Piao *et al.,* 2003). The physical structures of inclusions vary, for example skein like inclusions are fibrillar, while "bunina bodies" are amorphous (Wood *et al.,* 2003; Arai *et al.,* 2006; Okamoto *et al.,* 2008). Bunina bodies are thought to originate from the ER and are found in 80-100% of SALS cases, and have yet to be found in any other neurodegenerative disease (Bunina, 1962; Tomonaga *et al.,* 1978; Takahashi *et al.,* 1991; Piao *et al.,* 2003).

Immunohistochemistry studies have identified several proteins present in inclusions both from human ALS tissue and transgenic mice models. Most notably, inclusions immunoreactive for SOD1 are found in motor neurons and glial cells when SOD1 mutations are present (Bruijn *et al.,* 1997). In addition TDP-43 is present in all cases of SALS, but is low or undetectable in patients with SOD1 mutations (Mackenzie *et al.,* 2007). Two other proteins, FUS and optineurin have also been found in SALS inclusions (Mackenzie *et al.,* 2007; Deng *et al.,* 2010; Maruyama *et al.,* 2010). Immunohistochemistry studies are limited in terms of the range and scope of antibodies used. Proteomic technologies can be much more powerful at identifying changes in protein expression, identifying proteins, and looking at protein-protein interactions. Unfortunately there are a limited number of proteomic studies that use human tissue, as most investigations have focussed on proteins obtained from mouse tissues and cells (Jain *et al.,* 2008), and no quantitative comparisons between control and ALS tissues has been performed. Nevertheless proteomics have identified a vast array of proteins that make up the composition of inclusions from transgenic mice tissues, including the cytoplasmic chaperones αB-crystallin and Hsp70, cytoskeletal proteins, proteins that normally reside within the ER, intermediate filaments such as neurofilaments, vimentin, and GFAP (Bergemalm *et al.,* 2010; Basso *et al.,* 2009). While these studies identify proteins that may participate in the pathogenesis of ALS in mouse models, these results must be repeated using tissue obtained from ALS patients.

1.6 TRANSACTIVE RESPONSE DNA BINDING PROTEIN (TDP-43)

While the role of SOD1 in ALS pathogenesis has been extensively characterised, comparatively little is known about the role of TDP-43. It was only relatively recently that TDP-43 was identified as a major component of ubiquitinated inclusions in ALS, leading to the identification of several mutations in the *TARDBP* gene capable of causing the disease (Neumann *et al.,* 2006; Gitcho *et al.,* 2008; Sreedharan *et al.,* 2008; Van Deerlin *et al.,* 2008; Corrado *et al.,* 2009; Daoud *et al.,* 2008; Yokoseki

et al., 2008). It is uncertain what the normal functions of TDP-43 are, making it difficult to determine if this protein contributes to ALS pathogenesis via a toxic gain or loss of function.

1.6.1 Function, Structure and Location

The exact cellular function of TDP-43 is unclear, but it likely has diverse and important roles in several RNA processing mechanisms (Figure 1.6). TDP-43 was originally identified as a factor capable of binding to the transactive response (TAR) DNA sequence of human immunodeficiency virus type 1, repressing its transcription (Ou *et al.*, 1995), and was later shown to be involved in pre-messenger RNA (mRNA) splicing, exon skipping, and mRNA transport (Buratti *et al.*, 2001; Mercado *et al.*, 2005; Wang *et al.*, 2008). More recently it has been shown that TDP-43 is recruited to stress granules, suggesting that it may play a role in protecting the body against cellular insult (Colombrita *et al.*, 2009).

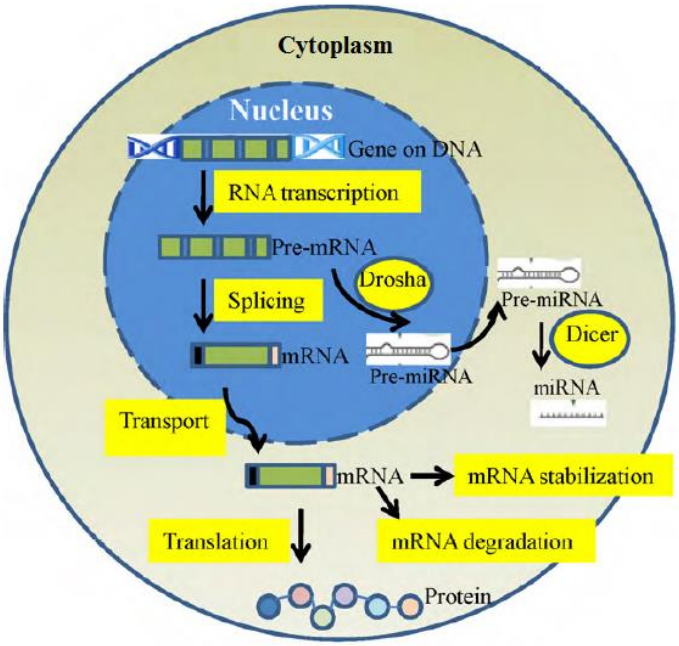

Figure 1.6: Known biological functions of TDP-43. TDP-43 has diverse roles in RNA processing (highlighted in yellow) in the nucleus and cytoplasm (modified from Warraich *et al.*, 2010).

TDP-43 is a highly conserved, intrinsically aggregation prone, ubiquitously expressed 43 kDa nuclear protein encoded by the 6 exon gene *TARDBP* (Figure 1.7; Ayala *et al.*, 2005; Sreedharan *et al.*, 2008). It has sequence homologs in mice, zebrafish, *Drosophila* and *C. elegans*. The protein contains two RNA recognition motifs (RRM1 and RRM2) which are involved in RNA and DNA binding (Ayala *et al.*, 2005; Ou *et al.*, 1995; Burrati *et al.*, 2005). At the C terminal domain is a glycine rich sequence

which may be required for mediating protein-protein interaction and exon skipping (Lagier-Tourene *et al.*, 2010; Wang *et al.*, 2004; Burrati *et al.*, 2005). TDP-43 also contains a nuclear export signal (NES) and a nuclear localisation signal (NLS) which allows the protein to shuttle between the nucleus and cytoplasm, possibly transporting mRNA (Banks *et al.*, 2008). TDP-43 is expressed in many tissues including the CNS, where it is mainly localised to the nucleus, with low levels in the cytoplasm. In ALS it is depleted from the nucleus and accumulates in insoluble, ubiquitinated inclusions in the cytoplasm of motor neurons (Neumann *et al.*, 2006). Under stress conditions TDP-43 can also be found in stress granules which have been observed in CNS tissue of ALS patients, but it is unknown whether they contribute to ALS pathogenesis (Liu-Yesucevitz *et al.*, 2010).

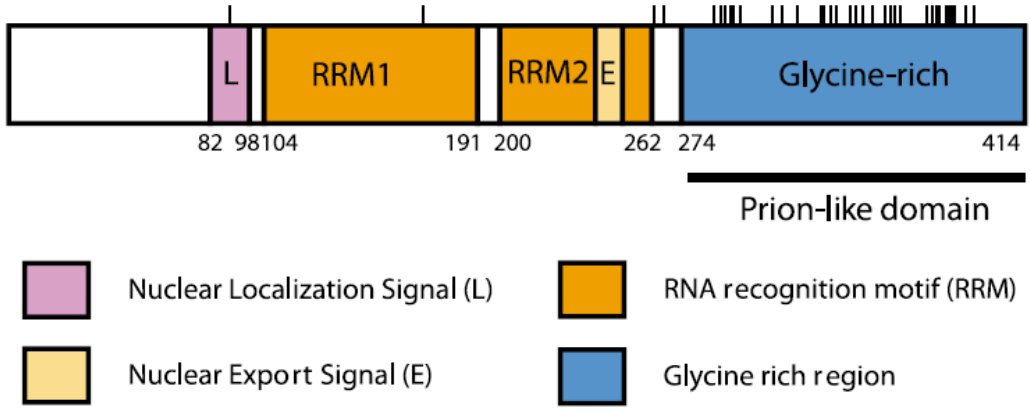

Figure 1.7: A schematic representation of the domain organisation of TDP-43, including the glycine-rich "prion-like" domain. Different protein domains are indicated in different colours (see key). The location of ALS associated mutations is depicted by small black bars immediately above the molecular representation (modified from Blokhuis *et al.*, 2013).

1.6.2 Pathological modifications of TDP-43

Since the discovery that TDP-43 is a major disease protein in ALS, there has been much interest in understanding the pathological modifications that lead to the formation of cytoplasmic inclusions containing TDP-43. In 2006 Neumann *et al*. examined the brains and spinal cord of ALS patients, and found that pathologic TDP-43 was ubiquitinated, hyper-phosphorylated, and cleaved to generate C and N terminal fragments. It is thought that while shuttling between the nucleus and cytoplasm, these fragments are unable to re-enter the nucleus if they lack the NLS, and accumulate in the cytosol where they form insoluble inclusions (Gendron *et al.*, 2010). Cleaved TDP-43 has been shown to contribute to ALS pathogenesis via both a toxic gain and loss of function. C terminal fragments expressed in neuroblastoma cell cultures or transgenic mice are toxic and reproduce the aggregation and

phosphorylation observed in ALS, while the cleavage of full length TDP into fragments that lack key functional domains leads to a loss of function (Zhang *et al.,* 2009; Nonaka *et al.,* 2009; Dormann *et al.,* 2009; Wils *et al.,* 2010; Buratti and Baralle, 2001). TDP-43 and the pathologic truncated TDP-25 are both degraded by the ubiquitin-proteasome system, and the presence of accumulated ubiquitin and proteasome in ALS neurons suggests that in ALS ubiquitinated protein aggregates are unable to be degraded, possibly due to the ubiquitin system being overwhelmed or dysfunctioning (Wang *et al.,* 2010; Mendonca *et al.,* 2006). Additionally, proteasome inhibition in cultured cells promotes cleavage of TDP-43, providing further links between ALS pathogenesis and disruption to proteostasis (Rutherford *et al.,* 2008). The precise effect of hyper-phosphorylation is not yet fully understood, but the C-terminus of TDP-43 has many phosphorylation sites, and it has been hypothesised that hyper-phosphorylation may contribute to disease pathogenesis by disrupting the normal functions of TDP-43, and promoting aggregation and oligomerisation (Dormann *et al,* 2009; Gendron *et al.,* 2010; Kametani *et al.,* 2009). Finally, many mutations in TDP-43 have been identified, accounting for ~ 5% of ALS cases (combining SALS and FALS; Gitcho *et al.,* 2008; Sreedharan *et al.,* 2008; Van Deerlin *et al.,* 2008; Corrado *et al.,* 2009; Daoud *et al.,* 2008; Yokoseki *et al.,* 2008; Da Cruz and Cleveland, 2011). The majority of these are missense mutations found in the glycine rich C terminal region (Kabashi *et al.,* 2008). The presence of these mutations in the highly conserved C terminal region, together with their absence in control patients suggests that they are pathogenic (Lagier-Tourenne and Cleveland, 2009). Some mutations have been shown to increase phosphorylation, increase the propensity of TDP-43 to become fragmented, or accelerate its aggregation (Johnson *et al.,* 2009; Corrado *et al.,* 2009).

1.6.3 Does TDP-43 contribute to ALS via a loss or gain of function?

1.6.3.1 Loss of function

Under normal conditions TDP-43 is primarily a nuclear protein which plays important roles in pre mRNA splicing and transcriptional repression. In ALS it is depleted from the nucleus and accumulates in ubiquitinated cytosolic inclusions (Neumann *et al.,* 2006), thus loss of nuclear function has been proposed as an underlying cause of ALS pathogenesis. To fully understand the consequences that result from this loss of function, the role of the protein must be better defined. *In vivo* studies using *Drosophila* showed that full knockdown of TDP-43 caused dramatic locomotive defects with spastic, uncoordinated movements, incapacity to fly or walk normally and reduced life span, phenotypes which could be partially rescued by expression of human TDP-43. This demonstrates that activity of TDP-43 in neurons is needed to regulate locomotive behaviors and indicates that the pathogenesis observed in ALS may result in part from loss of TDP-43 function in the nucleus (Feiguin *et al.,* 2009). In other studies using *Drosophila*, knockdown of TDP-43 caused decreased dendritic branching and reduced lifespan, suggesting that TDP-43 plays a role in dendrite

formation and structural integrity, and is required for normal viability of adult flies (Lu *et al.,* 2009). Additionally, knockdown of TDP-43 in zebrafish causes loss of motor function and altered motor neuron axon phenotypes (Kabashi *et al.,* 2010). Expression of WT, but not MT TDP-43 is partially able to rescue the motor function and axonal phenotypes observed in TDP-43 knockdown zebrafish. It has been hypothesised that loss of normal nuclear function of TDP-43 in neurons reduces dendritic complexity, leading to compromised neuronal connectivity contributing to ALS pathogenesis (Lu *et al.,* 2009). Finally, knockdown of TDP-43 in human and mouse cells results in dysmorphic nuclear shape, mis-regulation of the cell cycle, apoptosis and reduced neurite outgrowth (Ayala *et al.,* 2008; Iguchi *et al.,* 2009). These studies support the theory that loss of normal nuclear TDP-43 function contributes to neurodegeneration and ALS pathology. Genetic studies using transgenic mice are limited due to the fact that TDP-43 knockout is lethal for mice from embryonic day 3.5, demonstrating the essential function of TDP-43 in embryo development and survival, likely due to its role in a variety of cellular functions such as splicing and transcriptional regulation of various genes (Wu *et al.,* 2010; Kraemer *et al.,* 2010).

1.6.3.2 Toxic gain of function

Many studies examining the roles of TDP-43 in neurodegeneration support the idea that TDP-43 causes toxicity, at least in part, by a toxic gain of function. The overexpression of TDP-43, truncated TDP-43, and ALS associated TDP-43 mutants in yeast cells causes toxicity dependent on aggregation (Johnson *et al.,* 2009). Similarly the expression of C terminal TDP-43 fragments in human cell lines also causes aggregation and enhanced cellular toxicity. The expression of these TDP-43 fragments did not reduce the exon skipping activity of full length TDP-43 nor alter the nuclear distribution of TDP-43, suggesting that toxicity was a result of a toxic gain of function independent of normal TDP-43 function (Zhang *et al.,* 2009). *In vivo* studies also support the toxic gain of function of TDP-43. The overexpression of *TARDBP* mutations in zebrafish caused loss of motor function and altered motor neuron axon phenotypes, and the overexpression of human TDP-43 in transgenic flies is sufficient to cause protein aggregation and neurotoxicity in an age dependent manner, suggesting that aberrant regulation of TDP-43 expression or decreased clearance of TDP-43 may contribute to the pathogenesis of ALS (Kabashi *et al.,* 2010; Li *et al.,* 2010). Interestingly, studies using rat and mouse models of TDP-43 proteinopathy show that toxicity can occur even in the absence of large cytoplasmic aggregates, suggesting that soluble MT TDP-43 may also play a role in the pathogenesis of ALS either by directly or indirectly altering protein degradation pathways and promoting the accumulation of ubiquitinated proteins and neurodegeneration, similar to the way soluble oligomers of amyloid-β contribute to Alzheimer's disease (Tatom *et al.,* 2009; Wegorzewska *et al.,* 2009). It is likely that TDP-43 mediated neurodegeneration results from both the loss of normal nuclear functions and a toxic gain of function (Gendron *et al.,* 2010).

1.7 "PRION-LIKE" PROPAGATION MECHANISMS IN ALS

The term prion was first proposed in in 1982 to describe the enigmatic scrapie agent capable of causing a degenerative disease of the central nervous system in sheep and goats (Prusiner, 1982). The agent was shown to be small and resistant to heat, formalin treatment and most procedures that modify nucleic acids, indicating that the scrapie agent must be an infectious entity differing from viruses, parasites and plasmids. Several lines of evidence, including the sensitivity of the scrapie agent to proteases, suggested that it contained a protein that was required for infectivity (Prusiner, 1982). Thus the term "prion" was coined to describe the scrapie agent as a novel *pro*teinaceous *in*fectious particle. The progressive accumulation of misfolded proteins in motor neurons and associated cells is a key feature of ALS. Recent studies of various neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and ALS have provided evidence for unique mechanisms of protein propagation, termed "prion-like" due to their similarity to the self-perpetuating spreading characteristics of the prion protein. Such mechanisms provide an explanation for the typical outward spread of symptoms from a focal point of onset observed in ALS, and have implications for the development of new therapeutic strategies. This review will focus on studies that suggest prion-like propagation of SOD1 and TDP-43, two proteins involved in ALS.

1.7.1 Evidence of prion-like self-propagation and cell-to-cell spread of SOD1

Misfolding of recombinant SOD1 leads to the formation of amyloid fibrils which have been shown to act as seeds capable of accelerating the formation of new fibrils (Chia *et al.,* 2010), and aggregated recombinant MT SOD1 is capable of inducing misfolding of WT SOD1 *in vitro* (Grad *et al.,* 2011). Spinal cord homogenates from transgenic MT $SOD1^{G93A}$ mice have also been shown to contain amyloid fibrils which can seed the formation of new fibrils in both WT and MT SOD1 protein *in vitro* (Chia *et al.,* 2010). Similar results have been observed in human cells where the expression of a FALS associated SOD1 mutant induced misfolding of WT natively structured SOD1 in human mesenchymal and neuronal cell lines (Grad *et al.,* 2011). The conformational conversion of natively structured SOD1 to a misfolded form is comparable to the conversion of PrP^{C} to PrP^{Sc} in prion disease. Other studies have established that MT SOD1 exhibits prion-like properties. For example Munch *et al.,* (2011) showed that aggregates composed of an ALS-causing mutant can penetrate inside neuronal cells in culture via macropinocytosis, exit the macropinocytic compartment and trigger the self-perpetuating aggregation of the endogenous protein. The transfer of SOD1 aggregates between cells does not require cell-to-cell contact, but instead depends on their extracellular release, as transfer occurred between two co-cultured populations separated by a filter (Munch *et al.,* 2011). Secretion of SOD1 has been reported for various cell lines including fibroblasts, neuroblastoma, motor neuron cell lines and primary spinal cord cultures (Krauss and Vorberg, 2013; Mondola *et al.,* 1997; Urushitani *et al.,* 2006; Gomes *et al.,* 2007). It has also been demonstrated that SOD1 aggregates are able to

transfer between cultured NSC-43 cells via the release of protein aggregates from dying cells (the aggregates are subsequently taken up by other cells via micropinocytosis), or the secretion of exosomes which can be taken up by other cells (Grad *et al*., 2014; Gomes *et al*., 2007). Exosomes normally transport various cargoes between cells, but are being increasingly implicated in the spread of proteins in neurodegenerative diseases. Both MT and WT SOD1 can be secreted via exosomes and taken up by surrounding cells causing subsequent protein misfolding and death (Basso *et al*., 2013; Grad *et al*., 2015). Recently studies have provided the first evidence for the *in vivo* transmissibility of SOD1 pathology. Genetically vulnerable SOD1 mice were intraspinally injected with spinal cord homogenates from terminally sick MT SOD1 mice, which induced MND in 6/10 mice and caused redistribution of endogenous SOD1 to protein inclusions (Ayers *et al*., 2014). These results suggest that SOD1 can self-propagate and spread from cell-to-cell in a manner similar to the progression mechanisms of prion diseases.

1.7.2 Evidence of prion-like self-propagation and cell-to-cell spread of TDP-43

There is also convincing evidence suggesting that in ALS TDP-43 shares properties similar to prions. TDP-43 contains a prion like domain with a similar amino acid composition to yeast prion-forming domains. These domains are a glutamine/asparagine (Q/N)-rich region in the C-terminal domain of TDP-43 (Fuentealba *et al.,* 2010; Cascarina and Ross, 2014; Udan-Johns *et al.,* 2014), have a strong tendency to self-aggregate and are required for aggregation (Johnson *et al.,* 2008; Fuentealba *et al.,* 2010; Sun *et al.,* 2011). Several studies have also shown that TDP-43 can act as seeds for aggregation. When exogenous TDP-43 C-terminal aggregates were applied to cultured human embryonic kidney cells transiently transfected with TDP-43, the aggregates were taken up by the cells where they acted as seeds for the aggregation of endogenous TDP-43 (Furukawa *et al*., 2011) Additionally when TDP-43 fragments containing the C-terminal end of the RRM1 domain were expressed in NSC-43 cells, aggregation of the TDP-43 fragments drove co-aggregation with the full-length TDP-43 (Chunxing *et al.,* 2010). Interestingly TDP-43 prepared from dissected ALS brains has also been shown to have prion like properties. When insoluble TDP-43 prepared from the brains of ALS patients was applied to SHSY-5Y cells expressing HA-tagged TDP-43 it seeded the aggregation of the latter (Nonaka *et al*., 2013). Nonaka *et al*. (2013) also demonstrated propagation of TDP-43 aggregates between cultured cells. SHSY-5Y cells transiently expressing a red fluorescent protein were co-cultured with SH-SY5Y cells harbouring phosphorylated TDP-43 aggregates and incubated for 3 days. Confocal microscopy revealed phosphorylated TDP-43 aggregates in 2.9% of cells that also expressed the red fluorescent protein, indicating that aggregates had spread to surrounding cells. TDP-43 has been found to be enriched in exosome fractions isolated from SHSY-5Y cells treated with detergent insoluble brain homogenate from ALS patients (Nonaka *et al*., 2013), and in exosomes isolated from the blood and CSF of ALS patients (Feneberg *et al*., 2014) supporting the idea that as with SOD1,

exosomes may contribute to the release of TDP-43 aggregates. Currently *in vivo* spread between cells or tissues has yet to be demonstrated.

1.7.3 How might aggregates move from cell to cell?

The most popular hypothesis is that prion-like aggregates are released from cells and taken up by surrounding cells where they can be transported to the cytoplasm and promote further aggregation (Bowen *et al.,* 2007; Goedert *et al.,* 2010; Kanouchi *et al.,* 2012). Exosomes and tunnelling nanotubes are two mechanisms that have been reported to transfer prions between cultured cells.

Exosomes are small lipid membrane vesicles of endocytic origin secreted by most cells *in vitro* and *in vivo* under physiological and pathological conditions. Following their secretion they can be taken up by neighbouring cells via endocytosis. The role of exosome release is to secrete proteins in order to remove them and to deliver signals between cells (Goedert *et al.,* 2010; Kanouchi *et al.,* 2012; Schneider and Simons, 2013). Exosomes have been shown to play a role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and ALS. β-Amyloid peptides are released in association with exosomes, and amyloid plaques of Alzheimer's Disease (AD) brain sections are enriched in exosome-associated proteins (Rajendran *et al.,* 2006), while in NSC-34 cells overexpressing human WT and MT SOD1, SOD1 was secreted via exosomes (Gomes *et al.,* 2007). Studies are now beginning to investigate whether TDP-43 may also be transferred between cells via exosomes (Nonaka *et al.,* 2013).

Tunnelling nanotubes are channels made from extensions of surface membrane that connect cells over long distances and traffic proteins and organelles either inside or along their surface (Davis and Sowinski, 2008; Goedert *et al.,* 2010). Tunnelling nanotubes have been shown to mediate the transfer of PrP^{Sc} *in vitro* between cells (Gousset *et al.,* 2009) and it has been suggested that aggregates in ALS may also be trafficked from cell-to cell in this manner.

1.8 MODELS OF ALS

The discovery of mutations in the genes encoding SOD1 and TDP-43 have led to the development of a number of vertebrate and invertebrate models of ALS, including mouse, rat, zebrafish, *C. elegans* and *Drosophila melanogaster.* Due to the complex nature of ALS, cell culture models which allow specific cell types or cellular events to be tested are also of great importance. Parallel studies of both cell and animal models of ALS are needed to fully understand disease pathology, and develop a successful therapeutic strategy. This section aims to introduce to the reader some of the models available for the study of ALS, especially the *Drosophila* model as it is used extensively in this thesis.

1.8.1 Cell culture models of ALS

Cell culture models allow researchers to study the role of a specific cell type, for example motor neurons or astrocytes, under controlled and defined conditions, and investigate mechanisms of a possible protective or deleterious role for molecules or compounds (Schlachetzki *et al.*, 2013). A well designed cell model should be easy to use and complex enough to recapitulate at least one part of the disease pathology (Veyrat-Durebex *et al.,* 2014). The three main culture systems used include primary cultures, established cell lines, and cultures derived from human cells. Primary cultures are isolated from tissues excised from young animals and are advantageous as their morphology, behaviour and activity retain features similar to the parent tissue (Veyrat-Durebex *et al.,* 2014). However they are difficult and require skill to obtain and have a relatively short life span in culture, making them unsuitable for medium to large scale studies where long term experiments may be need to be undertaken. Established cell lines are immortal and are thus ideal for such experiments as they will proliferate indefinitely given adequate space and media. Cell growth is rapid, uncomplicated, inexpensive, and results can be reproduced across different laboratories using the same cell lines. Importantly cell lines can be easily modified by transfection or viral transduction to overexpress ALS related genes such as the genes encoding for TDP-43 and SOD1. The main disadvantage of established cell lines is that the process of immortalisation means they differ significantly from their cells of origin, and may behave differently in experiments. Recent advances in cultures derived from human cells has introduced a robust model for the study of human neurodegenerative processes. Pluripotent stem cells (PSCs) are derived from human embryos of foetal tissue and have the potential to differentiate into almost any cell in the body. Induced pluripotent stem cells (iPSCs) are adult cells that that have been genetically reprogrammed to an embryonic stem cell-like pluripotent state (Takahashi *et al.,* 2007). They are advantageous as they can be generated from adult patients with specific inherited diseases such as FALS, which would enable the large scale production of the cell types affected by that individual patient's disease, which could be used for disease modelling, drug testing, or even eventually cell replacement therapies (Dimos *et al.,* 2008). While the possibilities of iPSCs are exciting, the technology is still relatively new and has some disadvantages, for example generating iPSCs and subsequent differentiated cells is very time intensive, taking up to 2-4 months.

1.8.1.1 Mouse

Many mouse models exist for studying ALS and the most commonly used is the SOD1G93A, which overexpresses a mutated human SOD1 gene (Gurney *et al.,* 1994). In other mouse models the SOD1 gene is mutated at different positions and recently transgenic models of human TDP-43 associated FALS have been created (Moser *et al.,* 2013; Shan *et al.,* 2010; Wils *et al.,* 2010; Wegorzewska *et al.,* 2009). Transgenic mice recapitulate many of the clinical, neuropathological and molecular features of ALS patients and have been valuable for providing insights into the mechanisms underlying

neurodegeneration. The choice of therapeutic agents in many clinical trials of human ALS has been predicted on the efficacy of these drugs when studied in the SOD1 mouse (Benatar 2006). However to date there have been over 50 publications describing therapeutic agents that are effective in SOD1 mice but have completely failed in human trials (Greek and Hansen *et al.,* 2013; Corcia and Gordon, 2012). The failure to translate positive preclinical findings achieved in SOD1 mice to successful human trials has led some to conclude that this model has outlived its usefulness, and is in fact a poor model for developing therapeutic agents (Greek and Hansen *et al.,* 2013).

1.8.1.2 Drosophila melanogaster

Drosophila has been used in research for over 100 years. They are a popular species to use for the study of neurological diseases due to the similarity between gene response, behaviour, and mode of drug action with mammalian systems, and the existence of sophisticated genetic manipulations developed for them. They are cheaper to culture and easier to maintain than mice and the regulations regarding animal care and use that apply to vertebrate research do not apply to *Drosophila*. They have a short generation time, produce a large number of offspring, and possess a large range of phenotypical characteristics such as body colour and size, wing phenotype and bristle patterns. Fly behaviour can be easily quantified through various assays including larval crawling, adult mating, and adult negative geotaxis or climbing assays (Nichols *et al.,* 2012). In addition 75% of human disease genes have homologs in the *Drosophila* genome, and the *Drosophila* brain shares anatomical, functional and biochemical similarities with human brains. *Drosophila* TDP-43 (dTDP-43), like its human homolog is expressed in neurons, glia and muscle cells. Many studies have investigated the pathological role of TDP-43 in ALS using *Drosophila* models and will be discussed throughout this thesis (Li *et al.,* 2010; Hanson *et al.,* 2010*;* Estes *et al.,* 2011; Diaper *et al.,* 2013a; Diaper *et al.,* 2013b).

1.9 CHAPERONES AND ALS

1.9.1 Interactions between chaperones and proteins involved in ALS

As outlined in section 1.3.2 and 1.3.3, chaperones are a key component of both intra- and extracellular quality control systems, working to prevent irreversible protein aggregation and ensure proteins are kept on the correct folding pathway. ALS pathology has recently been attributed to protein aggregation and disruption to proteostasis, with SOD1 and TDP-43 being two of the major aggregating proteins associated with the disease. Studies have reported interactions between SOD1 and TDP-43 and various Hsps including αB-crystallin, Hsp70 and Hsp40, and Hsp27/25 as well as altered chaperone expression levels in transgenic SOD1 mice, suggesting that chaperones may influence ALS onset and progression. The majority of studies to date have focussed on the effect of

Hsps on the aggregation and toxicity of SOD1 in cell cultures and *in vivo* models of ALS, and relatively little is known about their effect on TDP-43.

1.9.2 SOD1 and chaperone

In vitro the sHsp αB-crystallin is able to inhibit the rate of aggregation of SOD1 by binding directly to and forming a stable high molecular mass complex with SOD1, thus preventing inappropriate self-assembly, a mechanism typical of sHsps (Wang *et al.,* 2005; Yerbury *et al.,* 2013). Similarly co-expression of αB-crystallin with MT SOD1 reduced MT SOD1 aggregation in cell culture (Karch and Borchelt, 2010). Knock down of αB-crystallin in SOD1 transgenic mice reduced the interval to disease end stage by 20-30 days, however, a dose-dependent effect on survival was not seen. In addition, knock down of αB-crystallin did not change the amount of detergent-insoluble SOD1 aggregates in the spinal cord, suggesting that in this case eliminating one element in the proteostasis network is not enough to alter the extent of protein aggregation (Karch and Borchelt, 2010). αB-crystallin is normally expressed in oligodendrocytes and is upregulated in symptomatic MT SOD1 mice (Wang *et al.,* 2002). In mouse models of ALS, MT SOD1 accumulates specifically in motor neurons where αB-crystallin is absent, but not in oligodendrocytes, suggesting that the availability of Hsps may influence the susceptibility of certain cell types (such as motor neurons) to MT SOD1 aggregation (Wang *et al.,* 2005).

MT SOD1 has also been found to interact with Hsp70. In primary motor neuron cultures and neuronal cell lines, the formation of MT SOD1 aggregates is reduced and survival prolonged by increasing the level of Hsp70 (Bruening et a., 1999; Takeuchi *et al.,* 2002; Patel *et al.,* 2005; Koyama *et al.,* 2006), and in transgenic SOD1 mice Hsp70 is able to form complexes with soluble MT SOD1 (Wang *et al.,* 2009). Despite these interactions, studies investigating the ability of Hsp70 to extend lifespan in transgenic SOD1 mice have yielded contrasting results. Liu *et al.* (2005) created three transgenic MT SOD1 mouse models that constitutively expressed Hsp70 at various levels. They found that even when Hsp70 was expressed at levels up to 10 times greater than endogenous Hsp70, it did not have a significant effect on disease onset or survival time. The authors offered several explanations for the differing results seen in mouse models and cell culture models. They first suggest that short term toxicity studies done in cell models do not adequately reflect the slow neurodegeneration and SOD1 accumulation observed *in vivo.* Secondly they hypothesise the likelihood that multiple chaperones will need to be upregulated at specific locations in order to ameliorate SOD1 toxicity (Liu *et al.,* 2005). In addition to these suggestions it is important to note that most studies using transgenic mice do not characterise changes in gene expression other than their gene of interest. Thus caution must be taken when interpreting results solely based on mouse studies, as compensatory changes in other genes may affect and possibly invalidate interpretation of the results observed.

Another study found that when recombinant human Hsp70 was intraperitoneally injected three times a week into MT SOD1 mice, it was effective at increasing lifespan, delaying symptom onset, preserving motor function, extending motor neuron survival, and increasing the number of innervated neuromuscular junctions (NMJs; Gifondorwa *et al.,* 2007). Injected Hsp70 was localised to skeletal muscles and the authors hypothesize that the injected Hsp70 provided a protective effect at the neuromuscular junction (NMJ) through an unknown peripheral mechanism or it could bind to growth factors promoting activity at the NMJ (Gifondorwa *et al.,* 2007; Gifondorwa *et al.,* 2012).

In vitro Hsp27 is also able to inhibit SOD1 aggregation and in one cell culture model can protect against MT SOD1 induced cell death; it has been also been shown to co-fractionate with SOD1 aggregates in ALS tissue (Yerbury *et al.,* 2013; Patel *et al.,* 2005; Wang *et al.,* 2003). However a second report showed that upregulation of Hsp27 alone was not sufficient to protect MT SOD1 expressing cells from death, but that activation of the heat shock response was sufficient to provide protection (Krishnan *et al.,* 2006). This could be simply due to different levels of MT SOD1 expressed. Motor neurons express and are able to upregulate Hsp27 (Plumier *et al.,* 1997; Costigan *et al.,* 1998), however in pre-symptomatic transgenic SOD1 mice expression of Hsp25 (mouse homologue of Hsp27) decreases in motor neurons, but not in other cell types (Maatkamp *et al.,* 2004). It is likely that reduced Hsp25 levels contribute to the neurodegeneration observed in SOD1 mice, as a reduction in Hsp25 levels means a reduction in the ability to scavenge misfolded proteins such as SOD1. Mice expressing both SOD1 and Hsp25 show a delayed decline in motor strength and increased survival of motor neurons, however this protective effect is only observed in the early stages of disease progression when Hsp25 is abundant in motor neurons, and does not improve total life span (Sharp *et al.,* 2008).

The above studies highlight the limitations of various experimental approaches and while some of the results may be conflicting, a common idea becomes apparent; if a successful therapeutic strategy involving chaperones is to be developed, a balanced upregulation of multiple chaperones may be needed. Several studies have tested this idea. In a SOD1 cell model of FALS the expression of Hsp40 alone was ineffective at supressing aggregate formation or cell death, but the combination of Hsp40 + Hsp70 reduced aggregates, improved neurite outgrowth and prevented cell death to a greater extent than when Hsp70 alone was expressed (Takeuchi *et al.,* 2002). Hsp40 is a co-chaperone essential for the optimal functioning of Hsp70. Similarly, while expression of either Hsp27 or Hsp70 has a protective effect against SOD1 associated cell death, co-expression of the two chaperones together has a greater effect (Patel *et al.,* 2005). Activation of the heat shock transcription factor (Hsf-1) can induce the heat shock response leading to the expression of multiple heat shock proteins. Motor neurons have a high threshold for induction of the heat shock response, and stress-induced activation

of Hsf-1 decreases in neurons during aging which may contribute to their vulnerability in ALS (Batulan *et al.,* 2006). The expression of activated Hsf-1 in cultured motor neurons induced the expression of multiple heat shock proteins including but not limited to Hsp70, 40 and 25, almost completely prevented aggregation of MT SOD1, and protected motor neurons from MT SOD1 toxicity (Batulan *et al.,* 2006). Consistent with the idea that the expression of multiple Hsps is needed, transgenic MT SOD1 mice treated with arimoclomal, an inducer of the heat shock response (by prolonging the activation of Hsf-1), showed delayed disease progression and improved muscle function and motor neuron survival in later stages of disease (Kieran *et al.,* 2004). A second drug, resveratrol can also protect motor neurons and extend the lifespan in transgenic MT SOD1 mice, via activation of Hsf-1 and upregulation of heat shock proteins (Han *et al.,* 2012).

1.9.3 TDP-43 and chaperones

While interactions between Hsps and SOD1 are very well studied, little is known about the potential effects of Hsps on the aggregation and toxicity of TDP-43. As previously mentioned, mutations in SOD1 occur in only 20% of FALS and 5% of SALS cases. TDP-43 on the other hand is present in inclusions in all cases of SALS and non-SOD1 related FALS, and is low or undetectable in patients with SOD1 mutations. Therefore studying SOD1 models of ALS only represents a small population of the disease, and there may be subtle differences between FALS and SALS pathology. In order to fully understand the disease, TDP-43 based models of neurodegeneration must be developed and studied. In the past few years interactions between TDP-43 and heat shock proteins have begun to be investigated. In a *Drosophila* model of ALS entailing overexpression of TDP-43, upregulation of the heat shock response or the chaperone GG14207 (which has sequence homology with human Aβ-crystallin) inhibited the aggregation of TDP-43 and partially reduced its neurotoxic effects on photoreceptors and motor neurons. Similarly, in flies that expressed the C-terminal fragment TDP-25, upregulation of the heat shock response and GG14207 expression completely rescued from toxicity and cleared TDP-25 from the brain (Gregory *et al.,* 2012). A recent study used cell culture models to demonstrate that heat shock induced aggregation of TDP-43 is regulated by Hsp40/70. In this study live HeLa cells expressing TDP-43-Cherry were heat shocked at 42 °C for 1 h, resulting in the formation of nuclear aggregates of TDP-43. Co-immunoprecipitation revealed that TDP-43 was constitutively bound to Hsp40 and Hsp70. Overexpression of Hsp40 was able to supress the formation of TDP-43 aggregates, while Hsp40 knock down led to an increase in TDP-43 aggregation (Udan-Johns *et al.,* 2014). Udan-Johns *et al.* hypothesise that chaperones are constitutively bound to TDP-43 but are lost during stress such as heat shock as they are bound to other misfolded proteins. This leads to reversible aggregation of TDP-43, which can directly affect its mRNA binding partners thus altering its function (2014, Figure 1.8).

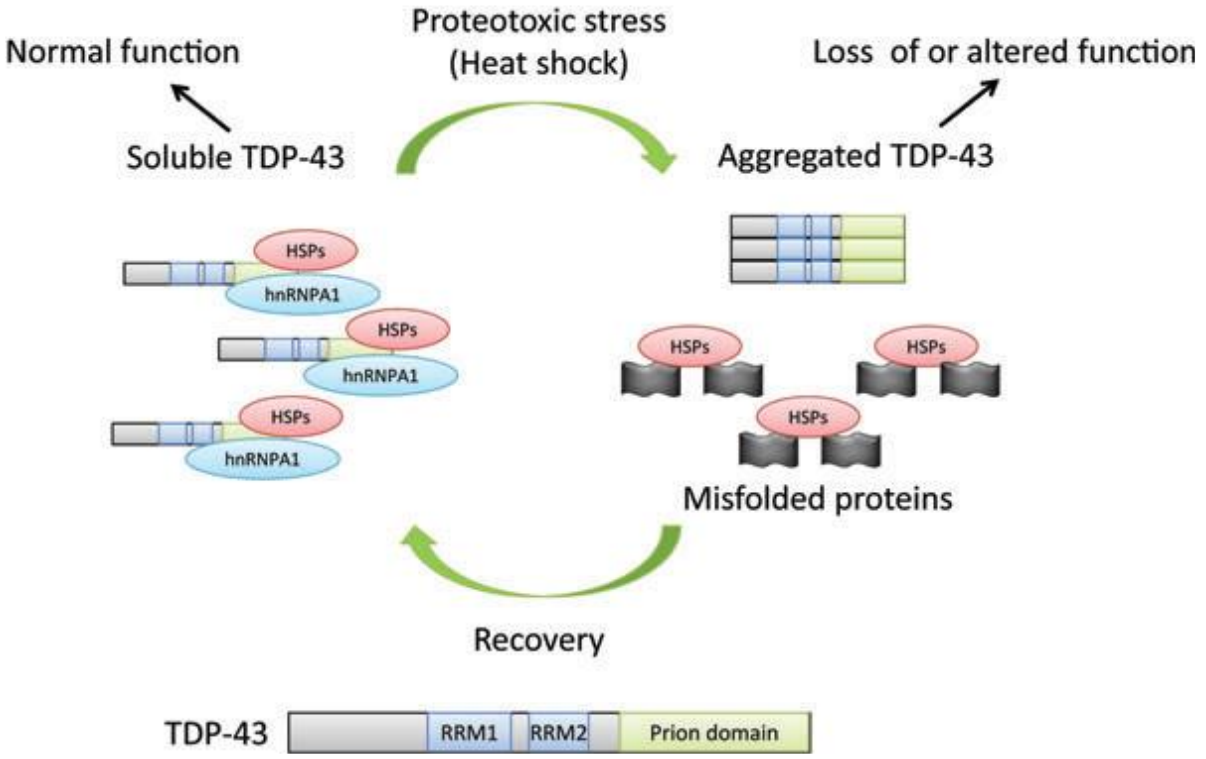

Figure 1.8: Model of the TDP-43 prion-like domain as a sensor of misfolded protein stress. TDP-43 is constitutively bound to heat shock proteins to keep the prion domain in a soluble conformation and able to interact with its normal binding partners. During heat shock chaperones are titrated away by increased amounts of misfolded proteins in the cell, leading to the self-aggregation of TDP-43, loss of interaction with its binding partners and loss of function. As cells recover from heat shock and new chaperones are translated, they disaggregate TDP-43 and return it to a normal state (Udan-Johns *et al.*, 2014).

While studies thus far have focussed exclusively on the role of intracellular chaperones, one paper published in 2013 has investigated the relevance of the extracellular chaperone clusterin to ALS, using cultures of mouse spinal cord and MT SOD1 transgenic mice (Zinkie *et al.*, 2013). Clusterin functions as a chaperone with mechanisms similar to that of heat shock proteins, and its transcription can be activated by the heat shock transcription factor Hsf-1 (Wilson and Easterbrook-Smith, 2000; Michel *et al.*, 1997), making it a good candidate for investigation as a potential therapeutic agent. Zinkie *et al.* (2013) examined mouse spinal cord dorsal root ganglion cultures and found that clusterin was constitutively expressed in astrocytes and motor neurons of healthy cells and was secreted into

the culture medium. Expression was increased by geldanamycin, a Hsp90 inhibitor that induces Hsps by releasing Hsf-1, with expression increasing more in astrocytes. Cell cultures expressing MT SOD1 and transgenic SOD1 mice were also created to investigate the effect of MT SOD1 expression. The expression of MT SOD1 had no effect on clusterin level in cell cultures, but *in vivo* clusterin was slightly increased in the spinal cord of symptomatic mice. Most importantly, clusterin was identified as a constituent of intracellular inclusions that also contained SOD1, supporting the Nizard *et al.*, (2007) study showing that clusterin can be retrotranslocated from the ER to the cytoplasm under some stress conditions. These results reveal similarities between Hsps and clusterin in ALS models, for example clusterin has a similar chaperone function to Hsps, and SOD1 containing inclusions in transgenic mice have been shown to contain clusterin and Hsc70 (a constitutively expressed form of Hsp70; Watanabe *et al.*, 2001; Zinkie *et al.*, 2013). The role of extracellular chaperones, particularly clusterin in ALS is thus worth pursuing.

Overall, it appears that chaperones are able to influence and slow the aggregation of proteins involved in ALS, at least in the early stages of disease, and that the availability of chaperones within motor neurons influences their susceptibility to neurodegeneration. Motor neurons are less able to upregulate some chaperones, and are thus more vulnerable to damage. While chaperones may be able to successfully prevent aggregation and neurodegeneration in the early stages of disease, the supply can become depleted as chaperones are overwhelmed, leaving motor neurons vulnerable and unable to maintain protein quality control, resulting in the formation of insoluble protein aggregates. It is also apparent that up regulating multiple chaperones has a greater protective effect than up regulating a single chaperone. Current studies have focused on the interactions between intracellular chaperones and SOD1, and very little has been done to examine the effects of chaperones on the aggregation and pathogenicity of TDP-43. In order to fully understand the role of TDP-43, more detailed *in vitro* and *in vivo* work needs to be undertaken. In light of the recent studies showing that clusterin is able to be retrotranslocated to the cytosol in times of stress such as ALS, where it may act as an intracellular chaperone, potential interactions between TDP-43 and clusterin present an exciting new area for study.

1.9.4 Significance and Implications in Disease Treatment

ALS is a common neurodegenerative disease in adults and is responsible for the death of ~ 10 Australians per week (MND Australia, 2013). There is currently no cure for ALS and treatment options are extremely limited. There has been little success in the search for neuroprotective agents. Clinical trials have investigated several novel ALS therapeutics including antioxidants, anti-inflammatory or immunomodulatory drugs, calcium regulatory drugs, anti-glutamate, energy metabolism, and trophic factors (Cleveland and Rothstein, 2001). Of all these therapeutics, only the

glutamate inhibitor riluzole has a modest effect, prolonging the life of ALS sufferers by ~ 3 months. As outlined in section 1.4.2, the pathogenic mechanisms mediating neurodegeneration in ALS are probably multifactorial, with glutamate induced excitotoxicity representing only one of many overlapping pathogenic mechanisms. It is possible that all of the pathogenic mechanisms involved in ALS will need to be targeted in order for an effective therapeutic treatment to be produced. This can only be achieved once these mechanisms are better understood. Studies investigating the interactions between chaperones, TDP-43 and SOD1 provide new insight into the mechanisms underlying ALS pathology and how the body has developed defences to inhibit protein aggregation. Increased knowledge of these interactions could lead to the development of new therapeutics for the treatment of ALS which may eventually include the use of chemical or peptide chaperones to prevent the aggregation or spread of proteins, or increasing the expression of chaperones and their receptors. Increasing the expression of multiple chaperones with different and complimentary cytoprotective functions could have a greater therapeutic potential then increased expression of one chaperone alone.

1.10 CONCLUSIONS

The folding of an amino acid sequence into its correct native three dimensional structure is vital for a protein to perform its normal function. As a result of mutations or various stress conditions, proteins may become unstable, unfold and form aggregates, a process associated with many harmful protein deposition diseases. The body has evolved intra- and extracellular systems of protein quality control to maintain proteostasis, with molecular chaperones being a key component in these systems. Protein aggregation has recently been implicated in the pathology of the incurable neurodegenerative disease ALS. ALS is characterised by the selective degeneration of upper and lower motor neurons, together with the accumulation of insoluble and ubiquitinated intraneuronal inclusions. Some proteins involved in the pathology of ALS have been shown to have “prion-like” properties, with SOD1 being capable of both self-propagation and cell-to-cell spread. TDP-43 is another protein that can self-propagate, but cell to cell spread has yet to be observed. Aggregating proteins can contribute to neurodegeneration by overwhelming chaperones and the ubiquitin-proteosome system and disrupting proteostasis. Manipulation of chaperone expression levels or the development of chemical or peptide chaperones could ultimately provide a novel therapeutic strategy for the treatment of ALS by either preventing protein aggregation, or limiting cell-to-cell spread of prion-like proteins. The intracellular chaperones αB-crystallin, Hsp70, and Hsp27/25 can all influence the aggregation of SOD1, a protein present in the intraneuronal inclusions of MT SOD1 associated FALS. More detailed analysis of the interactions between other chaperones and a wide range of aggregating proteins involved in ALS will provide a better understanding of the pathogenic mechanisms underlying the disease, and aid in the development of new therapeutics for its treatment.

The results in this thesis are divided into three separate chapters with each chapter containing a different approach undertaken to examine the effects of clusterin on the aggregation and pathogenicity of TDP-43. Each result chapter contains an individual introduction, methods and discussion section specific to the experiments performed. A general materials and methods chapter and general conclusions chapter are also presented. Additionally, the specific aims of this thesis are given in the introduction section of each results chapter, but overall this thesis aimed to;

1) Assess the ability of TDP-43 to spread from one cell type to another *in vivo* using a *Drosophila* model of ALS and *in vitro* using neuronal and astrocyte cell lines.
2) Investigate the extent to which non-neuronal cells contribute to disease progression *in vivo* by expressing TDP-43 in a subset of glial cells of *Drosophila* to determine if this resulted in motor neuron defects and premature mortality.
3) Investigate interactions between clusterin and TDP-43 *in vitro*, *in vivo* using the *Drosophila* model, and in post mortem human tissue from ALS patients.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 BUFFERS AND MEDIA

Buffers and media that were used throughout this project include: phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 11.6 mM Na_2HPO_4, pH 7.4), TAE buffer (40 mM TRIS, 20 mM acetic acid, 1 mM EDTA, pH 8), hypotonic lysis buffer (10 mM HEPES, pH 7.4), 10 x HEPES buffer (0.5 M HEPES, 1 M NaCl, pH 7.4), and Luria-Bertani (LB) broth (10% [w/v] NaCl, 10% [w/v] Tryptone, 5% [w/v] Yeast extract). Details of other less commonly used buffers and media are given in their respective sections.

2.2 MATERIALS

A list of materials and chemicals used in this thesis, along with full names, abbreviations and suppliers are given in Table 2.1. Details of any materials not listed in Table 2.1 are given in their respective sections.

Table 2.1: Materials and chemicals used

Name	Company/Supplier
Acetic acid (CH_3COOH)	Ajax Chemicals
Agarose	Promega
Ampicillin ($C_{16}H_{19}N_3O_4S$)	Sigma
Bovine serum albumin (BSA)	Sigma
Ready to use Target Retrieval Solution	Dako
di-Sodium Hydrogen Orthophosphate (Na_2HPO_4)	Ajax Chemicals
DMEM/F-12 Media	Invitrogen
Ethanol	Ajax Chemicals
Ethylenediaminetetraacetic Acid (EDTA)	Sigma
Foetal calf serum (FCS)	Bovogen
HEPES ($C_8H_{18}N_2O_4S$)	Sigma
Methanol (CH_3OH)	Unichrom

Potassium chloride (KCl)	Ajax Chemicals
Potassium di-hydrogen orthophosphate (KH_2PO_4)	Ajax Chemicals
Schneider's medium	Sigma
Sodium chloride (NaCl)	Biochemicals
Sudan Black B ($C_{29}H_{24}N_6$)	Sigma
Thioflavin-T (ThT; $C_{17}H_{19}CIN_2S$)	Sigma
Tris ($C_4H_{11}NO_3$)	Sigma
Triton X-100 (C_2H_4O)n$C_{14}H_{22}O$	Sigma
Trypsin-EDTA 0.05% (1X)	Gibco by Life Technologies
Tryptone	Sigma
Tween-20 ($C_{58}H_{114}O_{26}$)	Sigma
Xylene substitute	Sigma
Yeast extract	Sigma

2.3 HUMAN SPINAL CORD TISSUE

Human thoracic spinal cord tissue sections from sporadic amyotrophic lateral sclerosis (SALS; n = 2) and control (n=3) individuals were obtained from the Victorian Brain Bank Network. The specimens had been embedded in paraffin and cut into 8 µm sections and mounted on Superfrost Plus slides. The tissue reference number (Ref #), age, gender, post mortem interval (PMI) times and clinical diagnosis for each are given in Table 2.2.

This work has been approved by the Human Research Ethics Committee, University of Wollongong (UoW). The UoW Human Ethics approval number is: HE11/217 - Proteomic analysis of protein inclusions in tissues from frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) patients.

Table 2.2: Spinal cord tissue samples used for immunohistochemical analysis of motor neurons.

Ref #	Age (years)	Gender	PMI (hours)	Diagnosis
09/066	78.1	Male	14	SALS
10/223	62	Male	13	SALS
04/273	52.1	Male	33	Control
05/418	75.9	Male	50	Control
07/743	63.9	Male	68	Control

2.4 IMMUNOHISTOCHEMISTRY

2.4.1 Human tissue

The tissue sections were deparaffinised and rehydrated by sequential immersion in xylene substitute (3 x 5 min), then graded concentrations of ethanol/water mixture (3 x 2 min in 100% ethanol, 2 min in 90% ethanol, 2 min in 70% ethanol) and finally milli Q water (2 min). Tissue sections were then submerged in ready-to-use Dako Target Retrieval Solution and heated at 94-96 °C for 30 min. Blocking was performed overnight by submersing the tissue sections in filtered 1% (w/v) BSA/PBS at 4 °C. The sections were then incubated with polyclonal goat anti-clusterin (1/200; Rockland) and polyclonal rabbit anti-TARDBP (1/500; Proteintech Europe) in 1% BSA/PBS overnight at 4 °C and subsequently washed 3 times in PBS for 30 seconds. The sections were then incubated with Alexa Fluor® 594 donkey anti-rabbit Ig (2.5 µg/mL; Life Technologies) and biotinylated donkey anti-goat Ig (1/400; Sigma) in 1% BSA/PBS for 1 h at room temperature. Tissue sections were then washed 3 times for 5 min with 0.02% (v/v) tween/PBS, followed by incubation with streptavidin Alexa Fluor® 488 conjugate (2.5 µg/mL; Life Technologies) in 1% BSA/PBS for 30 min at room temperature (RT). The sections were then washed 3 times for 10 min with 0.02% tween/PBS. Background fluorescence was quenched by incubating for 3 min in filtered 0.5% (w/v) Sudan Black in 70% ethanol. The tissue was then dipped 4 times in PBS followed by 4 x 5 min washes in 1% (v/v) triton X-100/PBS (PBST) with shaking and 3 x 5 min washes in PBS. Mounting was performed using Vectashield hard set mounting medium for fluorescence (Vector). Confocal microscopy of the sections was performed using a Leica SP2-405 inverted microscope equipped with 488 and 594 nm laser lines. Images were captured using a 20 and 63 X lens. Following microscopy, the images were merged using Image J (National Institutes of Health) and re-sized using Adobe Photoshop and Illustrator CS6 software (Adobe Systems).

2.4.2 *Drosophila*

Brains from 3rd instar larvae (Figure 2.1) as well as adult female brains were dissected in cold Schneider's insect medium and immediately transferred to ice cold 3.7% formaldehyde in 0.05% (v/v) PBST (fixative solution). Brains were fixed within half an hour of dissection by incubating in fixative solution at RT for 30 min, then washed (2 x 1 min then 2 x 20 min) using 0.5% (v/v) PBST at RT. Methanol was then added to the samples and left overnight on a bench-top rotator (~ 5 rpm) at 4 ºC. After discarding the methanol, the samples were washed again (2 x 1 min then 2 x 20 min) using 0.05% (v/v) PBST at RT followed by incubation with primary antibody diluted in 5% (w/v) BSA in 0.05% (v/v) PBST on a bench-top rotator (~ 5 rpm) at 4 ºC overnight. When staining for HA tagged TDP-43 the primary antibody used was anti-HA high affinity (Roche, details given in Table 2.3). Motor neurons were detected using anti-Elav-9F89 (Developmental studies hybridoma bank; details given in Table 2.3) to label the nuclei and F43 anti-discs large (Developmental studies hybridoma bank; details given in Table 2.3) to stain for cell membranes. After removal of primary antibody, brains were washed again (3 x 1 min then 3 x 10 min) with 0.05% PBST at RT, followed by incubation with the secondary antibody in 5% (w/v) BSA in 0.05% (v/v) PBST on a on a bench-top rotator (~ 5 rpm) at 4 ºC overnight. Secondary antibodies used were either anti-rat Alexa Fluor® 594 or anti-mouse Alexa Fluor® 488. The secondary antibody was removed and a final wash (3 x 1 min then 3 x 10 min) with 0.05% (v/v) PBST was performed at RT. Brains were then transferred to a clean microscope slide (Fronine) and mounted using Vectashield hard set mounting medium for fluorescence (Vector). Clear nail varnish was used to seal the edges of the coverslip. Following mounting, brains were imaged within 48 h using a Leica SP2-405 inverted confocal microscope.

Table 2.3: Antibodies used for *Drosophila* immunohistochemistry

Antibody	Dilution	Animal	Company
anti-HA high affinity	1/1000	Rat	Roche
anti-rat Alexa Fluor® 594	1/1000	Donkey	Life Technologies
anti-Elav-9F8A9	1/100	Mouse	Developmental studies hybridoma bank (University of Iowa, USA)
anti-mouse Alexa Fluor® 488	1/1000	Goat	Life Technologies
F43 anti-discs large	1/100	Mouse	Developmental studies hybridoma bank (University of Iowa, USA)

2.5 *DROSOPHILA*

2.5.1 *Drosophila* maintenance and lifespan

Fly stocks were cultured according to the "Basic Methods of Culturing *Drosophila*" guidelines provided by the Bloomington *Drosophila* stock centre (Bloomington, Indiana). Briefly, flies were cultured in 4 inch glass vials containing cornmeal media (104.33 g/L agar, 875 g/L dextrose, 875 g/L maize, 175 g/L yeast, 29.16% (v/v) nipagin) at 25 ºC and 65-75% humidity unless otherwise stated. Flies were anaesthetised using CO_2 and examined under a dissecting microscope. Virgin flies were collected within 8 hours of eclosion at 25 ºC, or within 16 hours at 18 ºC over the course of 1 or 2 weeks. Generally for each cross performed 20 virgin females were collected and mated with 5 males. The lifespan of *Drosophila* is split into three main developmental stages; embryonic, larval and adult, and the reproductive life cycle changes depending on what temperature they are kept at. Briefly, at 25 °C the reproductive life cycle of a *Drosophila* is about 10 days, with a newly laid egg undergoing embryogenesis after ~ 24 hours, pupa forming at ~ day 5-6, and adults emerging from the pupal case (eclosion) at ~ day 10. Females are able to mate 12 hours after eclosion (Figure 2.1). When flies are kept at 18 °C, each developmental stage takes twice as long. For example an adult will take 20 days to emerge from the pupal case.

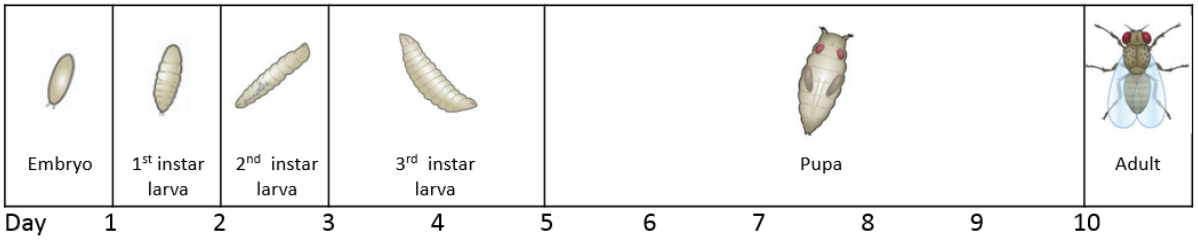

Figure 2.1: Overview of the various developmental stages of the *Drosophila* lifespan (modified from Ong *et al.*, 2015).

2.5.2 *Drosophila* stocks used

Fly stocks used and their origin are summarised in Table 2.4. Full names of fly lines (where available) are given, with any short hand names used throughout this thesis in bold in brackets. Flies were either obtained from Bloomington, self-generated by members of the Dobson lab, or gifted from other members of the University of Cambridge Chemistry Department. Detailed descriptions of what each fly line does and how they were used in experiments are given in the methods section of the respective chapters.

Table 2.4: Fly stocks used.

Bloomington	Self-generated	Gift
P{GAL4-elav.L}2	deaat-1-GFP;Gal80	UAS-clusterin (**Clu;** Sarah Meehan)
UAS-mCD8::RFP		UAS-TDP-43;clusterin **(TDP;Clu;** Leila Luheshi**)**
dEAAT1-Gal4::UAS-GFP		HA tagged UAS-TDP FL **(TDP-43;** Leila Lueshi)
P{Eaat-Gal4.R}2 **(dEAAT1-Gal4)**		UAS-mCD8::GFP (Teresa Pereira de Barros)
51D(yM{int.Dm}ZH2Aw*;M{3x P3RFP.attP}ZH-51D) **(51D)**		
W[1118]		

2.6 QUANTITATIVE COLOCALISATION ANALYSIS OF MULTICOLOUR CONFOCAL MICROSCOPY IMAGES

Quantitative colocalisation analysis was used to determine the degree of colocalisation in multicolour fluorescence microscopy images. Colocalisation was determined by calculating values which represent the proportion of colocalised pixels in an image. These values were calculated using the Pearson's coefficient and the overlap coefficient, whose formulas are given below.

2.6.1 Pearson's correlation coefficient (R_r)

$$R_r = \frac{\sum_i (S1_i S1_{\text{ave}}) \times (S2_i - S2_{\text{ave}})}{\sqrt{\sum_i (S1_i - S1_{\text{ave}})^2 \times \sum_i (S2_i - S2_{\text{ave}})^2}}$$

Where $S1$ and $S2$ represent signal intensity of pixels in channels 1 and 2 respectively; and $S1_{ave}$ and $S2_{ave}$ are the average intensities of these channels (Demandolx and Davoust, 1997).

2.6.2 Overlap coefficient

$$R = \frac{\sum_i S1_i \times S2_i}{\sqrt{\sum_i (S1_i)^2 \times \sum_i (S2_i)^2}}$$

Where *S*1 and *S*2 represent signal intensity of pixels in channels 1 and 2 respectively (Manders *et al.*, 1993).

2.7 IN VITRO PROTEIN AGGREGATION ASSAY

Clusterin was purified from human plasma obtained from Wollongong Hospital (Wollongong, NSW, Australia) as previously described (Wilson and Easterbrook Smith, 1992). The synthetic peptide corresponding to residues 286-331 of TDP-43 was obtained from China Peptides and Thioflavin T from Sigma**.** TDP-$43_{286\text{-}331}$ was dissolved in water (pH 11.3), followed by the addition of 10 X HEPES buffer (0.5 M HEPES, 1 M sodium chloride, 0.001% azide, pH 7.4), so that the final concentration of TDP-$43_{286\text{-}331}$ was approximately 448 µM. TDP-$43_{286\text{-}331}$ (224 µM) was incubated at 37 °C in 1 X HEPES buffer (0.05 M HEPES, 0.1 M NaCl, pH 7.4) with no additions, or containing SOD1 at 2 µM or clusterin at 0.22, 0.022, 0.011, or 0.008 µM whilst shaking for 16 h in a 384 well plate. SOD1 was also incubated without the addition of TDP-43 (data not shown). Thioflavin T (20 µM) was added to each well prior to incubation in a FLUOstar OPTIMA with an excitation filter of 440 +/- 10 nm and an emission filter of 490 +/- 10 nm.

2.8 TISSUE CULTURE

2.8.1 Cultured cell lines

Murine motor neuron-like hybrid cells (NSC-34), murine astrocyte cells (Ast-1) and murine Neuro-2a (N2a) neuroblastoma cells were obtained from American Type Culture Collection. The three cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS; Bovogen Biologicals) at 37 °C in a humidified, 5% (v/v) CO_2 atmosphere unless otherwise stated.

2.8.2 Transient transfection of cultured cells

DNA for use in transfections (see section 2.8.3) was purified using a QIAGEN Maxi plasmid purification kit, according to the manufacturer's instructions. DNA concentration and purity was determined by UV spectrophotometry at 260 and 280 nm. One day before transfection, cells were

plated so that they were 70-95% confluent at the time of transfection. Cells were transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions, using a Lipofectamine™ 2000: DNA ratio of 4 µL:1 µg. Transfected cells were incubated at 37 °C for 48 hours prior to experimental use.

2.8.3 Plasmids

The following 3 plasmids were used for the transient transfection of cultured cells;

1) pEGFP-N1 (Invitrogen). This plasmid was used as a positive control for all transfections performed, and encodes enhanced green fluorescent protein (EGFP).
2) pEGFP-N1-TDP-CTF (Addgene, #28197). This plasmid carries an insert for amino acids 216-414 of human TDP-43, tagged with EGFP at the carboxy terminus.
3) M337V TDP-43-TurboGFP (Origene, #RG210639). Originally encoding for WT TDP-43 (GFP tagged), the M337V mutant was created and gifted by Associate Professor Ian Blair.

2.8.4 Cell lysis

Cells to be lysed were removed from tissue culture flasks with 0.05% Trypsin-EDTA (1x; Gibco by Life Technologies), washed with DMEM supplemented with 1% (v/v) FCS, and then centrifuged at 300 x g for 5 min at RT. The pellet was then resuspended in sterile ice cold lysis buffer (10 mM HEPES, pH 7.4) with cOmplete protease inhibitor cocktail (Roche) and 0.1 mg/mL DNAse (Sigma) and kept on ice for 20 min. Cells were then lysed in a gentleMACS™ dissociator (Miltenyi Biotec) using the "Homogenization of tissue for protein extraction" protocol and the "Protein_01" program.

2.8.5 Cell stress and calcein orange labelling

Cells transfected with TDP-43-GFP were incubated overnight (16 h) in 20 µM MG-132 (Sigma) to inhibit the proteasome. They were then detached with 0.05% Trypsin-EDTA (1x; Gibco by Life Technologies), washed with DMEM supplemented with 1% (v/v) FCS, and centrifuged at 300 x g for 5 min at RT. The pellet was then resuspended in 20 µM Calcein Orange™ (AAT Bioquest) for 30 min at 37 °C, followed by washing with PBS. Cells were either analysed by flow cytometry as detailed below or imaged by confocal microscopy. Cells that were destined to be analysed by confocal microscopy were grown on round coverslips placed inside the wells of tissue culture plates. Coverslips were removed from the wells, washed 3 X with PBS and then mounted on a clean microscope slide using Vectashield hard set mounting medium. Confocal microscopy of the slides was performed using a Leica TCS SP5 II microscope. Following analysis, images were merged using Image J software.

2.9 FLOW CYTOMETRY

Following detachment by 0.05% Trypsin-EDTA (1x; Gibco by Life Technologies), cells were centrifuged as described above and washed with PBS. Pellets were resuspended in 400 µL of PBS and transferred to FACS tubes. Before analysing each sample, 1 µL of the nuclear stain RedDot™2 (Biotium) was added to the samples and allowed to incubate for 10 min at RT. This was to stain the nuclei of dead cells, which were electronically excluded from the analysis. Flow cytometry was performed on a BD LSR II or BD LSRFortessa X-20 flow cytometer (Becton Dickinson). Excitation was at 488 nm (blue laser) and emissions were collected at 695 +/-40 nm (RedDot2), 575 +/-26 nm (Calcein Orange) and 525 +/-20 nm (pEGFP). Data was collected using FACSDiva software (Becton Dickinson) and analysed using FlowJo software (TreeStar Inc).

CHAPTER 3: INVESTIGATING THE SPREAD AND TOXICITY OF TDP-43 IN A LARVAL *DROSOPHILA* MODEL OF ALS

3.1 INTRODUCTION

ALS is a common neurodegenerative disease characterised by a focal point of onset and contiguous spread of symptoms. It has recently been proposed that "prion-like" mechanisms of protein propagation represent a pathogenic pathway which could explain the outward pattern of spread observed in ALS. Prions are proteins that can propagate a self-replicating conformation, spread between cells and transmit a disease phenotype. Many studies have been conducted to investigate prion-like properties of SOD1, and have found it capable of both self-propagation and cell-to-cell spread. TDP-43 contains a glycine rich C-terminal domain that resembles prion domains and studies have shown that TDP-43 is capable of aggregating in a self-templating manner and cell-to-cell spread in cultures of SH-SY5Y cells (Nonaka *et al*., 2013). However it remains to be determined if it can spread between cells or tissues *in vivo.* This study aimed to investigate for the first time whether TDP-43 is able to spread cell-to-cell *in vivo* by utilizing a *Drosophila* model in which TDP-43 is expressed in a specific subset of glial cells, and if the co-expression of the extracellular chaperone clusterin can influence any spread.

Clusterin was introduced briefly in Chapter 1 as a key component of the extracellular protein quality control system, with the ability to ATP-independently inhibit aggregation of a wide range of proteins. The clusterin gene is located on chromosome 8p21-p12 in humans and is ubiquitously expressed during both development and in adults (Trougakos, 2013). Clusterin is first expressed in the ER as a glycosylated precursor protein, which undergoes further N-linked glycosylation and intracellular cleavage producing a 75-80 kDa heterodimer of α and β subunits linked in anti-parallel by 5 disulfide bonds, which is then secreted into the extracellular space (sClu; DeSilva *et al*., 1990; Burkey *et al*., 1991). There is currently no X-ray crystallography data available for clusterin but it is predicted to contain three amphipathic α-helices, and two coiled-coil α-helices (Figure 3.1; Jenne and Tschopp, 1989; DeSilva *et al*., 1990). It is thought that these amphipathic helices mediate the binding of clusterin to a diverse array of molecules (Bailey *et al*., 2001). A nuclear localisation signal has also been identified (Jones and Jormay, 2002).

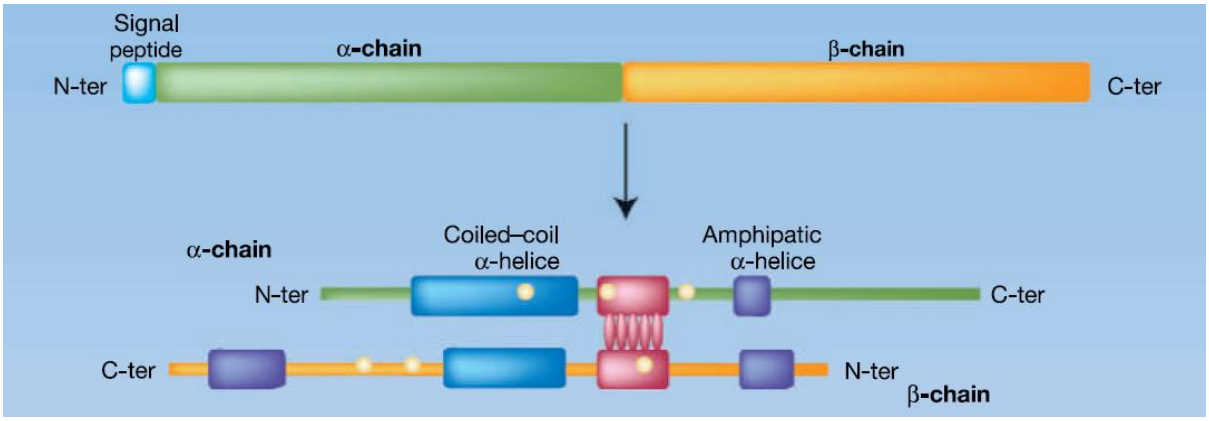

Figure 3.1: Schematic representation of the predicted structure of human clusterin. The precursor peptide is cleaved between amino acids 22 and 23 to remove the signal peptide (light blue) and between residues 227 and 228 to generate the α (green) and β (orange) chains. These chains are assembled in antiparallel to form a mature heterodimer consisting of a cysteine rich centre (red) linked by 5 disulfide bridges (red ovals). The bridges are flanked by two coiled-coil α-helices (blue) and three amphipathic α-helices (purple). Yellow dots indicate N-glycosylation sites (Zoubeidi *et al*., 2010).

In addition to secreted clusterin, alternative isoforms of clusterin have also been proposed, including a truncated form which localises to the nucleus, although the existence of this alternative isoform and its relevance are controversial. Blot analysis of nuclear lysate show a 45-55 kDa band identified as nuclear clusterin (nClu) by several papers (Reddy *et al*., 1996; Yang *et al*., 2000) and clusterin has been imaged in the nucleus of stressed and dying cells by immunofluorescence microscopy (Leskov *et al*., 2003; Yang *et al*., 2000), but more recent work could not confirm the nuclear localisation of clusterin, even in apoptotic cells (Prochnow *et al*., 2013) and the presence of nClu has yet to established in live cells. While sClu is cytoprotective, enhanced expression and accumulation of nClu has been proposed to promote cell death, for example during apoptosis (Reddy *et al*., 1996), and treatment with ionizing radiation (Yang *et al*., 2000), tumor necrosis factor alpha or anti-estrogen (O'Sullivan *et al*., 2003). However the amount of endogenous clusterin isoforms present (even in stressed cells) is very low compared to sClu, suggesting that the "alternative isoforms" are physiologically irrelevant and do not account for the reported changes in subcellular distribution of clusterin (Prochnow *et al*., 2013). Greater detail about why clusterin was chosen for this study will be given in the introduction of Chapter 4 (4.1).

Drosophila was an attractive model to use for this study, as they are already extensively used to study many neurodegenerative diseases including Alzheimer's, Parkinson's, Huntington's and of course

ALS. Expressing TDP-43 in *Drosophila* can recapitulate many aspects of ALS disease pathology. For example the expression of full length, mutant, or a C terminal fragment of hTDP-43 in the motor neurons of flies causes loss of nuclear TDP-43, accumulation of cytoplasmic TDP-43, and motor dysfunction (Voigt *et al*., 2010). It is possible to express a wide variety of TDP-43 variants (for example human or *Drosophila*, various mutations, truncated, or tagged) in any number of expression sites such as the eye, sensory neurons, motor neurons, all neurons, all glia or subsets of glia. Additionally the nervous system of *Drosophila* shares many functional and structural features with that of the vertebrate nervous system, with its neurons and glia organised into centres with specific functions. Glial cells make up ~90% of cells in the vertebrate nervous system and have important roles in nourishing, supporting and insulating neurons, and are also required for the proper function and development of the nervous system (Shin *et al*., 2005; Awasaki *et al*., 2008). In vertebrates glial cells are divided into the following types, astrocytes (provide support for neuronal growth), oligodendrocytes (ensheath and myelinate axons), microglia (immune cells that respond to infection and trauma) and Schwann cells (Doherty *et al*., 2009). *Drosophila* provide an easily genetically manipulated system for the study of neurodegenerative diseases as their glia are divided into distinct types that share many morphological and molecular similarities to those in vertebrates, and their central nervous system (CNS) provides a relatively simple but well characterised model (Doherty *et al*., 2009). Glial cells in the CNS are classified into three types; surface, cortex and neuropil associated glial cells. Surface glia, as the name suggests, are closely associated with the CNS surface and form a sheath around the brain, isolating it from the haemolymph of the fly's open circulatory system. Sometimes these cells protrude into the cortex and fill the space between outer neurons; they also play a role in regulating CNS permeability and act as the blood brain barrier. Cortex glia enwrap individual neurons in the cortex in a three-dimensional scaffold. They may have roles in providing trophic support for neurons as well as modulating neuronal functions. Cortex glia share structural and developmental characteristics with vertebrate astrocytes. Neuropil glia lie further inside the brain and surround the neuropil, and play important roles in axon path-finding (Doherty *et al*., 2009; Awasaki *et al*., 2008; Ito *et al*., 1995). Neuropil glia are comparable with oligodendrocytes in vertebrates (Pereanu *et al*., 2005).

Glial cells have an important role in protecting against excitotoxicity by clearing excess excitatory neurotransmitters, such as glutamate, in the mammalian CNS. Increased levels of glutamate are seen in various neurodegenerative diseases including ALS. In vertebrates the clearing of neurotransmitters is carried out by excitatory amino acid transporters (EAATs), which are expressed mainly in glial cells or in neurons (Besson *et al*., 1999). *Drosophila* has a single high affinity glutamate transporter termed dEAAT1, which is selectively expressed in a subset of differentiated glia in the CNS and not in neurons. It is expressed in 10% of glial cells in the larval and adult CNS (Rival *et al*., 2004). In the

Drosophila model of ALS used in this study, TDP-43 was expressed in these dEAAT1 expressing glial cells. Although pan-glial drivers such as the REPO-Gal4 line exist, TDP-43 was only expressed in the dEAAT1 positive subset of glial cells for several reasons. Firstly, it was hoped that by expressing TDP-43 in a smaller subset of glial cells, any potential toxicity would be reduced, making any phenotypic effects of clusterin co-expression easier to observe. Secondly, it was not known how much if at all TDP-43 would spread from the glial cells where it was expressed. Again the aim was to make any spread of TDP-43 and potential effect of clusterin more visible and easy to image by limiting expression to a smaller subset of cells. Finally, a system was desired that would give the highest insurance that the proteins were expressed in glial cells only and not in neurons. In *Drosophila* glia and neurons have a common precursor, neuroglioblasts. Various transcription factors are responsible for determining the fate of these precursor cells, including the glial differentiation factor *reversed polarity* (REPO), which promotes differentiation to form glia, while dEAAT1 is expressed only in subsets of glia that are already differentiated (Lee and Jones, 2005; Soustelle, 2002; Yuasa *et al*., 2003). It would be ideal to have a driver protein found in glia whose expression was as far removed from the common precursor cells as possible, thus the dEAAt1 driver line was chosen. In summary, a *Drosophila* model of ALS where TDP-43 and/or clusterin could be expressed in a subset of glial cells was created and used to address the following aims:

1) Determine if expression of human TDP-43 (hTDP-43) in a subset of glial cells from the embryonic life stage onwards was sufficient to cause premature lethality and locomotor defects.
2) Determine if the co-expression of clusterin was able to influence lethality or locomotion.
3) Determine if hTDP43 could spread from glia to surrounding cells and if clusterin could influence any spread.
4) Identify cell types that hTDP-43 was spreading to.

3.2 METHODS

There are many systems available to control the expression of both endogenous and transgenes in *Drosophila*. This study utilised the GAL4/UAS system, which allows precise spatial control of any given transgene (Brand and Perrimon, 1993). A DNA plasmid containing a tissue-specific promoter region able to promote expression of the GAL4 gene is stably integrated into the genome of one fly line. In this "driver" fly line, the GAL4 protein is produced only in the tissue of interest but has no target gene to activate. In a second ("reporter") fly line, a DNA plasmid containing a GAL4 dependant gene of interest downstream of a unique GAL4 binding site (or upstream activation sequence, UAS) is stably integrated into the genome. Every cell has the UAS but the gene of interest is silent in the absence of the GAL4 activator. When the GAL4 and UAS fly lines are crossed, in the offspring

inheriting both engineered sequences, GAL4 will be expressed in a cell or tissue specific manner and bind to the UAS, thus activating expression of the target gene in the desired tissue (Figure 3.2; Brand and Perrimon, 1993; Konsolaki, 2013; Nichols, 2006). A description of the fly lines used and crosses performed in this study are given below. Flies were maintained according to the methods outlined in section 2.5.1 unless otherwise stated.

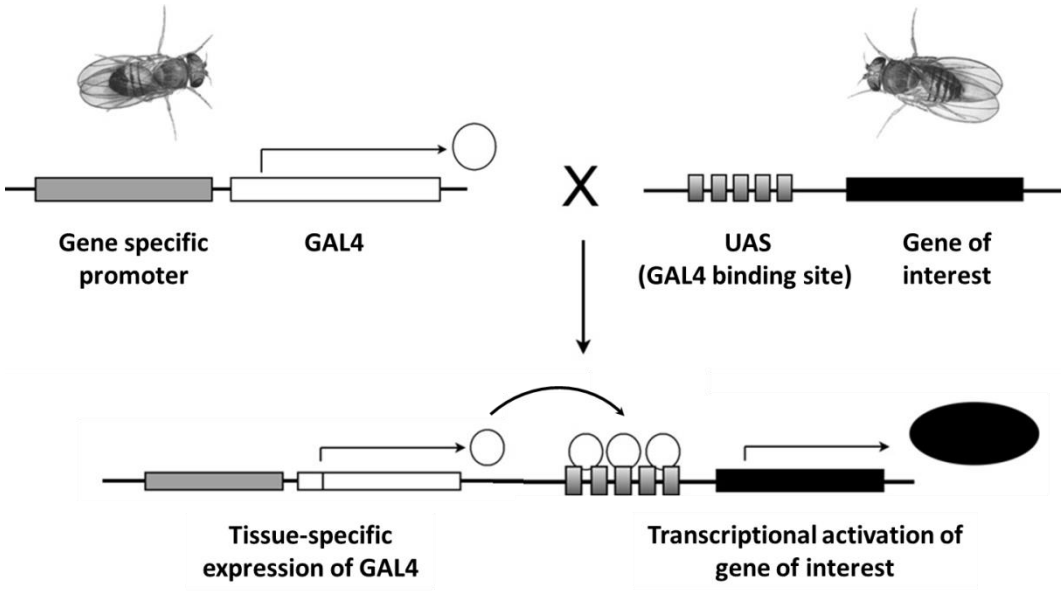

Figure 3.2: The GAL4/UAS expression system. One fly line is generated to express the yeast transcription factor GAL4 under the control of a gene specific promoter. A second line is generated that has a gene of interest behind the Gal4 binding site (UAS). When these two strains are mated, the progeny express the gene of interest exclusively in the tissues defined by the gene specific promoter (modified from Nichols, 2006).

3.2.1 dEAAT1-Gal4 driver line crosses

As discussed in section 3.1, the dEAAT1 protein is expressed in about 10% of glial cells in the larval CNS. The dEAAT1-Gal4 driver line produces the Gal4 protein only in dEAAT1 expressing cells. Male dEAAT1-Gal4 driver line flies were crossed with virgin female "gene of interest" flies. In this case these were UAS-TDP-43, UAS-clusterin, or UAS-TDP-43;clusterin flies (TDP-43, Clu, or TDP;Clu respetively). The progeny of these crosses will have inherited a copy of the gene of interest from their mother and a copy of the driver line from their father, and will thus express the gene of interest in dEAAT1 positive cells only. As a negative control, UAS-51D (51D) flies were crossed with the dEAAT1-Gal4 driver line. 51D flies do not have a "gene of interest" insert, but have the same genetic background as the TDP-43 flies. Once the progeny reached the third instar larval stage they were dissected and immunohistochemistry performed according to the methods outlined in

section 2.4.2. Additionally, to test for any non-specific interactions of the secondary antibodies used, progeny from the above four crosses were stained for TDP-43 as outlined in section 2.4.2, but with the omission of primary antibodies. For each cross 10 larvae brains were dissected for staining. This was the maximum number possible as each brain had to be fixed within 30 minutes of being dissected. Of the 10 brains dissected for each cross, normally between 2-3 brains would be lost or damaged during staining and washing. All remaining brains were examined by confocal microscopy and ~5-12 images were collected from ~2-3 different brains per genotype, with each image containing ~40 cells or more within the field of view, depending on the objective used. The various anatomical regions of a *Drosophila* larval brain are shown below in Figure 3.3 for future reference.

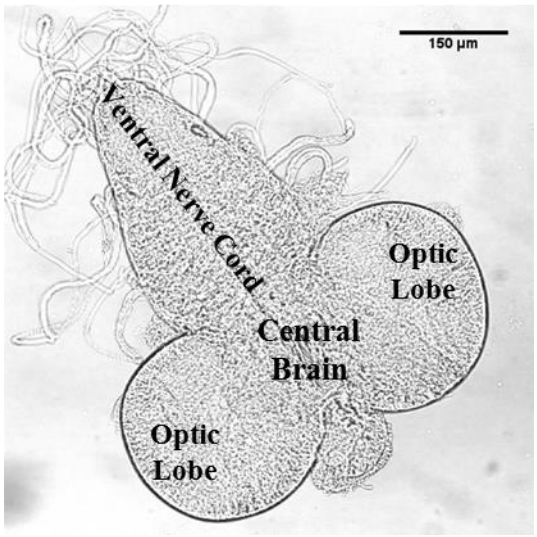

Figure 3.3: Confocal microscopy image of a 3rd instar larval brain showing annotated anatomical regions.

3.2.2 dEAAT1-Gal4::UAS-GFP driver line crosses

Like the dEAAT1-Gal4 driver line discussed above, the dEAAT1-Gal4::UAS-GFP line produces the Gal4 protein in dEAAT1 expressing cells. However in this line the UAS-GFP reporter has also been recombined onto the same chromosome as the dEAAT1-Gal4, meaning that dEAAT1 positive cells will also express cytosolic GFP. Male driver line flies were crossed with virgin female "gene of interest" (TDP-43, Clu, 51D or TDP;Clu) flies. The progeny of these crosses express the gene of interest in dEAAT1 positive cells, which also express cytosolic GFP. Once the progeny reached the third instar larval stage they were dissected and immunohistochemistry performed according to the methods outlined in section 2.4.2. 10 brains were dissected for each cross and following washing and staining all remaining brains were imaged by confocal microscopy; ~5-10 high power images were collected from ~3 different brains per genotype, with each image containing ~30 cells of more within

the field of view, depending on the objective used. One low power Z stack (16 slices, with each slice consisting of an average of at least 12 images) containing the entire brain within the frame of the image was also obtained for each of the 4 genotypes. Additionally, two control crosses were performed. Firstly male dEAAT1-Gal4::UAS-GFP flies were crossed with virgin UAS-mCD8::RFP females. mCD8::RFP is a fusion protein between mouse lymphocyte marker CD8 and red fluorescence protein. The progeny of this cross should have dEAAT1 positive cells expressing cytosolic GFP, as well as RFP localised predominantly to the cell membrane, as mCD8 is a transmembrane protein (Lee and Luo, 1999). Secondly, as a negative control UAS-w1118 (w1118) flies were crossed with the dEAAT1-Gal4::UAS-GFP driver line. W1118 flies do not have a "gene of interest", but have the same genetic background as the Gal4 driver line and UAS lines (except for TDP-43, which as previously mentioned has a 51D background). For each cross performed 10 brains were dissected, all remaining brains were examined by confocal microscopy and ~3-5 images (of whole brains) were collected from each of ~3 different brains per genotype.

3.2.3 Uncrossed TDP-43 and TDP;Clu reporter lines

As an additional control third instar larvae from uncrossed reporter TDP-43 and TDP;Clu lines were dissected and stained for TDP-43 as outlined in section 2.4.2. This was to ensure that the uncrossed fly lines were not expressing any TDP-43, to confirm that any expression and spread observed in the above crosses was genuine. For each line 10 brains were dissected, all remaining brains following washing and mounting were examined by confocal microscopy and 3 images (of whole brains) were collected from each of 3 different brains per fly line.

3.2.4 Quantitative co-localisation analysis of confocal microscopy images

dEAAT-Gal4::UAS-GFP driver line crosses were prepared according to section 3.2.2. For each genotype (TDP-43 or TDP;Clu) 10 larval brains were dissected according to section 2.4.2 and mounted onto microscope slides. Approximately 7-8 brains per genotype remained intact throughout this process and were examined by eye under the confocal microscope to ensure there were no large discrepancies between samples. One Z stack (consisting of 16 slices, with each slice consisting of an average of at least 12 images) was obtained from one whole brain, as well as 6-8 high magnification images from ~2-3 different brains. These 22-24 images (each containing a red and green channel) were then imported individually into ImageJ and the "just another colocalisation plugin" (JACoP) used to calculate the Pearson's and Overlap coefficients as described in section 2.6. The means for each coefficient were then calculated, and a t test performed to determine if the mean coefficients of the TDP-43 and TDP;Clu flies were significantly different.

3.3 RESULTS

3.3.1 Expression of TDP-43 in a specific subset of glial cells in *Drosophila* larval CNS results in spread to other cell types

Male dEAAT1-Gal4::UAS-GFP flies were crossed with virgin female TDP-43, TDP;Clu, Clu, or 51D flies to create progeny that expressed the gene of interest in dEAAT1 positive glial cells (which also expressed cytosolic GFP). Following dissection, immunohistochemistry was performed to label HA-tagged TDP-43 and the brains imaged on an inverted confocal microscope. A Z stack consisting of 16 slices was obtained to collect images from different focal planes within the brain (with each slice consisting of an average of at least 12 images). The Z stack was then flattened into a single image using the "Z-project" tool in ImageJ. In all 4 crosses GFP florescence (shown in green) was detected in cortical cell bodies at the periphery of the larval CNS, in a pattern corresponding to dEAAT1 expressing cells previously published (Figure 3.4; Rival *et al*., 2004). As expected TDP-43 (shown in red) was only detected in progeny from the TDP-43 and TDP;Clu crosses (Figure 3.4 top left and right respectively), and was completely absent from the Clu and 51D control crosses (Figure 3.4 bottom left and right respectively). The progeny expressing TDP-43 alone or co-expressing TDP-43 and clusterin (Figure 3.4 top left and right respectively) both showed many cells containing TDP-43 but not GFP, suggesting that TDP-43 had spread from dEAAT1 positive glial cells to other surrounding cell types. This was particularly evident in the optic lobes. Both crosses also had a portion of TDP-43 which remained within the dEAAT1 positive glia where it was expressed, seen as an overlap of the red TDP-43 and green glia (yellow), best seen in the ventral nerve cord of the TDP;Clu fly.

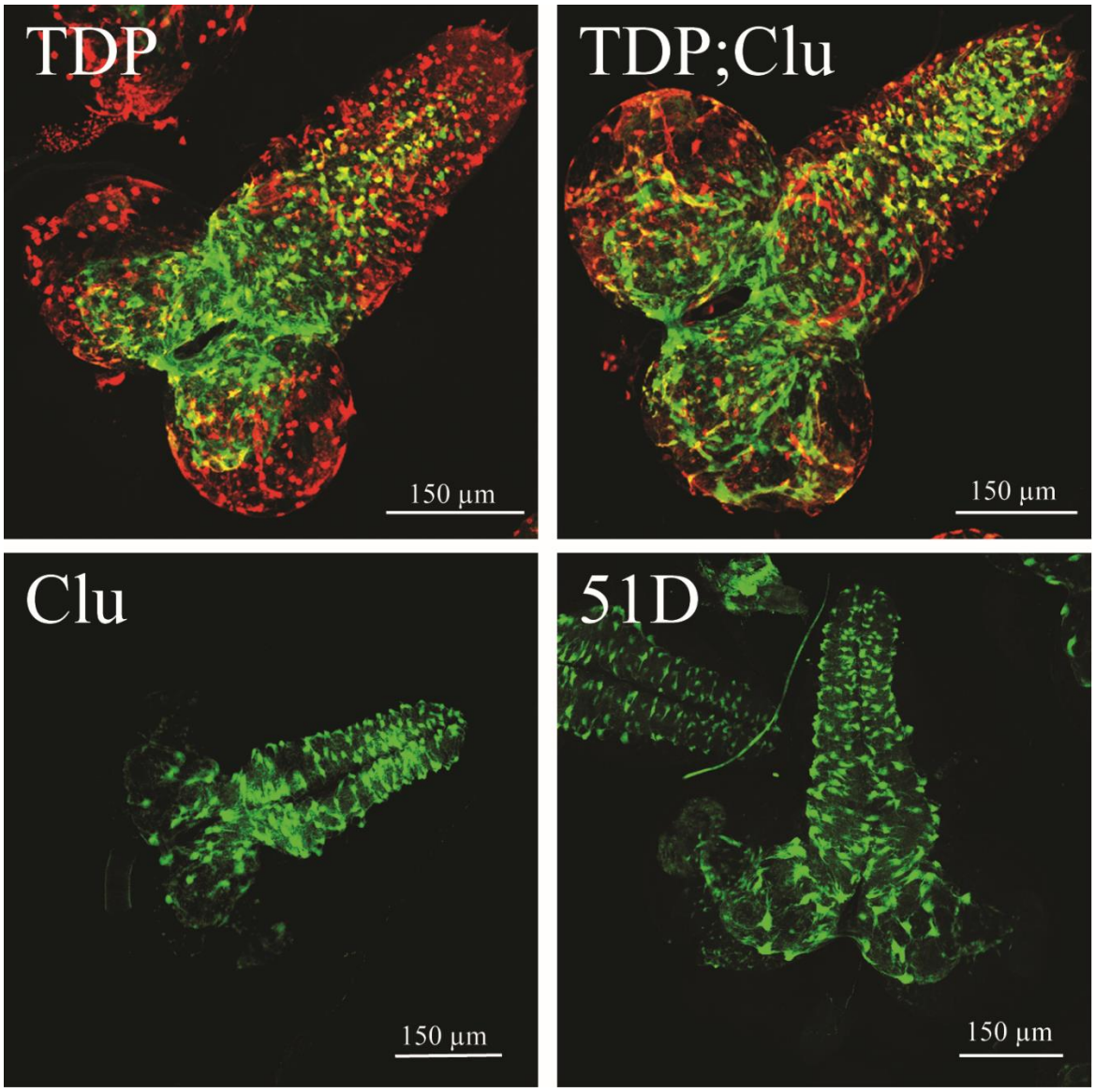

Figure 3.4: Glial expression of TDP-43 results in the spreading of TDP-43 to non-glial cells. Confocal microscopy images of third instar larval brains of *Drosophila melanogaster* expressing TDP-43, co-expressing TDP-43 and clusterin, or clusterin in glial cells under control of the dEAAT1-Gal4::UAS-GFP driver. TDP-43 is shown by anti-HA (red) staining, while glial cells are green. Yellow indicates co-localisation of TDP-43 and glial cells. Control used was the non-transgenic 51D. Images presented were obtained by flattening a single Z stack (consisting of 16 individual images) from one whole brain per genotype into a single image using the "Z-project" tool in ImageJ.

In an attempt to quantify the spread of TDP-43 the "Just another colocalisation plugin" (JACoP) for ImageJ was used to calculate The Pearson's coefficient (PC) and overlap coefficient (OC). The brains from flies expressing TDP-43 (TDP-43) or TDP-43 and clusterin (TDP;Clu) were dissected and

prepared according to section 2.4.2. Images (24 for TDP;Clu, and 22 for TDP). each containing a red (TDP-43) and green (glia) channel, were individually imported into ImageJ as described in section 3.2.4. Threshold values used for the calculations were those set automatically by JACoP. PC values range from +1 to -1, where +1 is perfect co-localisation, and 0 is no or random co-localisation, and a negative value indicates that the distribution of the two colours are inversely related. OC gives values between 1 and 0 and indicates an actual overlap of signals, for example an OP of 0.5 implies that 50% of both red and green channels overlap. Note that co-localisation represents TDP-43 that has remained in the glial cells where it was originally expressed, and thus a higher coefficient means less spread of TDP-43 to non-glial cells. Both the PC and OC were significantly higher in the TDP;Clu flies than in the TDP-43 flies ($P<0.05$), meaning that the TDP;Clu flies had a higher % of overlap between TDP-43 and glia (Figure 3.5). These results suggest that in the larval brains of *Drosophila*, clusterin co-expression is able to significantly reduce the spread of glial TDP-43.

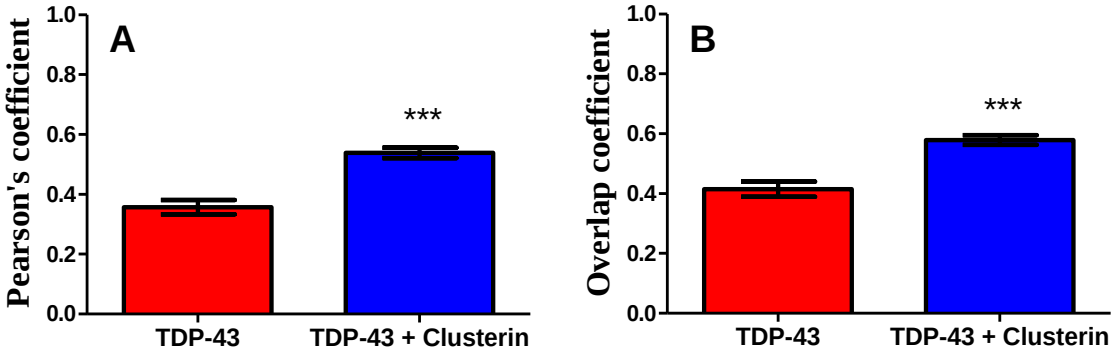

Figure 3.5: (A) Pearson's coefficients and (B) Overlap coefficients calculated from images of flies expressing TDP-43 or co-expressing TDP-43 and clusterin. A value of 1 indicates perfect co-localisation of TDP-43 and glial cells, where 0 is no or random co-localisation. Values were calculated using the JACoP plugin in ImageJ. *** = significant difference ($P<0.05$). Error bars represent SEM. N = 22 (TDP-43) and 24 (TDP-43 + clusterin).

A second cross was then set up to ensure that the dEAAT1-Gal4::UAS-GFP fly line was driving expression of the gene of interest in the appropriate dEAAT1 positive cells. To do this a RFP tagged control protein (mCD8) was selected that once expressed, should remain in the same location. Male driver line flies were crossed with virgin female UAS-mCD8::RFP flies, and the brains of third instar larva imaged on an inverted confocal microscope. In this case only one image from a single plane of focus was obtained to visualise individual cells more clearly, and thus no Z-stack was created (12 averages were still obtained). Figure 3.6A shows the progeny of this cross. The low power image on the left reveals that RFP was not detected in areas distinct from GFP expression, suggesting that the

dEAAT-1-Gal4::UAS-GFP driver is correctly driving expression of the RFP tagged mCD8 protein in dEAAT1 positive cells. As mentioned in section 3.2.2, the mCD8::RFP protein is localised predominantly to the cell membranes, while the GFP should be expressed throughout the entire cell. The high power image in Figure 3.6A showing the ventral nerve cord confirms the membrane localisation of mCD8::RFP. In addition to the RFP fluorescence observed in the membranes of dEAAT1 cells, faint fluorescence was seen throughout the entire brain. To check whether or not this was the result of incorrect RFP expression or background fluorescence, dEAAT1-Gal4::UAS-GFP flies were crossed with w[1118] flies. W[1118] flies do not have a "gene of interest" insert but have the same genetic background as the dEAAt1-Gal4::UAS-GFP and UAS-mCD8::RFP flies. The progeny of this cross have a pattern of GFP fluorescence indicative of dEAAT1 cells, and the same faint autofluorescence throughout the entire brain (Figure 3.6B), with no specific localisation pattern for RFP. To ensure that the secondary antibodies used were not binding non-specifically, an extra control experiment was performed, where dEAAT1-Gal4 flies were crossed with reporter line flies, and the larvae stained for TDP-43, but with the primary antibody omitted. As expected no TDP-43 staining was observed (Appendix 1). Finally, uncrossed TDP-43 and TDP;Clu reporter line larvae were stained for TDP-43, resulting in no specific detection of TDP-43 (Appendix 2).

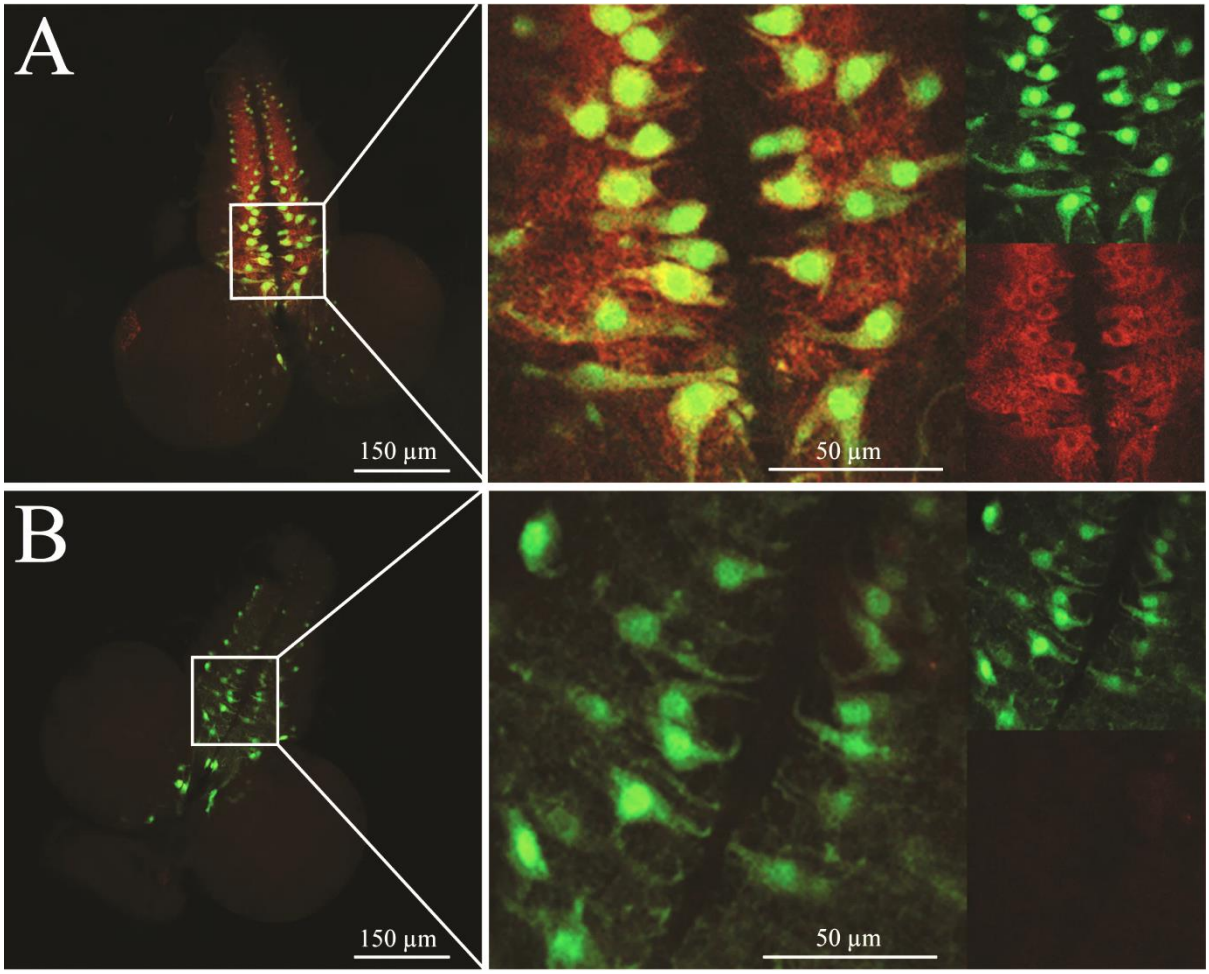

Figure 3.6: Immunohistochemistry images of *Drosophila* third instar larva expressing mCD8::RFP (A), or w1118 (B) in glial cells under the control of the dEAAT1-Gal4::UAS-GFP driver. w1118 flies do not have a "gene of interest" and were used as a negative control. Low power images showing a merge of red and green florescence are shown on the left panels, while the right panels show high power images of the red and green channels both separately (right) and merged (left). mCD8::RFP is red while GFP is green. Images presented are representative of multiple images obtained from ~2-3 brains.

3.3.2 Neurons were identified as one cell type to which TDP-43 had spread from glia

The results above suggested that when expressed in dEAAT1 positive glial cells, TDP-43 was able to spread to other cell types. A new set of crosses was then performed to attempt to identify what some of these cells were. First, male dEAAT1-Gal4 flies were crossed with virgin female TDP-43, TDP;Clu, Clu, or 51D flies to create progeny that expressed the gene of interest in dEAAT1 positive glial cells. Unlike in the crosses above, these glial cells did not express GFP. Following dissection, immunohistochemistry was performed to label HA-tagged TDP-43 and Discs large (Dlg). Dlg is a

member of a group of proteins called membrane-asssociated guanylate kinase homologs (MAGUKs) and is localised at synaptic junctions in neurons (Hough *et al*., 1997). It was used to stain the membranes of neurons to determine if they contained TDP-43. Figure 3.7 shows both high (right panel) and low (left panel) power microscopy images of the ventral nerve cord (VNC) of third instar larvae CNS. Neuronal cell bodies (green) can be seen in all 4 crosses. The long thin "fingers" coming from the sides of the ventral nerve cord likely represent axon projections, which extend out to the abdominal peripheral nervous system (Grueber *et al*., 2007). Again as expected, only the TDP and TDP;Clu crosses show TDP-43 (red) expression. Both with and without clusterin co-expression, axonal distribution of TDP-43 was observed. The high power images reveal instances of TDP-43 co-localising with axonal projections of neurons, indicated by the co-localisation of TDP-43 (red) and Dlg (green) indicated by white arrow heads. Co-expression of clusterin did not stop the spread of TDP-43 to the axonal projections of neurons.

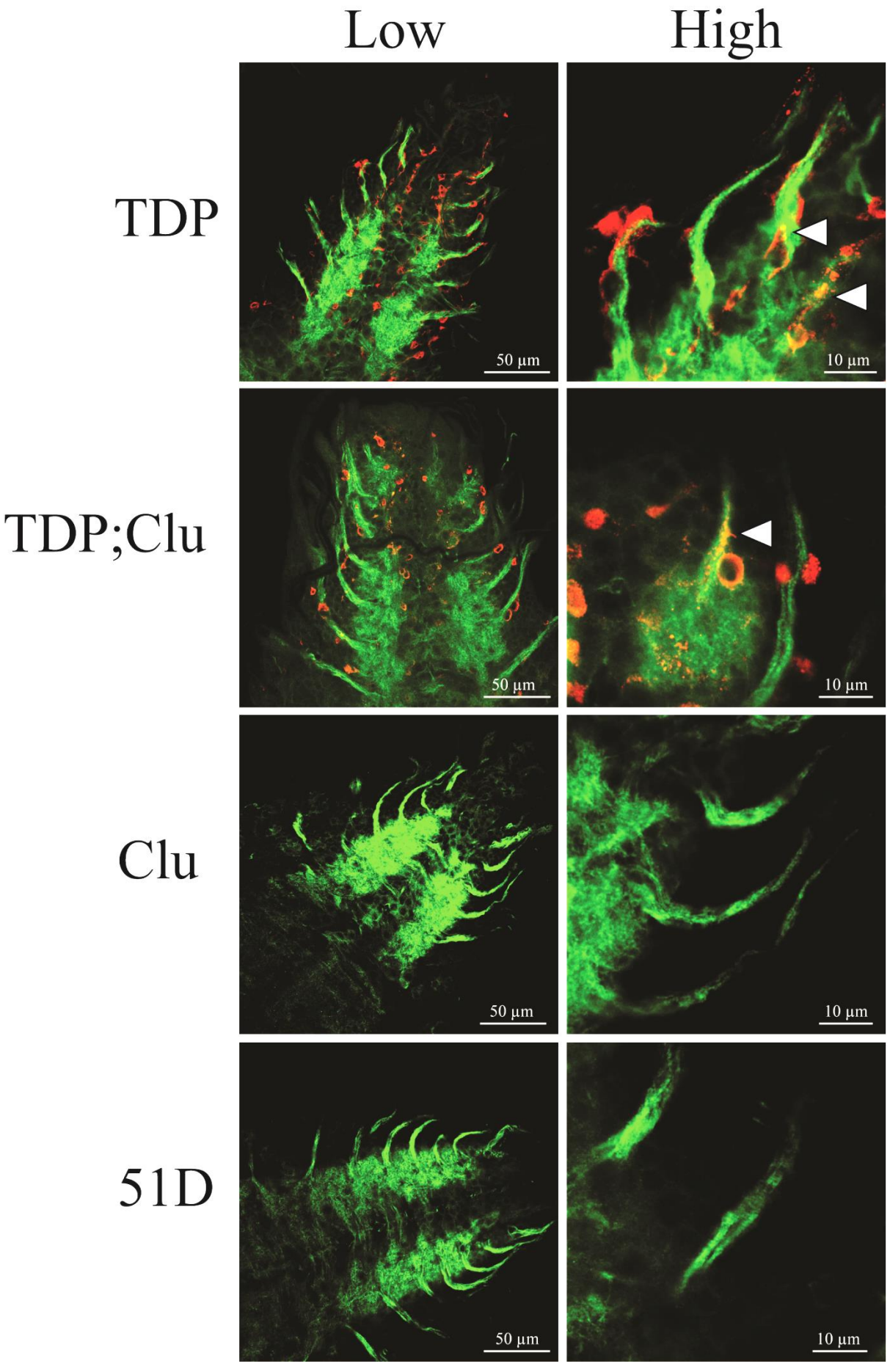
Low
High
TDP
TDP;Clu
Clu
51D
50 µm
10 µm

Figure 3.7: TDP-43 is able to spread from glial cells to the axonal projections of neurons. Low (left) and high (right) power confocal microscopy images of third instar larval brains of *Drosophila* expressing TDP-43, co-expressing TDP-43 and clusterin, or clusterin in glial cells under control of the dEAAT1-Gal4 driver. TDP-43 is shown by anti-HA (red) staining, while anti-discs large (green) detects the membranes of motor neurons. Yellow indicates co-localisation of TDP-43 and the membrane of motor neurons (indicated by white arrow heads). 51D is the non-transgenic control. Images presented are representative of multiple images obtained from ~2-3 brains.

To confirm that TDP-43 was able to spread to neurons, crosses (described in section 3.2.1) were set up to produce flies that expressed TDP-43 or TDP-43 and clusterin in dEAAT1 positive glial cells. As previous results showed no expression of TDP-43 in the Clu and 51D flies, these crosses were not repeated. Following dissection of the CNS from third instar larvae, immunohistochemistry was performed to label HA-tagged TDP-43 and the protein ELAV. The ELAV protein is the product of the *embryonic lethal abnormal visual system (elav)* gene. ELAV is found in all immature and mature neurons and is not detected in neuroblasts or glia, making it an excellent neuronal marker (Robinow and White, 1991). In addition ELAV is localized to the nucleus meaning it can be used to determine the cellular location of TDP-43. Figure 3.8 contains images from a single Z-plane of the ventral nerve cord, with the nuclei of neurons shown in green, and TDP-43 in red. In the TDP-43 cross, the high power image on the right clearly shows an instance of co-localisation between the nucleus of a neuron and TDP-43 (yellow, indicated by white arrow head in upper right panel). The red fluorescence in the nucleus of this cell is much brighter and stronger than in the surrounding area of the cytosol, where fluorescence appears duller and punctate. There are also cells in the low power image of Figure 3.8 which show the same dull punctate staining of TDP-43 surrounding an ELAV labelled nucleus, but without any co-localisation between the two (white arrow head). The flies expressing TDP-43 and clusterin (TDP;Clu) have similar types of ELAV and TDP-43 staining to those observed in the flies expressing TDP-43 only (TDP), i.e. duller punctate staining in the cytosol surrounding a more intense area of TDP-43 in the nucleus, although no co-localisation of TDP-43 and ELAV was observed in the brains imaged (blue arrow head in lower right panel).

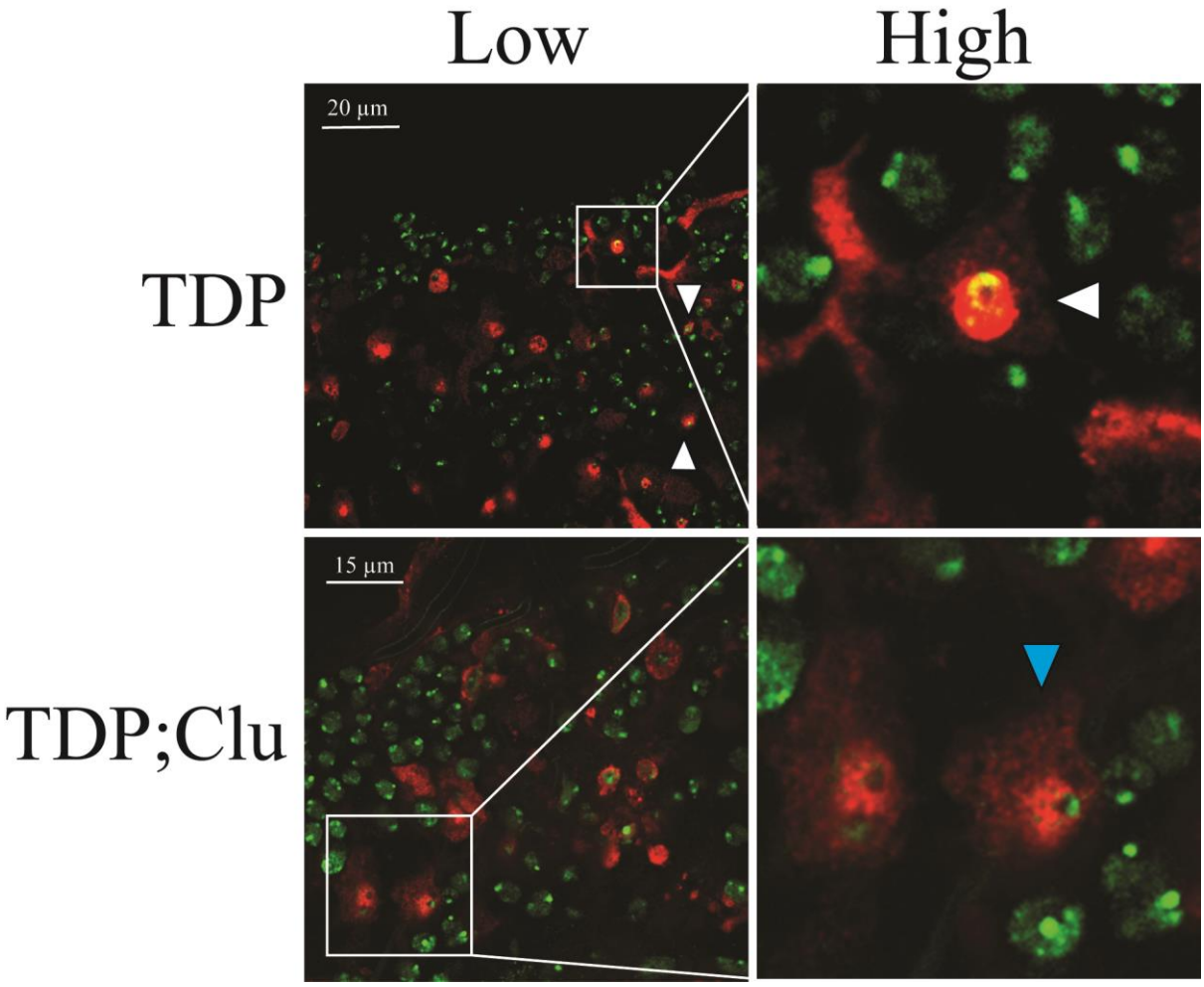

Figure 3.8: TDP-43 is able to spread from glial cells to co-localise with the nucleus of motor neurons. Low (left) and high (right) power confocal microscopy images of third instar larval brains of *Drosophila melanogaster* expressing A) TDP-43, or co-expressing B) TDP-43 and clusterin, under control of the Deeat-1-GAL4 driver. TDP-43 is shown by anti-HA (red) staining, while anti-ELAV (green) detects the nucleus of motor neurons. Yellow indicates co-localisation of TDP-43 and the nucleus of motor neurons (upper left and right panel, white arrowheads). Blue arrowhead indicates where TDP-43 was not found to co-localise with the nucleus of motor neurons. Images presented are representative of multiple images obtained from 2 brains.

3.3.3 Expression of TDP-43 in a specific subset of glial cells in *Drosophila* larval CNS causes premature lethality, regardless of clusterin co-expression

To test the toxicity of TDP-43 expression, virgin female gene of interest flies (TDP-43, TDP;Clu, Clu, 52D) were crossed with both dEAAT1-Gal4 and dEAAT1-Gal4::UAS-GFP male driver lines. In each cross, including those where clusterin was co-expressed in glial cells, the expression of TDP-43 was extremely toxic, as the vast majority of progeny (> 95%) did not reach the pre-pupal or pupal stage of the *Drosophila* life cycle, which occurs approximately 5-6 days after fertilisation (at 25 ºC). Of the flies that did reach the pupal stage, none reached adulthood which normally occurs approximately 10 days after fertilisation.

3.4 DISCUSSION

TDP-43 is present in all cases of SALS and *in vitro* is able to act as seeds triggering the aggregation of native TDP-43. Cell-to-cell spread of TDP-43 has yet to be demonstrated *in vivo*. The purpose of this study was to create a *Drosophila* model of ALS which could be used to determine if TDP-43 is capable of cell-to-cell spread *in vivo*, if the expression of TDP-43 in glial cells was toxic and if the co-expression of the extracellular chaperone clusterin could reduce spread and toxicity. The ability of TDP-43 to spread cell-to-cell was tested by creating a fly line in which TDP-43 was expressed in a subset of dEAAT1 positive glial cells, which also expressed cytosolic GFP. The brains of third instar larvae revealed that a surprisingly large amount of TDP-43 had spread to non-glial cells (Figure 3.4). Clusterin appeared to reduce the spread of TDP-43, at least in the cases of the particular brains imaged, as suggested by significantly higher ($P<0.05$) Pearson's and Overlap coefficients in the flies co-expressing clusterin compared to those expressing TDP-43 only (Figure 3.5), but it was not enough to stop the spread of TDP-43 to neurons. Although the Pearson's and overlap coefficients indicated that clusterin was able to influence the spread of TDP-43, the methods of quantitative co-localisation analysis used in this chapter have several disadvantages. For quantitative co-localisation analysis of microscopy images to be accurate, the images collected have to be suitable. For example the signals collected in each image must be easily distinguished from noise and background, uncontaminated by auto fluorescence generated by the sample itself, and free of signal bleed through between fluorophores (Dunn *et al*., 2011). As mentioned previously, however, auto fluorescence is a common problem when imaging *Drosophila* tissue. The PC was first used to quantify co-localisation as it can be measured without any form of pre-processing, is simple to use and is thus generally safe from user bias. It is also not overly sensitive to the intensity of background fluorescence. However this method is not without its disadvantages. It is generally recommended that PC be measured for individual cells by hand drawing a region of interest over the image, and is thus limited for use in simple images. It is also recommended to use in images where the intensity of each fluorescent channel (ie red and green) are similar (Zinchuk *et al*., 2007). Thus the PC is not ideal for this study as co-localisation within the

entire larva brain (eg Z stacks), not within single cells, was desired. The overlap coefficient was also used, which indicates an overlap of the red and green fluorescence channels and so represents the true degree of co-localisation. It has the advantage of being applicable even when fluorescence of one channel is stronger than the other, but also has the drawback of being very sensitive to background fluorescence. This method can also produce false co-localisation when applied to images with high molecular density or poor resolution (Wu *et al*., 2012). In summary, quantitative co-localisation analysis is difficult. There are multiple methods available, all which have advantages and disadvantages. There is no universal method used to deal with background fluorescence in images. Analysis of whole fly brains is also not ideal, as most coefficients are accurate only when used on a region of interest containing individual cells.

To confirm that the spread observed in the fly larvae was genuine, an extra cross was set up. The dEAAT1-Gal4::UAS-GFP line was crossed with both a line expressing an RFP tagged membrane protein, and a control line lacking a gene of interest but having the same genetic background as the Gal4 and UAS lines. Figure 3.6A shows that when the RFP tagged membrane protein was expressed in the dEAAT1 positive cells, it remains in those cells, as seen by the co-localisation between RFP and GFP. There is also faint red fluorescence throughout the brain, but this also appears in the w1118 control cross (Figure 3.6B). This suggests that the red fluorescence throughout the brain seen in both Figure 3.6A & B is due to autofluorescence, and not incorrect expression of RFP. These results indicate that the dEAAT1-Gal4::UAS-GFP driver is working correctly, and the spread of TDP-43 between cells is genuine.

Once it had been established that TDP-43 had spread from the dEAAT1 positive subset of glial cells, this study then sought to identify what cells TDP-43 had spread to. Flies expressing the gene of interest in dEAAT1 positive cells were generated, and motor neurons were labelled using either an antibody to stain the cell membranes or nucleus. Figure 3.7 remarkably shows that TDP-43 is able to spread from glia cells to axon projections of motor neurons. This was also observed in flies that co-expressed clusterin (Figure 3.7). The extent to which TDP-43 localises to axons has been a topic of interest, and recently studies have begun to show that it is recruited to motor axons where it co-localises with mRNA binding proteins. In primary cultures of motor neurons TDP-43 is localised in discrete, highly mobile granules along motor neuron dendrites, axons, and growth cones leading to the suggestion that TDP-43 has a potential role in axonal mRNA regulation, and enhanced axonal localisation may contribute to disease pathology. In support of this, alterations to TDP-43 levels in motor neurons caused a severe reduction of axon outgrowth and branching, and ALS-derived mutations in TDP-43 increased both the cytoplasmic and axonal levels of the protein (Fallini *et al*., 2012). Insoluble TDP-43 aggregates have also been seen localised to axons in photoreceptors (Estes *et*

al., 2013), and C-terminal fragments of TDP-43 form aggregates in dendrites and axons of motor neurons in culture (Fallini *et al*., 2012). Additionally, when TDP-43 was overexpressed in the spinal motor neurons of chick embryos, TDP-43 progressively mislocalised in the cytoplasm and axons (Tripathi *et al*., 2014). Results from the above literature and those reported in this thesis support the idea that TDP-43 regulates axon outgrowth, and enhanced axonal localisation may interrupt vesicle, protein, and mRNA trafficking, cause truncation of axon projections and contribute to disease pathology. Understanding the functions and mechanisms involved in axonal TDP-43 may help gain new insight into ALS pathology, and perhaps ultimately in the development of new therapeutic strategies.

To confirm that TDP-43 was reaching motor neurons, the brains of 3rd instar larvae were then stained for TDP-43 and ELAV, a protein localised to the nucleus of neurons. In the TDP-43 flies, TDP-43 was found co-localised with the nucleus of neurons and in cytoplasmic inclusions. The TDP;Clu flies also showed cytosolic TDP-43, as well as TDP-43 that appeared to be in the nucleus of neurons, as the TDP-43 fluorescence was seen surrounding the ELAV (nuclear) fluorescence, but no direct co-localisation was observed, at least in the brains imaged. The results of this study demonstrate that when TDP-43 is expressed in a subset of glial cells, it is able to spread to surrounding cell types. Although some of these cell types were revealed to be neurons, Figure 3.8 also shows a number of TDP-43 positive cells that do not correspond to neurons. The question remains then, where is TDP-43 spreading to? As previously mentioned, the dEAAT1-Gal4 drivers were used here to drive protein expression in dEAAT1 positive glial cells, which only make up about 10% of total glia. In future experiments the glia specific anti-Repo antibody could be used to label most glial cells, to determine if TDP-43 is spreading throughout the glia as well as to neurons.

Finally, this study showed that expression of TDP-43 in a subset of glial cells in *Drosophila* larvae was toxic regardless of clusterin co-expression. However a proper survival assay was not able to be performed, as expression from the embryo stage onwards was so toxic that flies failed to reach the pupal stage, making meaningful analysis of lifespan during the 5 day larval stage difficult. Additionally, in *Drosophila*, glial cells contribute to almost all aspects of nervous system development. In embryos glia regulate neuronal viability, and during subsequent development they secrete neurotrophic factors, aid in axon guidance, participate in neurotransmitter recycling, and remove apoptotic cells. Disruption of glial function can result in severe neurological defects (Booth *et al*., 2000; Lee at al., 2005). Thus the toxicity observed in our study could be a result of disrupting important developmental processes. ALS is most commonly a disease of late onset, once glial and neuronal cells are fully developed. In order to create an accurate model of ALS, in which glia and neurons can develop normally allowing an adult survival assay to be performed, an inducible system

needed to be established, where TDP-43 expression could be "switched on" in adult flies. This was the next step undertaken in this study with the generation of an inducible Gal80 expression system and will be discussed in Chapter 4.

Due to the premature lethality that resulted when TDP-43 was expressed throughout the embryonic and larval stages of the *Drosophila* lifecycle, it was impossible to perform survival and locomotor assays. Larval locomotor assays are done by placing an individual larva in a petri dish containing 2% agarose over graph paper with a 0.2 cm^2 grid, and counting the number of grid lines crossed in 1 min. Larvae that were expressing TDP-43 under the Gal4/UAS expression system used were so compromised that they did not travel enough to allow comparison between TDP-43 and TDP;Clu genotypes. The inducible Gal80 system described in the following chapter was used to overcome this problem too, as locomotor assays could be performed on adults to assess the decline in motor function over time as a result of TDP-43 expression.

To summarise, the results presented above suggest that in a *Drosophila* larval model of ALS, TDP-43 was able to spread from the subset of glial cells where it was initially expressed, to surrounding cell types, a result previously only observed for SOD1. It appears that co-expression of the extracellular chaperone clusterin was able to significantly reduce this spread ($P<0.05$), however as discussed above the methods used to quantify the spread were subject to several disadvantages.

Using immunohistochemistry it was established that TDP-43 was able to spread from the dEAAT1 positive glial cells to a number of neurons, reaching as far as their axons regardless of clusterin co-expression. It has been suggested that "prion-like" transfer of protein could be achieved through exosomes, tunnelling nanotubes, vesicle mediated exocytosis, or through direct release from damaged cells. Additionally, expression of TDP-43 in glial cells was lethal to flies, and co-expression of clusterin was unable to rescue flies from this toxicity. Previous studies have observed that TDP-43 expression in glia is toxic and affects locomotor function, but the molecular mechanisms behind this were unknown (Estes *et al*., 2013). Survival and locomotor assays were performed on adult flies and are described in Chapter 4. Results in this chapter support the growing theory that glial cells play important roles in ALS pathology. Studies often examine and compare the effects of TDP-43 expression in glia and neurons separately, but it may be possible that the phenotypic consequences of the two overlap as TDP-43 spreads between cell types. It remains important however that any effect of clusterin on the spread of TDP-43 should be quantified, and an inducible system developed to examine the effect of TDP-43 on mature glia.

CHAPTER 4: INVESTIGATING THE SPREAD AND TOXICITY OF TDP-43 IN AN ADULT *DROSOPHILA* MODEL OF ALS

4.1 INTRODUCTION

In Chapter 3 it was demonstrated that TDP-43 expression in the glial cells of *Drosophila* larvae resulted in both premature lethality and the spread of TDP-43 to non-glial cells (including motor neurons) regardless of clusterin co-expression. As a logical next step, an inducible system which would allow neurons and glia to develop normally before TDP-43 and/or clusterin expression was induced in the glia of adult flies was developed. Such a system would permit a survival assay to be performed to determine if TDP-43 was still toxic when expressed in mature glial cells, and if the co-expression of clusterin affected this toxicity. The development of an adult model would also allow the performance of a climbing assay to assess the effects of transgene expression on locomotor function. Since the results in Chapter 3 showed that TDP-43 could spread to motor neurons in larvae, it was of interest to examine whether TDP-43 expression in adult glial cells resulted in the same spread and caused locomotor dysfunction, and if so, whether the co-expression of clusterin could inhibit the loss of locomotor function.

Many studies have examined the toxicity of SOD1 and TDP-43 expression in various cell types and both autonomous and non-autonomous mechanisms of toxicity have been observed. The expression of human SOD1 in the motor neurons of adult *Drosophila* causes increased expression of chaperones including Hsp70 in glial cells which do not express SOD1 (Watson *et al.*, 2008), an effect referred to as non-cell autonomous. The expression of SOD1 also caused cell autonomous damage to motor neurons, meaning that the motor neurons which expressed SOD1 exhibited a disease phenotype, in this case progressive accumulation of SOD1 and a progressive loss in motor function. Other studies have identified different consequences of SOD1 expression in either motor neurons or glial cells. For example, expression of human (hSOD1) or *Drosophila* (dSOD1) SOD1 in motor neurons of *Drosophila* did not cause a reduction in lifespan yet expression of dSOD1 in glial cells did (Watson *et al*., 2008; Kumimoto *et al*., 2013). Interestingly the expression of MT dSOD1 in either motor neurons or glia reduced the lifespan of the flies, but when expressed in both the glial and motor neurons actually increased fly lifespan (Kumimoto *et al*., 2013). It seems that at least in the case of SOD1, expression of protein may have a beneficial or harmful effect depending on which cell type it is expressed in.

Studies have been performed expressing TDP-43 in specific cell types, for example in astrocytes or motor neurons *in vitro* and *vivo*, but these have yielded conflicting results. Non-cell autonomous mechanisms of toxicity have been observed in rats that were engineered to express the TDP-43 mutant

M337V. When expressed only in astrocytes, motor neuron degeneration and atrophy of skeletal muscles was observed (Tong *et al*., 2013). Another study generated astroglia derived from human iPSCs which carried the M337V TDP-43 mutation and had increased levels of TDP-43. When these cells were co-cultured with iPSC-derived motor neurons they did not have an adverse effect on motor neuron survival and no non-cell autonomous TDP-43 proteinopathy was observed (Serio *et al*., 2013). In *Drosophila*, pan-neuronal expression of *Drosophila* or human TDP-43 (hTDP-43) resulted in a reduced adult lifespan, motor neuron death, formation of cytoplasmic aggregates, and reduced locomotor ability or motor neuron dysfunction (Diaper *et al*., 2013b; Estes *et al*., 2011; Li *et al*., 2010; Hanson *et al*., 2010). When expressed in larvae or adults with a pan-glial driver, hTDP-43 hindered motor function and mis-localised from the nucleus to form cytoplasmic puncta, yet did not have a lethal effect on larvae or adults (Estes *et al*., 2013). In yet another study, pan-glial expression of dTDP-43 was lethal to larvae, with flies never reaching adulthood (Diaper *et al*., 2013a). It appears that there are not only differences between how each cell type responds to expression, but also differences between the expression of either human or *Drosophila* TDP-43. TDP-43 is a highly conserved protein in human and *Drosophila* with the most conserved regions being the RNA binding domains. Amino acid alignment shows 59% identity and 77% similarity between the N-terminals and RNA binding domains of hTDP-43 and dTDP-43 (Wang *et al.,* 2004), and both are interchangeable in *in vitro* splicing assays. The C-terminal domain of *Drosophila* TDP-43 is larger than in human TDP-43 and has fewer glycine residues (Ayala *et al.,* 2005). Perhaps the conflicting results described above are due to the differences in the C-terminal domains of human and *Drosophila* TDP-43, as the majority of mutations associated with ALS are located here and this glycine rich domain is also considered the prion-like domain. Regardless, it is clear that astrocytes and glial cells do play an important role in ALS pathogenesis, and studies which focus on the functions of astrocytes and microglia will help further our understanding of the disease.

The experiments described in the following section of this thesis were designed with the aim of creating a TDP-43 *Drosophila* model of ALS that could be used to examine both the role that glial cells play in disease pathogenesis, and the potential of the extracellular chaperone clusterin as a therapeutic. It has been demonstrated that the up-regulation of intracellular chaperone expression reduces TDP-43 aggregation and toxicity. For example the expression of hTDP-43 in adult motor neurons of flies causes locomotor defects and reduced lifespan, but chaperone expression significantly reduces the rate of locomotor decline and rescues lifespan. Up-regulated chaperone expression also reduced the number of insoluble cytoplasmic aggregates that formed as a result of expressing TDP-43 in the *Drosophila* eye (Gregory *et al*., 2012). Furthermore, overexpression of HSP70 protects from the rough eye phenotype caused by overexpression of WT or MT TDP-43 (Estes *et al*., 2011). The current study chose to examine the effects of clusterin expression on TDP-43 proteinopathy in *Drosophila* for

several reasons. Firstly clusterin has been associated with a number of neurodegenerative and protein deposition diseases - overexpression has been observed in diseases such as scrapie, Pick's disease, dementia, and SALS, and it has been found co-localised with deposits in AD, macular degeneration, dementia, and Down syndrome (Calero *et al*., 2000; Grewal *et al*., 1999; Wyatt *et al*., 2013). Clusterin gene polymorphisms are also associated with AD (Jun *et al*., 2010; Gu *et al*., 2011). Secondly clusterin is a potent chaperone that can inhibit protein aggregation at highly sub-stoichiometric molar ratios, for example it is able to inhibit lysozyme fibril formation at clusterin:lysozyme ratios of 1:80 (Kumita *et al*., 2007). It is also has a broad specificity and can inhibit aggregation of a range of proteins with differing structures (Poon *et al*., 2000). Lastly, as discussed in section 1.3.3, although clusterin is an extracellular chaperone, during ER stress it can be retrotranslocated from the secretory system to the cytosol, where it may act as an intracellular chaperone (Nizard *et al*., 2007; Li *et al*., 2013). Clusterin has also been shown to promote autophagy under stress conditions. Autophagy involves the disassembly and recycling of cellular components, is usually cytoprotective and helps maintain homeostasis (Zhang *et al*., 2014). Clusterin has the potential to be used, either alone or in combination with other chaperones, as a therapeutic tool for the treatment of ALS, and is thus worthy of investigation. A *Drosophila* model of ALS in which hTDP-43 is expressed in a small subset (10%) of adult glial cells was used to determine if:

1) Expression in a subset of adult glial cells was sufficient to reduce lifespan and cause locomotor defects.
2) The co-expression of clusterin was able to influence toxicity or locomotion.
3) hTDP-43 could spread from glial cells to other cells, and if clusterin could influence any spread.

4.2 METHODS

In Chapter 3 it was explained how the GAL4/UAS system can be used to express a transgene in a tissue specific manner. When a GAL4 driver line and a UAS reporter line are crossed, expression of the transgene in the desired tissue is activated. This means that expression occurs during the embryo stage and throughout the larval, pupal, and adult stages of the *Drosophila* life cycle. While the GAL4/UAS system allows for the precise spatial control of a transgene, it does not allow for any temporal control. To allow for temporal control during development the GAL4/GAL80 system was used. In this system GAL4 driver lines are created which ubiquitously express the temperature sensitive GAL80 protein ($GAL80^{ts}$); $GAL80^{ts}$ reversibly inhibits GAL4 function. At 18 °C $GAL80^{ts}$ functions normally and binds to the transcriptional activation domain of GAL4, preventing GAL4 mediated transcriptional activation. This process has no harmful phenotype of its own. At 25 °C

GAL80^{ts} is dysfunctional and thus GAL4 is able to function normally (Martin *et al*., 2011; Wolf and Rockman, 2011; del Valle Rodríguez *et al*., 2012; Figure 4.1).

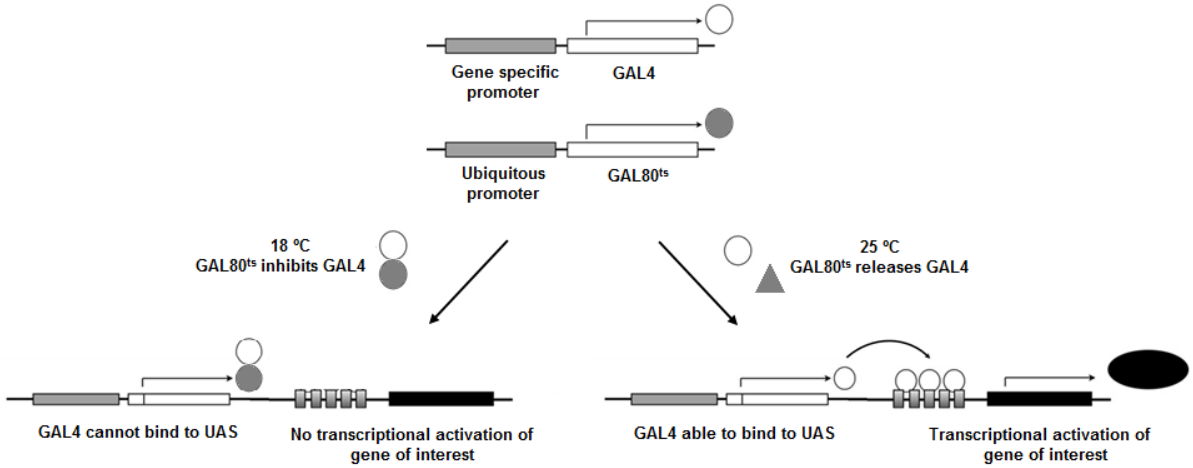

Figure 4.1: The GAL4/GAL80 expression system. Ubiquitous expression of the temperature sensitive GAL80^{ts} is incorporated into the GAL4/UAS system. At 18 °C GAL80^{ts} reversibly represses activation of GAL4 by binding specifically to the GAL4 activation domain. At 25 °C GAL80^{ts} is dysfunctional and GAL4 can function normally again (modified from Nichols, 2006).

4.2.1 dEAAT-1-GFP;Gal80 driver line crosses

The dEAAT-1-GFP;Gal80 driver line was derived from the dEAAT-Gal4::UAS-GFP line described in Chapter 3. The dEAAT-1-GFP;GAL80 driver line is similar to the dEAAT-Gal4::UAS-GFP line in that it produces the Gal4 protein in dEAAT1 expressing cells which also express cytosolic GFP. As outlined in section 3.1, the *Drosophila* glutamate transporter dEAAT1 is expressed in about 10% of *Drosophila* glial cells at all developmental stages, thus progeny generated using dEAAT lines will express the protein of interest in this small subset of glial cells that express the glutamate transporter. The dEAAT-1-GFP;GAL80 driver line differs in that it also includes ubiquitous expression of GAL80^{ts}. This line was used to create a system where the expression of the genes of interest could be "switched on" by moving the progeny of a cross from 18 °C to 25 °C after 20 days, once they had reached eclosion and hatched into adults. Male driver line flies were crossed with virgin female "gene of interest" (TDP-43, Clu, TDP;Clu or 51D) flies. These crosses were performed at 18°C. After 20 days the flies reached eclosion and were collected after hatching and immediately moved to 25 ºC where they were allowed to mate for 24 h. Following mating, non-virgin females were kept and maintained at this temperature for the rest of their development, where they were used for either survival assays, negative geotaxis (climbing) assays, or immunohistochemistry.

4.2.2 Gal80 Survival assay

dEAAT-1-GFP;Gal80 driver line crosses were performed as described above in section 4.2.1. Following mating, non-virgin female flies were collected and kept at no more than 10 flies per vial. 311 (TDP-43), 118 (TDP;Clu), 246 (Clu) and 229 (51D) flies were collected, individually counted, and transferred to fresh food 3 times a week over the course of the 80+ day experiment. A survival curve was generated and the median survival of each genotype was compared and analysed using one-way ANOVA followed by Tukey's post test.

4.2.3 Negative geotaxis (climbing) assay

A climbing assay was performed to assess motor function. Flies expressing TDP-43, clusterin, or co-expressing TDP-43 and clusterin in glial cells were generated as described above using the heat inducible Gal80 system (see section 4.2.1). 51D flies were used as a control. After 20 days at 18°C adults were collected and allowed to mate at 25 °C for 24 h. Following mating non-virgin female flies were collected and used for the experiment. For each of the 4 genotypes, 10 flies were placed into separate 25 mL tissue culture pipettes (Greiner Bio-One) that were plugged at the bottom and 15 mL mark (top) with cotton wool. After being moved to the tubes, flies were placed at 25 ºC for 10 min to allow for recovery. Following recovery their climbing ability was analysed each day for 10 days, with flies returned to glass vials (containing media) after each assay was performed. Analysis was performed at the same time each day to avoid any variation that may arise due to differences in activity of flies at different times of the day. For each genotype, 3 repeat measurements were obtained. A climbing index score was calculated based on the formula below where ntotal represents the total number of flies in the tube (10), and ntop and nbottom represents the number of flies in the top or bottom third of the tube 30 seconds after the flies were gently tapped to the bottom. The average climbing index of the 3 repeats was calculated and plotted. If a fly died or was lost during transfer it was replaced with a fly of the same age and genotype, from a stock that had been kept in identical conditions. Motor function was only assessed until day 10 to avoid replacing too many of the TDP-43 flies that were dying, which could skew the results to favour more healthy/living TDP-43 flies.

Formula: ½ [(ntotal + ntop – nbottom)/ntotal]

4.2.4 Immunohistochemistry

For immunohistochemistry, non-virgin females were collected at the following 4 time-points: pre-induction (PI), day 0, day 5, and day 10. For the PI time point, female flies were collected immediately after hatching (20 days after cross was set up) before they were moved to 25 ºC. "Day 0" was counted as the first day after mating at 25 ºC, and so it should be noted that although referred to as day 0, the flies had already been at 25 ºC for 24 h. Day 5 and 10 refer to 5 and 10 days post mating.

For each of the 4 time-points of the 4 genotypes, 5 adult brains were dissected (~ 80 brains total). Dissecting adult brains takes more time and skill than dissecting larvae, and thus only 5 brains per genotype could be dissected in the allocated time. Of the 5 brains dissected, usually no more than one would be lost or damaged during staining and washing. All remaining brains were examined under the confocal microscope, and ~5-10 high power images were collected from ~2-3 brains (on average) per genotype. Additionally for each genotype one Z stack consisting of 16 slices (with each slice consisting of an average of 12 individually acquired images) was obtained. The Z stack was then flattened into a single image using the "Z-project" tool in ImageJ unless otherwise stated. Details of antibodies and staining procedures used are given in section 2.4.2.

4.3 RESULTS

4.3.1 Induced expression of TDP-43 in a specific subset of glial cells in *Drosophila* CNS reduces lifespan and decreases locomotor ability

Using the temperature sensitive dEAAT-1-GFP;GAL80 expression system the genes of interest were expressed in adult dEAAT1 positive glial cells. Expression of TDP-43 in these cells resulted in a significant reduction of mean lifespan compared to WT flies (12 days v 46 days; $p < 0.0001$; Figure 4.2). Co-expression of clusterin with TDP-43 did not significantly increase the mean lifespan compared to flies expressing TDP-43 alone (13 days v 12 days; $p < 0.9888$). Interestingly, expression of clusterin alone caused a significant increase in mean longevity of *Drosophila* compared to WT flies (61 days v 46 days; $p < 0.0001$).

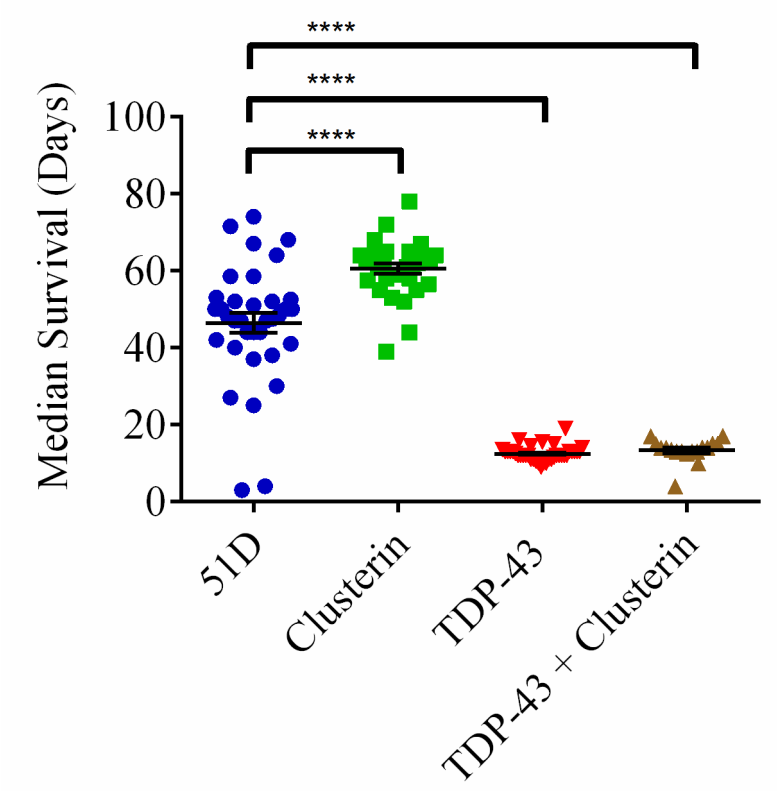

Figure 4.2: Expression of TDP-43 in adult glial cells results in significant reduction of medial lifespan. Plot showing the median survival of adult flies expressing TDP-43, TDP;Clu, or clusterin in dEAAT1 positive glial cells compared to non transgenic wild type (WT, 51D) flies. Expression was controlled using a temperature sensitive dEAAT-1-GFP;GAL80 driver. Lifespans were analysed by one-way ANOVA. N = 311 (51D), 118 (TDP;Clu), 246 (Clu), and 229 (51D) grouped into tubes of no more than 10 flies. Each data point represents the median survival of one tube of (no more than) 10 flies. **** = significant, $p < 0.05$. Error bars = SEM.

Next the sum of all the data from individual tubes was plotted to give an overall survival curve (Figure 4.3). The curve shows that flies expressing TDP-43 had a maximum lifespan of 20 days, while those expressing TDP;Clu lived to a maximum of 29 days. Clusterin flies lived up to 81 days, compared to WT which lived up to 80 days.

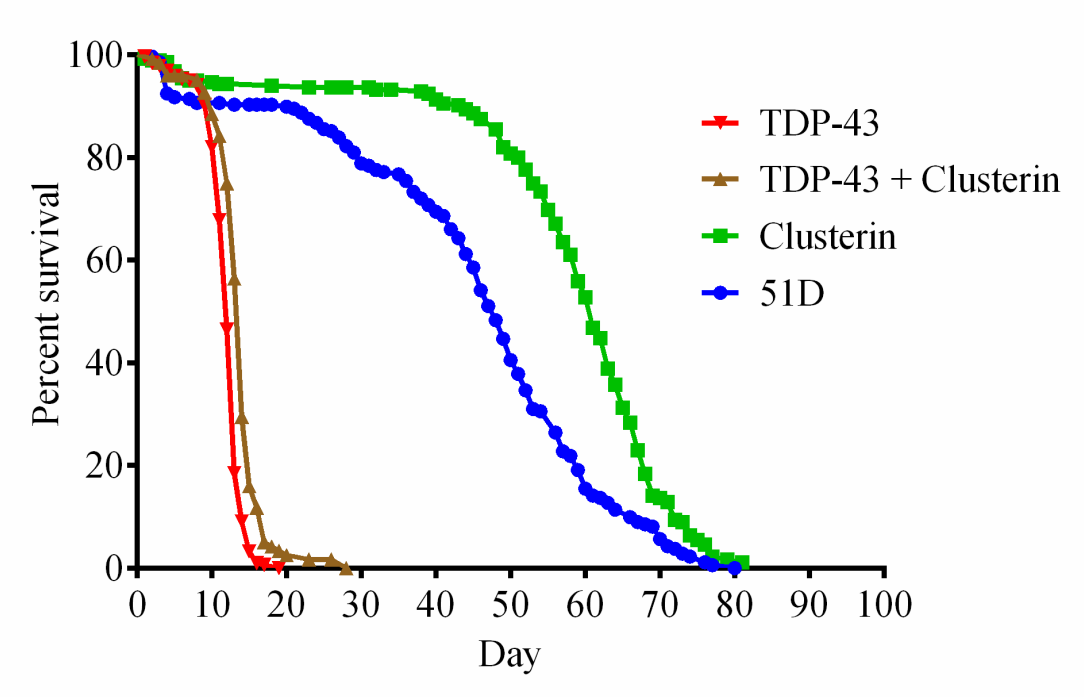

Figure 4.3: Survival data demonstrates that expression of TDP-43 in adult glial cells reduces longevity, which is not significantly affected by clusterin co-expression. Plot showing the survival of *Drosophila* expressing TDP-43, TDP;Clu, and clusterin in adult dEAAT1 positive glial cells compared to non-transgenic wild type (WT, 51D) flies. N = 311 (51D), 118 (TDP;Clu), 246 (Clu), 229 (51D) grouped into tubes of no more than 10 flies per genotype. Each data point represents the total percentage of flies alive on a particular day.

A climbing assay was also performed to assess deterioration of motor neurons associated with TDP-43 expression and any effect of clusterin co-expression. Flies expressing TDP-43 showed a decrease in locomotor ability from day 5 onwards and a 29.5 % reduction in climbing ability compared to age-matched non-transgenic flies at the 10 day end point of the assay (Figure 4.4A). One-way ANOVA analysis of data from the 10 day time point indicated that the mean climbing index of the TDP-43 flies was significantly lower than that of control flies ($P < 0.05$; Figure 4.4B). In contrast, the mean climbing indices of clusterin and TDP;Clu flies were not significantly different from the control. Thus, at the 10 day time point, clusterin co-expression maintained locomotor ability at a level comparable to that of control and clusterin(only)-expressing flies. The results shown are from a single experiment; this assay could not be repeated in the available time frame due to problems with temperature control in the incubators housing the flies.

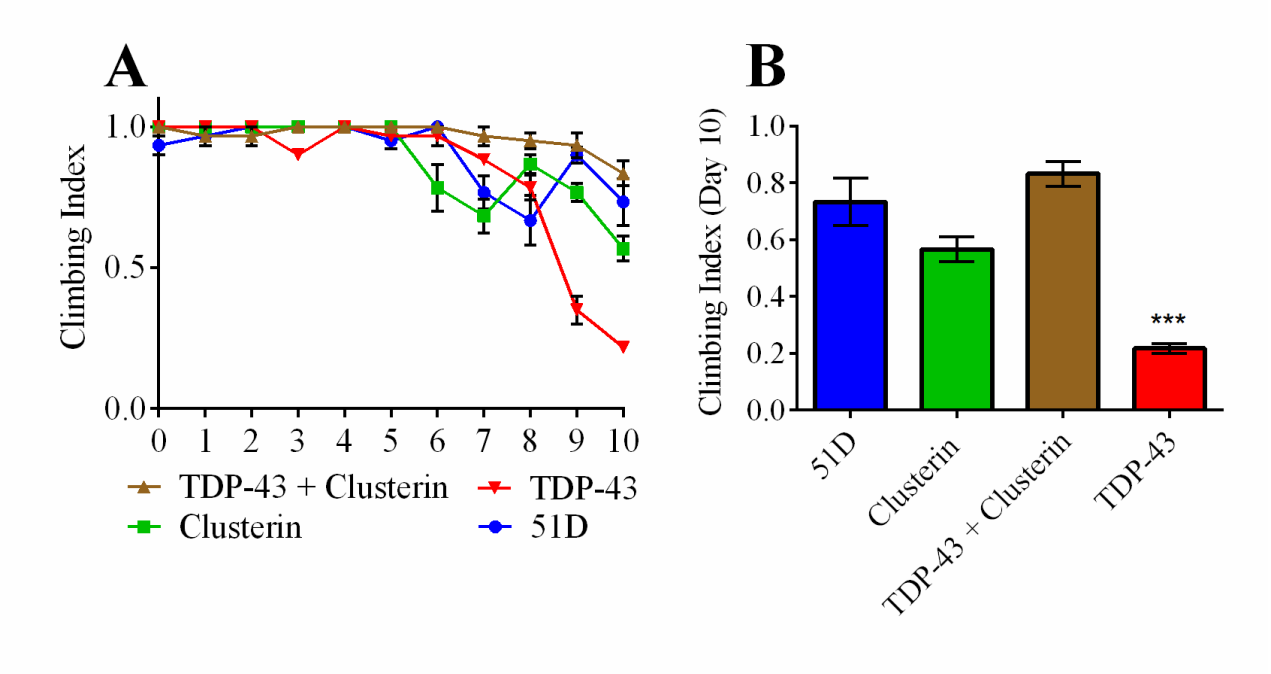

Figure 4.4: Climbing assay data demonstrates that expression of TDP-43 in adult glial cells results in reduced locomotor ability, which is able to be rescued by the co-expression of clusterin. A) Locomotor assay comparing control flies (51D) with flies expressing (in adult glial cells) TDP-43, TDP-43 and clusterin, or clusterin alone. Each data point represents the mean of 30 measurements. Error bars represent SEM. B) Comparison of day 10 climbing indices for control flies (51D) versus flies expressing (in adult glial cells) TDP-43, TDP-43 and clusterin, or clusterin alone. Differences between genotypes were analysed by a one-way ANOVA. The mean climbing index for TDP-43 flies was significantly lower than 51D controls *** = $P < 0.05$. Error bars represent SEM, N=30.

The primary goal of the work described in this chapter was to generate flies that could be used to examine the phenotypic consequences in adults of TDP-43 and clusterin expression. The number of progeny generated, however, was only sufficient to allow the dissection of brains at 4 time points and their analysis by immunohistochemistry to examine spreading of TDP-43 (as done with the larvae in Chapter 3). Not knowing how rapid spread might be, 4 time points during the first ten days of expression were selected, with the earliest time point being 24 h post-induction (Day 0).

4.3.2 Expression of TDP-43 in a specific subset of glial cells in the adult *Drosophila* CNS results in rapid spread to other cell types

Male dEAAT-1-GFP;GAL80 flies were crossed with virgin female TDP-43, TDP;Clu, Clu, or 51D flies to create progeny that expressed the gene of interest in dEAAT1 positive glial cells, which also expressed cytosolic GFP. Brains were dissected at four time points; PI (i.e. before progeny were moved to 25 °C), day 0, day 5 and day 10 after being moved to higher temperatures to induce expression. Immunohistochemistry was performed to label HA-tagged TDP-43 and the brains imaged on an inverted confocal microscope as outlined in section 2.4.2. Gains for each image acquired were identical. In all 4 crosses GFP fluorescence was detected in neuropil areas in a pattern corresponding to dEAAT1 expressing cells previously published (Figure 4.5 & Figure 4.6; Rival *et al*., 2004). The intensity of GFP fluorescence can be seen to increase over time, from almost nil at the PI time point (Figure 4.5), to a bright strong punctate signal at day 10 (Figure 4.6). The diffuse green fluorescence seen in each of the 4 crosses at the PI time point is due to auto-fluorescence, a common complicating factor when imaging *Drosophila* brain tissue (Berg *et al*., 2010; Figure 4.5). As expected TDP-43 (shown in red) was only detected in progeny from the TDP-43 and TDP;Clu crosses, and was completely absent from the Clu and 51D control crosses at all time points (Figure 4.5 & Figure 4.6) In both the flies expressing TDP-43, and those expressing TDP-43 and clusterin, TDP-43 was not detected at the pre-induction time point, indicating that when progeny were kept at 18 °C Gal80 was efficiently binding to Gal4 and preventing TDP-43 expression (Figure 4.5). By day 0 however, both GFP (green) and TDP-43 (red) were detected indicating that after being moved to 25 °C, the expression of TDP-43 and GFP was successfully induced. In addition, the day 0 time point shows instances where TDP-43 did not co-localise with GFP, suggesting that after only 24 h TDP-43 had spread from the glial cells (which contain GFP) where it was originally expressed to other cell types (Figure 4.5).

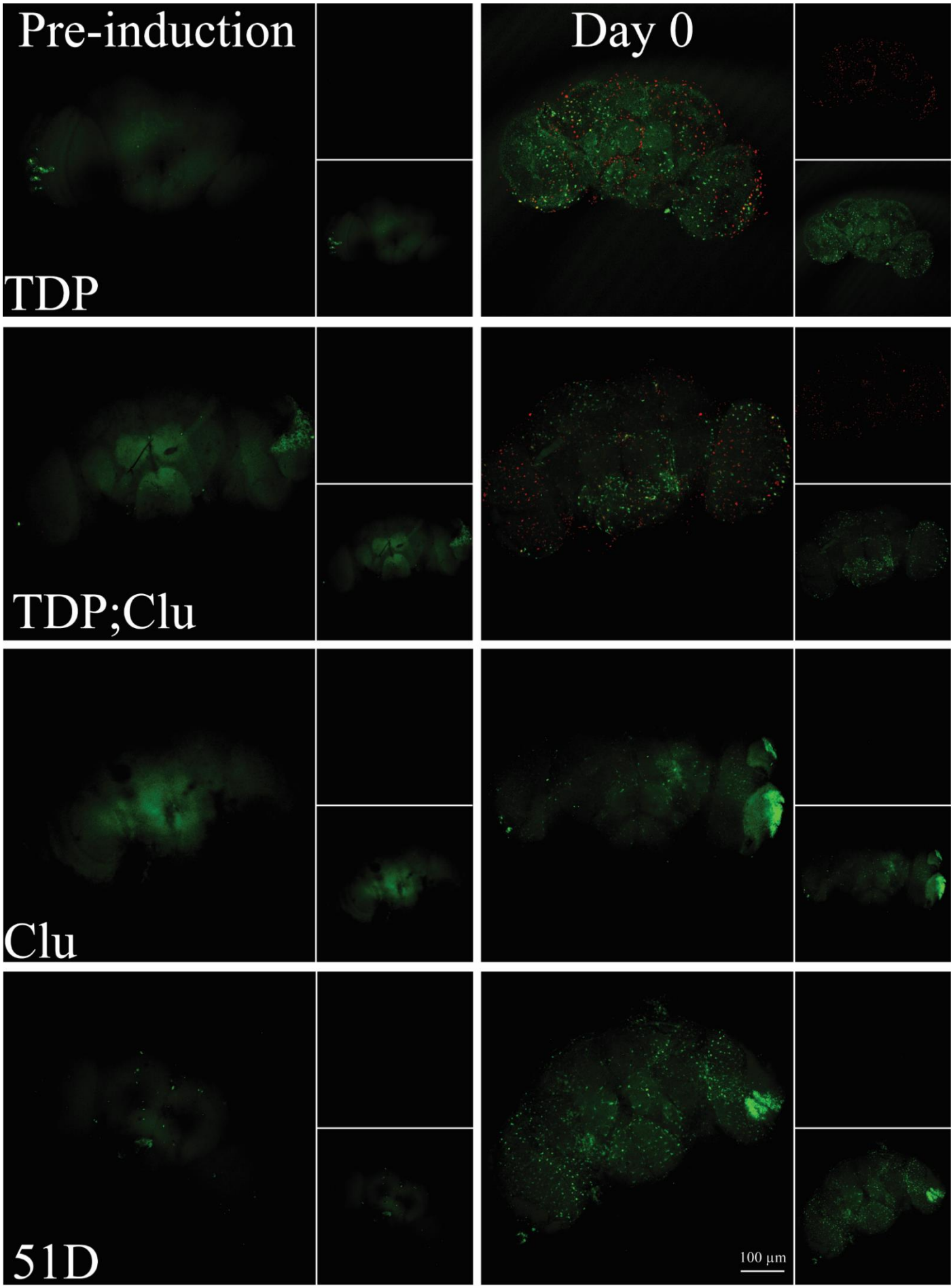
Pre-induction
Day 0
TDP
TDP;Clu
Clu
51D
100 µm

Figure 4.5: Expression of TDP-43 and GFP throughout the brains of adult *Drosophila* over a 24 h time period following induction. Confocal microscopy images of the brains of adult *Drosophila* expressing in glial cells either TDP-43, TDP-43 and clusterin (TDP;Clu), or clusterin alone. In the smaller panels TDP-43 is shown by anti-HA staining (red), while glial cells are green. Large panels show the merged image. 51D is a non-transgenic control. The results for the pre-induction time point show that TDP-43 and GFP expression was successfully supressed when progeny were kept at 18 ℃. The day 0 time point shows the induction of TDP-43 and GFP expression. At the day 0 time point TDP-43 was observed in cells that did not contain GFP, suggesting that TDP-43 had spread from where it was originally expressed. Images presented were obtained by collecting one Z stack (consisting of 16 individual images) from one brain per genotype, which was then flattened into a single image using the "Z-project" tool in ImageJ.

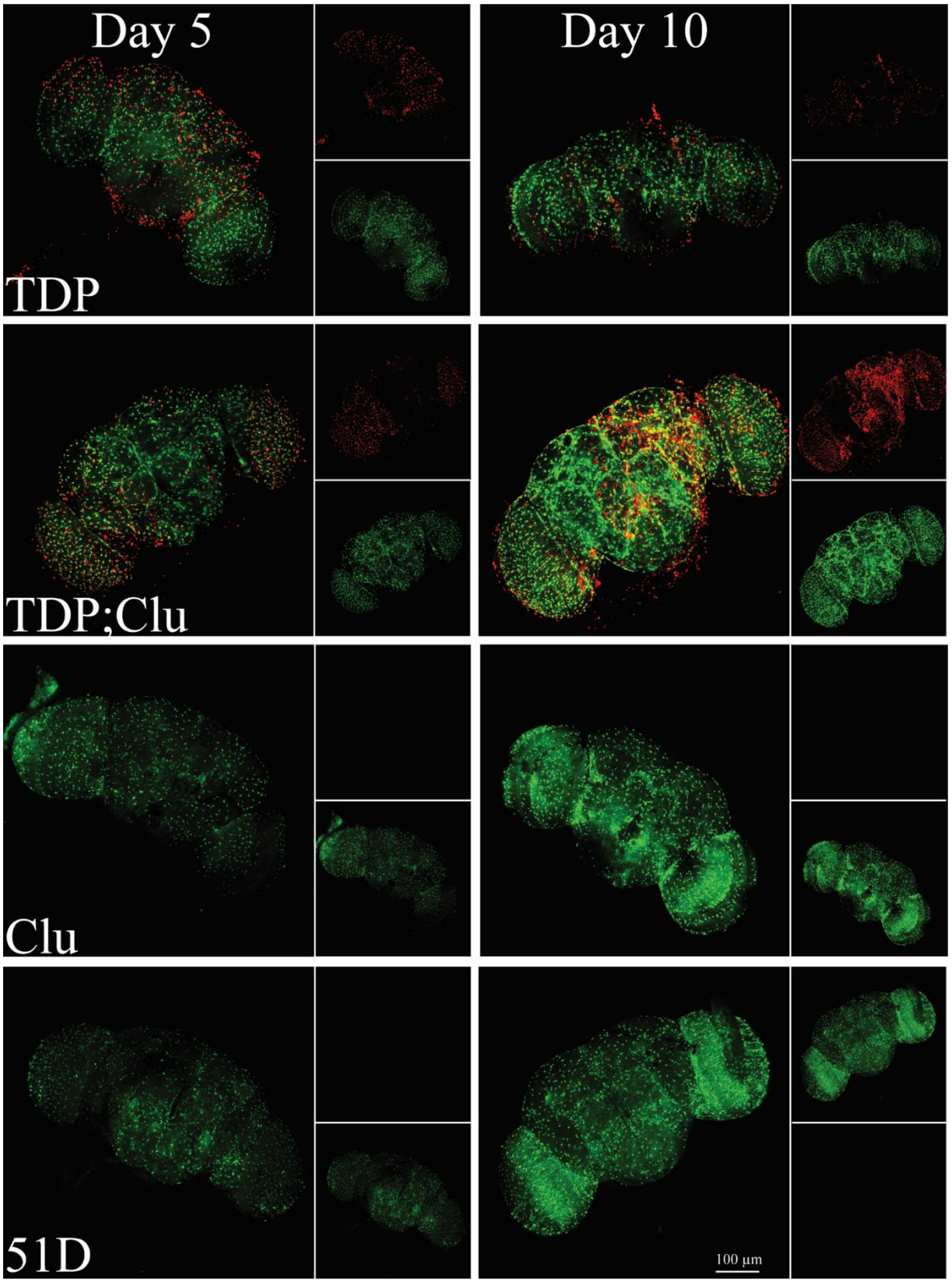
Day 5
Day 10
TDP
TDP;Clu
Clu
51D
100 µm

Figure 4.6: The spread of TDP-43 throughout the brains of adult *Drosophila* over a 10 day time period. Confocal microscopy images of the brains of adult *Drosophila* expressing in glial cells either TDP-43, TDP-43 and clusterin (TDP;Clu), or clusterin alone. In the smaller panels TDP-43 is shown by anti-HA staining (red), while glial cells are green. 51D is a non-transgenic control. Large panels show the merged image. The regions appearing as yellow in the image of TDP;Clu brain indicate co-localisation of TDP-43 and glial cells at day 10. Images presented were obtained by collecting one Z stack (consisting of 16 individual images) from one brain per genotype, which was then flattened into a single image using the "Z-project" tool in ImageJ.

When examining the spread of TDP-43 in the brains of flies expressing either TDP-43 or co-expressing TDP-43 and clusterin, after 24 h at 25 °C (day 0), high power images reveal the majority of TDP-43 (red) observed was not co-localised with the glia (green), suggesting that TDP-43 is able to move from these cells extremely quickly, within 24 hours of being expressed (Figure 4.7).While the majority of TDP-43 was observed to be outside the glia, it was still possible to see some glial cells that retained TDP-43 throughout the brain. This was most clearly seen in the optic lobes of the flies, and is indicated by white arrow heads in Figure 4.7A. The white boxes indicate the area of the brain which has been magnified, in this case the right optic lobe. At this time point of the experiment no obvious differences between the flies expressing TDP-43 and those expressing TDP-43 and clusterin could be observed. While control experiments described in Chapter 3 showed that in the larval system used the protein of interest is correctly expressed in the glial cells, due to time constraints no equivalent experiments to re-confirm that TDP-43 is initially correctly expressed in the glia were performed for the adult inducible system. However the driver line used in this chapter (dEAAT-1-GFP;Gal80) was derived from the driver line used in Chapter 3 and should thus function the same.

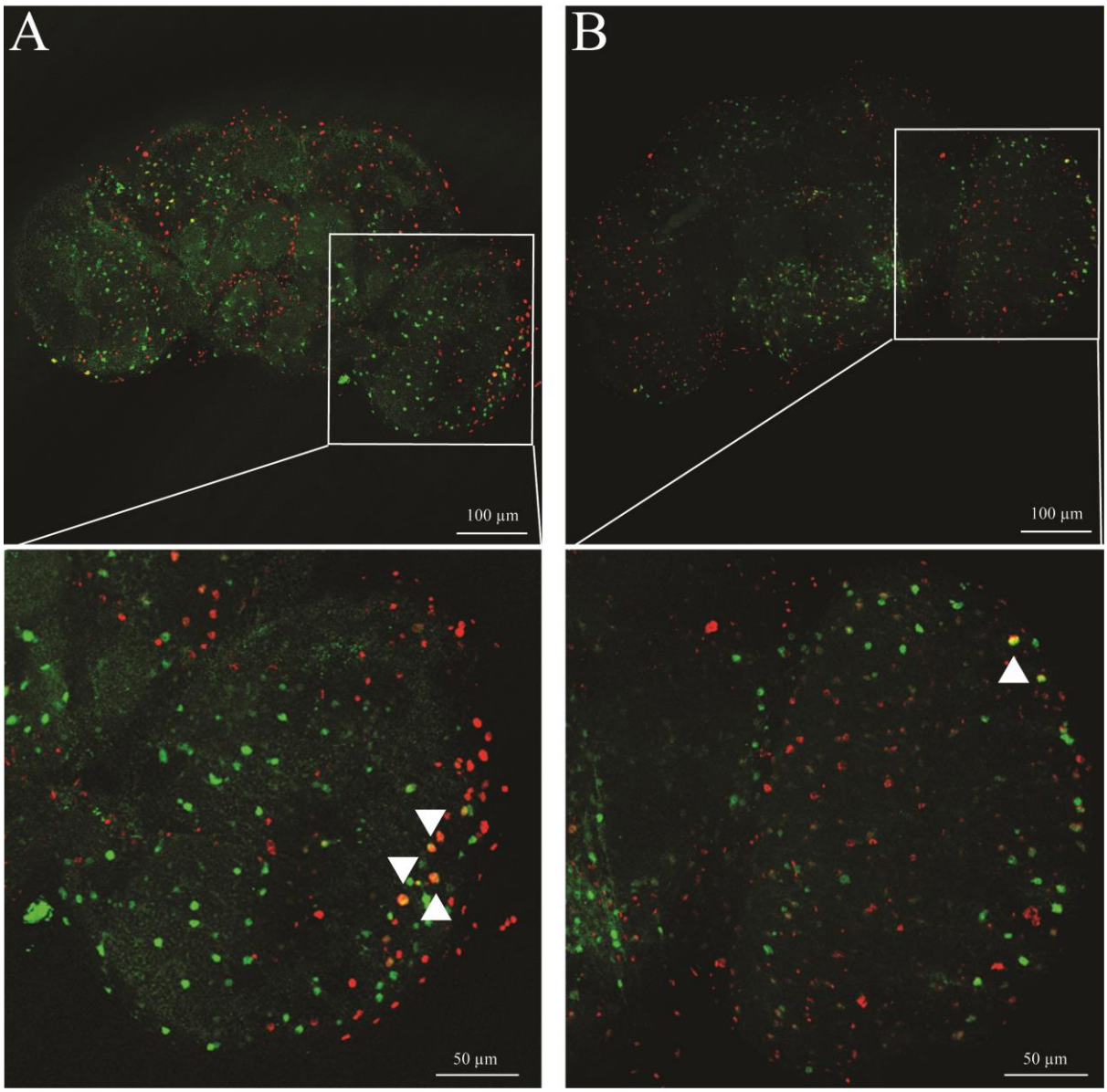

Figure 4.7: Immunofluorescence images of the brains of adult *Drosophila* at day 0 expressing TDP-43 (A) or co-expressing TDP-43 and clusterin (B). Low power images (whole brain) are in the top panel, and high power images (primarily right optic lobe) are at the bottom. TDP-43 is shown in red while GFP is shown in green. The clearest cases of glial cells containing TDP-43 were in the optic lobes, indicated by white arrow heads pointing to yellow areas of TDP-43 and glia overlap. The images presented are not a Z stack but a single image from one plane of focus.

Interestingly, while very little TDP-43 was detected in the glia at day 0, this increased over time. This may, however, be due to the fact that at day 10 expression of both TDP-43 and GFP are at a maximum level, and thus any overlap between the two will be most easily detected. By day 5, differences in TDP-43 expression patterns between TDP-43 and TDP;Clu flies were observed. For example, in the flies expressing TDP-43 only, TDP-43 was located mainly in the centre of the brain, and was less abundant in the optic lobes, while in flies expressing TDP-43 and clusterin the converse was true (Figure 4.8).

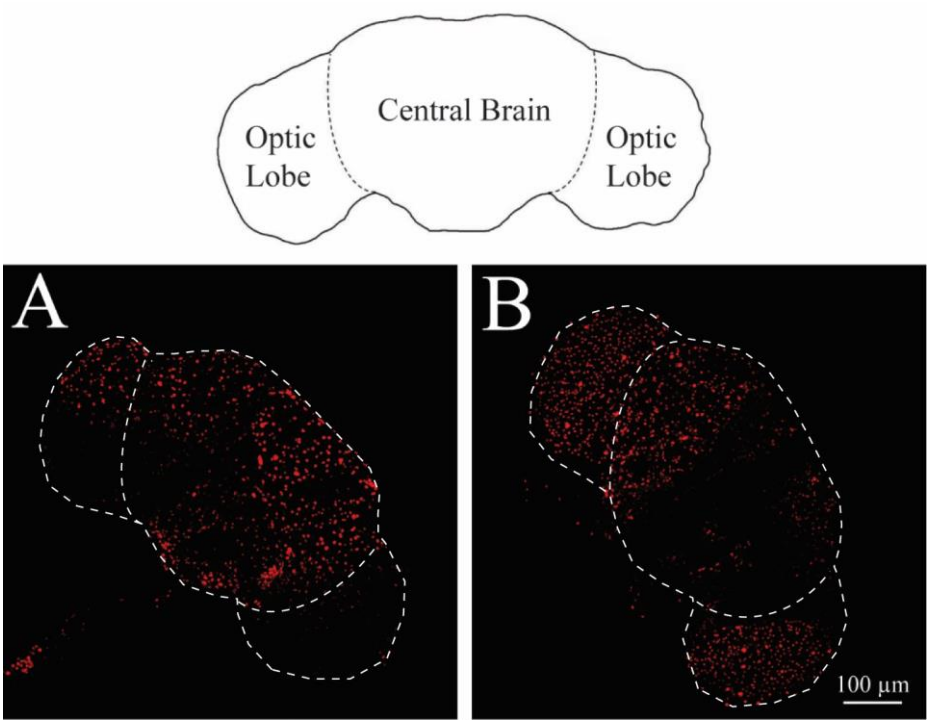

Figure 4.8: Expression of TDP-43 (red) in the brains of 5 day old adult *Drosophila* expressing A) TDP-43 or B) TDP-43 and clusterin. White dotted lines indicates regions of the brain as outlined in the anatomical cartoon. The images presented were obtained by collecting 1 Z stack which was then flattened into a single image using the "Z-project" tool in ImageJ.

Higher magnification images of the optic lobes and central regions of day 5 brains revealed that the flies which co-expressed TDP-43 and clusterin contained a greater amount of TDP-43 which remained in the glial cells where it was originally expressed, when compared to flies that expressed TDP-43 only. This is visualised as "yellow cells" due to the co-localisation of TDP-43 (red) and GFP (green) expressed in glia (Figure 4.9; white arrow heads). In the TDP;Clu flies, co-localisation of TDP-43 and GFP was particularly prominent in the optic lobes, although some instances of lo-localisation were also observed in the central brain region.

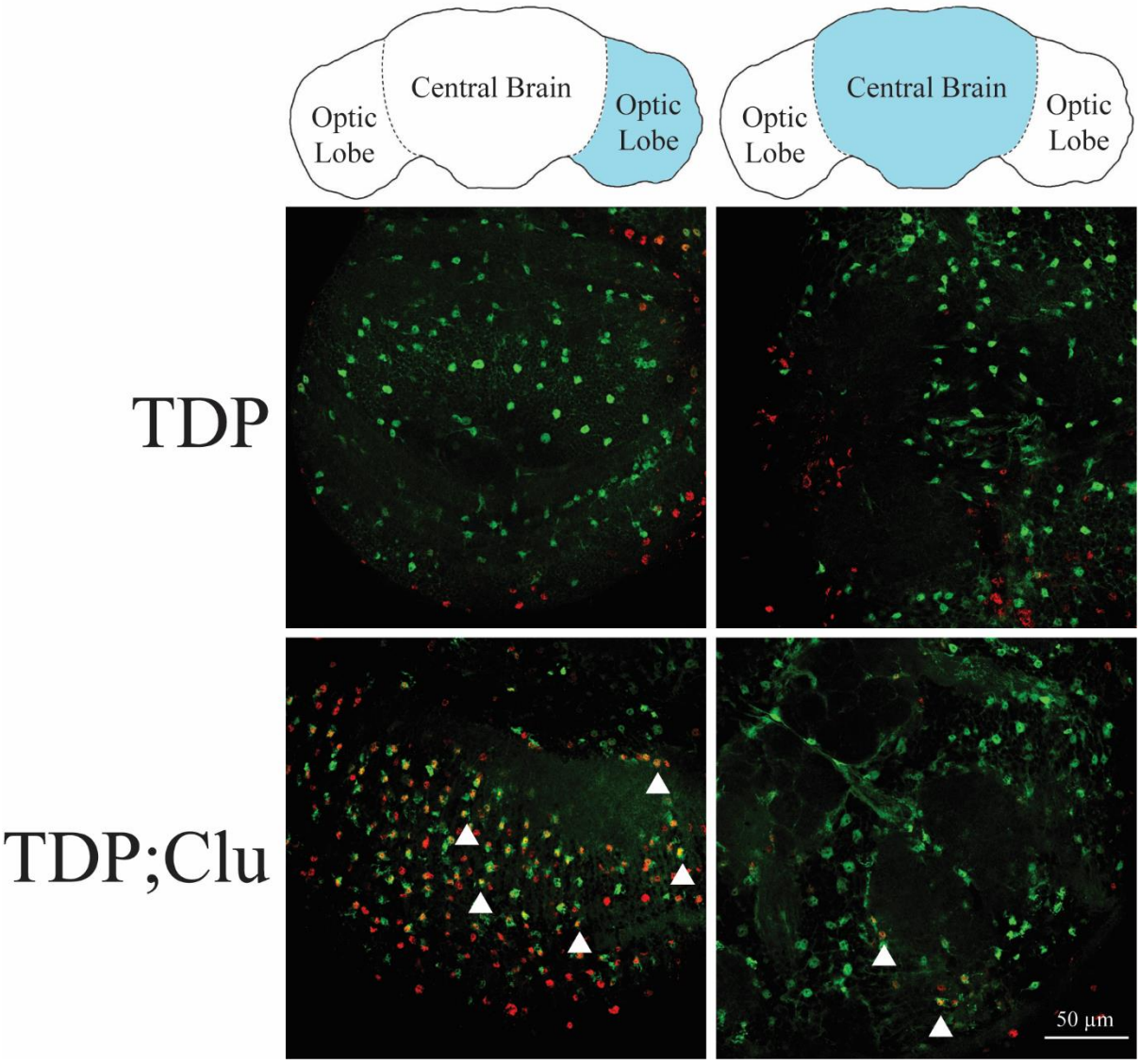

Figure 4.9: Immunofluorescence images of the optic lobe and central brain regions of 5 day old adult *Drosophila* expressing TDP-43 or co-expressing TDP-43 and clusterin (TDP;Clu). TDP-43 is shown in red and glial cells are shown in green. Instances of TDP-43 detected in glial cells are shown by white arrow heads. Areas highlighted in blue shading in the brain schematic (above) indicate the area of the brain being imaged in the panels below. The images presented are not a Z stack but a single image from one plane of focus.

At day 10, TDP-43 and TDP;Clu brains also showed considerable differences in the distribution of TDP-43, particularly in the optic lobes. Figure 4.10 shows higher magnifications of both the optic lobes and central brain regions of TDP and TDP;Clu flies. Again, in flies expressing TDP-43 and clusterin, but not in flies expressing TDP-43 only, TDP-43 was detected in optic lobe glial cells. Examples of TDP-43 detected in glial cells of the central brain region of TDP-43 and TDP;Clu flies

are shown in Figure 4.10. While the results presented here are consistent with the theory that TDP-43 is able to spread from glial cells to surrounding cell types, as seen in the larval system, this data is only preliminary and more work will be needed to both confirm the same is happening in adults and to develop a method of accurately quantifying spread.

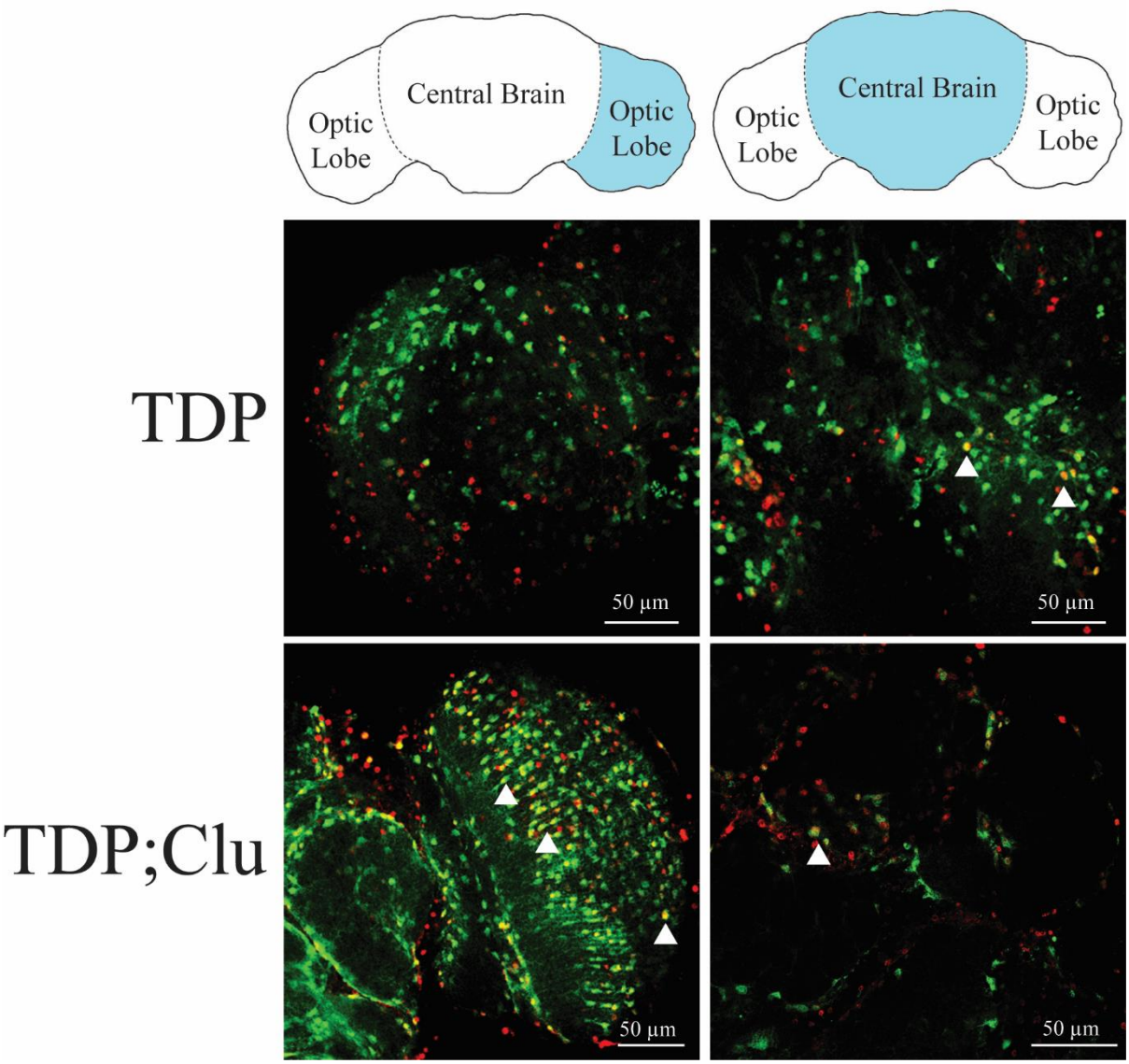

Figure 4.10: Immunofluorescence images of the optic lobe and central brain regions of 10 day old adult *Drosophila* expressing TDP-43 or co-expressing TDP-43 and clusterin (TDP;Clu). TDP-43 is shown in red and glial cells are shown in green. Instances of TDP-43 detected in glial cells are shown by white arrow heads. Areas highlighted in blue shading in the brain schematic (above) indicate the area of the brain being imaged in the panels below. The images presented are not a Z stack but a single image from one plane of focus.

4.4 DISCUSSION

4.4.1 Expression of TDP-43 in a subset of glial cells in *Drosophila* reduces lifespan

The need for an inducible *Drosophila* expression system arose from the observation that when expressed from the embryonic stage of development onwards, glial hTDP-43 was extremely toxic, causing premature lethality and making survival and locomotor assays difficult. An inducible system would allow the nervous system to develop normally before the expression of TDP-43. Furthermore, since ALS is a late onset disease, the inducible system would provide a more relevant *Drosophila* model of ALS.

Using a temperature sensitive inducible system it was found that expression of hTDP-43 in dEAAT1 positive glial cells caused a significant reduction in mean lifespan compared to WT flies (12.49 days v 46.46 days; $p < 0.0001$; Figure 4.2). The toxicity observed in the adults, and the larvae (section 3.3.3) is comparable to that seen in experiments performed by Diaper *et al*. (2013a), in which pan-glial expression of dTDP-43 caused premature lethality and age-related motor abnormalities. It was initially surprising that despite expression in only a subset of glial cells (representing 10% of the total glial population) lethality comparable to that resulting from pan-glial expression was observed. This led to the question of how this particular subset of cells could play such a large role in toxicity.

4.4.2 How might glutamate transporters contribute to toxicity?

As outlined in Chapter 3, a fly line was used in which expression of the protein of interest could be driven in only glial cells that expressed the high affinity glutamate transporter dEAAT1. Glutamate is the primary excitatory neurotransmitter in the central nervous system. In a normal synapse, glutamate is released from the presynaptic terminal where it activates specific receptors resulting in an influx of Na^+ and Ca^{2+} ions into the post-synaptic element and ultimately an action potential. Glutamate transporters are required for glutamate clearance in the synaptic cleft and protect neurons from glutamate excitotoxicity and neurodegeneration. In humans five transporters have been identified; EAAT1 and EAAT2 are astroglial type transporters, with EAAT2 (expressed mainly by astrocytes) being responsible for 90% of clearance from motor neurons. EAAT3 and EAAT4 are neuron type transporters, and EAAT5 expression is retina specific (Foran and Trotti, 2009; Sasaki *et al*., 2000). *Drosophila* have a single transporter called dEAAT1 which is expressed by a subset of glial cells. There is much evidence that glutamate induced excitotoxicity contributes to ALS and neuronal death. ALS patients show a large increase in plasma and cerebrospinal fluid glutamate levels, decreased glutamate uptake and decreased EAAT2 expression levels (Andreadou *et al*., 2008; Rothstein *et al*., 1992; Lin *et al*., 1998). SOD1 mouse models have also shown down regulation of glutamate transport activity and decreased glutamate transporter levels (Canton *et al*., 1998; Wilson *et al*., 2003). Glutamate transporters are also vital in flies, as RNA interference (RNAi)-mediated loss of

Drosophila transporter dEAAT1 in adult flies results in a shortened lifespan (10-13 days at 29 °C) and deficits in escape and flying behaviours, which can be rescued by the expression of the human transporter EAAT2 or the administration of the antiglutamateric drug riluzole, which is the only drug currently approved to treat ALS patients and regulates glutamate release and postsynaptic receptor activation (Rival *et al*., 2004; Foran and Trotti, 2009).

More recently links between TDP-43 expression and EAAT levels in ALS have been identified, suggesting that TDP-43 either directly or indirectly regulates transporter mRNA levels via distinct cell type specific mechanisms. mRNA of human EAAT1 and EAAT2 has been identified as potential TDP-43 targets, and pan-glial over expression of dTDP-43 in *Drosophila* results in a down regulation of dEAAT1 levels (Tollervey *et al*., 2011; Diaper *et al*., 2013a). In the current study it appears that expressing TDP-43 in dEAAT1 expressing cells shortens lifespan to the same extent as complete knockdown of dEAAT1. These results, together with those of the studies described above, suggest that in the adult *Drosophila* model used in this study the observed toxicity may be caused by a reduction in glutamate transporter levels caused by over-expression of TDP-43. An interesting extension of the experiments performed in this chapter would be to treat TDP-43 expressing flies with riluzole in food to determine if this could improve lifespan thus confirming whether glutamate induced excitotoxicity was occurring, and to measure glutamate and glutamate transporter levels in flies that express TDP-43 and/or clusterin.

4.4.3 Co-expression of clusterin with TDP-43 did not result in an increase in lifespan

When co-expressed with TDP-43 in motor neurons, clusterin has been previously shown to increase the average longevity of flies, compared to flies expressing TDP-43 alone (20 ± 0.53 days vs 15 ± 0.39 days; p=0.0006; Gregory *et al*., 2015). The current work tested whether clusterin would have a similar effect when expressed with TDP-43 in a subset of glial cells. While the co-expression of clusterin with TDP-43 increased lifespan by 1 day, this increase was not statistically significant and could be a result of clusterin having an effect on general aging rather than TDP-43 induced toxicity (Figure 4.2, 13.35 days v 12.49 days; $p < 0.9888$). As previously mentioned, the retrotranslocation of clusterin to the cytosol is a phenomenon dependant on ER stress. In the study by Gregory *et al*. (2015), the expression of TDP-43 in the neurons was shown to induce ER stress, demonstrated by higher levels of an ER stress marker in homogenates of the heads of flies expressing TDP-43 versus control flies. It may be that expressing TDP-43 in the dEAAT1 positive glia does not induce ER stress, and thus clusterin is not able to be translocated to the cytosol where it can act as an intracellular chaperone. Testing homogenates of fly heads for ER stress markers would determine if ER stress was induced in the flies and, if it was not, ER stress could be artificially induced to determine if this enabled clusterin to work as an intracellular chaperone and increase the lifespan of the TDP-43 flies.

Furthermore, more generally, it is possible that neurons and glia differ substantively in terms of their relative vulnerability to TDP-43 expression and their responses to it. Glial transporters are responsible for the majority of glutamate transport, and when in rats these are impaired, increased levels of extracellular glutamate, neurodegeneration and paralysis result; in contrast, loss of neuronal transporters produced only mild neurodegeneration and did not increase extracellular glutamate levels (Rothstein *et al*., 1996). Thus, if over-expression of TDP-43 is capable of disrupting the function of glutamate transporters, the toxic effects may be greater when expressed in glial cells compared to neurons, over-powering any protective effect of clusterin.

4.4.4 The expression of clusterin alone caused an increase in lifespan

Finally, the survival assay revealed that the expression of clusterin alone in glia caused a significant increase in mean longevity compared to WT flies (Figure 4.2; 60.56 days v 46.46 days; $p < 0.0001$), suggesting that in this model clusterin is able to delay aging in *Drosophila*. A number of animal models support the role that chaperones play in extending longevity. For example, the expression of Hsp22 in *Drosophila* increases both lifespan and resistance to oxidative stress (Morrow *et al*., 2004; Morrow and Tanguay, 2015), while in *C. elegans* the up-regulation of stress response genes including those for Hsps results in increased longevity (Walker *et al*., 2003). Results observed in this study are comparable with a previous study in which the expression of secreted clusterin both ubiquitously and restricted to the motor neurons of *Drosophila* resulted in an extension in lifespan as well as greater tolerance to heat shock and oxidative stress (Lee *et al*., 2012). Lee *et al*. (2012) also demonstrated that flies which overexpressed secreted clusterin had reduced levels of reactive oxygen species (ROS). Chronic damage cause by ROS-induced oxidative stress and dysregulation of the proteostasis network are features common to the multi-factorial process of aging. The results of the survival assay reported here and that performed by Lee *et al*. (2012) confirm that clusterin may play an important role in aging and age related diseases by acting to reduce both (i) the accumulation and toxicity of protein aggregates, and (ii) ROS levels by functioning as an antioxidant protein.

4.4.5 Expression of TDP-43 in a specific subset of glial cells in *Drosophila* decreased locomotor ability

There is an increasing volume of evidence suggesting that glial cells are significant contributors to motor neuron degeneration in ALS through non-cell autonomous mechanisms. Various *in vivo* and cell co-culture experiments have demonstrated that astrocytes expressing MT SOD1 are able to trigger ALS symptoms and induce motor neuron death (Papadeas *et al*., 2011; Marchetto *et al*., 2008; Nagai *et al*., 2007) and extracellular MT SOD1 can induce microgliosis resulting in the death of motor neurons (Urushitani *et al*., 2006). We aimed to test *in vivo* whether human TDP-43 expression in a small subset of glial cells was sufficient to cause motor neuron degeneration in *Drosophila* and if co-

expression of clusterin was able to prevent or delay this degeneration by performing a climbing assay. The first result that this assay revealed was that glial expression of TDP-43 resulted in an age dependant decrease in locomotor ability with a significantly lower climbing index at the end point of the assay compared to control flies (Figure 4.4B, $P < 0.05$), suggesting that glial TDP-43 expression, even in a small subset of glial cells, is sufficient to result in motor neuron degeneration. This is consistent with several published *in vivo* studies; for example, in rats the expression in astrocytes of MT TDP-43 resulted in paralysis and non-cell autonomous motor neuron death (Tong *et al*., 2013), and *Drosophila* larvae which expressed TDP-43 in all glial cells had significantly impaired locomotor function comparable to larvae which expressed TDP-43 in motor neurons (Estes *et al*., 2013). The current findings do, however, differ from a previous study in which astrocytes generated from ALS patients carrying a TDP-43 mutation were grown in co-culture *in vitro* with motor neurons. In this study the motor neurons did not suffer from any adverse effects as a result of being in co-culture with MT TDP-43 astrocytes (Serio *et al*., 2013). These differences could be the result of several factors. Firstly, *in vitro* co-culture models may not be able to fully recapitulate the interactions that occur between glia and motor neurons *in vivo*, and thus may not represent the complex disease mechanisms that occur during ALS progression. Similarly astrocytes that have been differentiated *in vitro* may behave differently to those that have matured in *in vivo*. Lastly, the differentiated astrocytes used by Serio *et al*. (2013) have been reported to express TDP-43 at moderate levels which may be insufficient to affect motor neurons (Tong *et al*., 2013). In the *in vivo* model of ALS used in the current study the glial cells are able to develop and interact with neuronal cells normally, and should thus avoid the first two issues outlined above. The *Drosophila* model also overexpresses human TDP-43 at high levels which may explain why expression in glial cells was sufficient to induce motor neuron degeneration, but not in the experiments performed by Serio *et al*. (2013). The results outlined above provide additional evidence to support the theory that non-neuronal cells can contribute to neurodegeneration, but relatively little is known about the mechanisms involved.

4.4.6 How might glial cells contribute to motor neuron degeneration?

It is thought that glial cells can contribute to motor neuron degeneration in two main ways. The first is by the release of various toxic factors; this was demonstrated by several studies which expressed SOD1 in astrocytes (Papadeas *et al*., 2011; Marchetto *et al*., 2008; Nagai *et al*., 2007). Similarly, astrocytes that express TDP-43 produce increasing amounts of pro-inflammatory cytokines, chemokines, free radicals and neurotoxic mediators proportional to the amount of TDP-43 they express, and can provoke a hyperactive innate immune response which increases neurodegeneration (Swarup *et al*., 2011). Microglia can also contribute to neurodegeneration, as early in ALS they undergo a process called reactive microgliosis and release a variety of substances that worsen neurodegeneration. The second major mechanism involves the loss of critical glial function, for

example via the loss of the glutamate transporters, altered potassium buffering and metabolic support (Valori *et al*., 2014). Loss of glutamate transporters is the most well understood and the only confirmed pathogenic mechanism in both humans and animal models. In rats the expression of MT TDP-43 in astrocytes causes a progressive loss of the glutamate transporters EAAT1 and EAAT2 in the spinal cord and is sufficient to kill spinal motor neurons (Tong *et al*., 2013), and the ubiquitous overexpression of MT SOD1 in rat spinal cord causes a loss of EAAT2 (Howland *et al*., 2002). The effect of TDP-43 expression on glutamate transporters is discussed above.

4.4.7 Co-expression of clusterin with TDP-43 protected locomotor function

The most intriguing result revealed by the climbing assay was that the co-expression of clusterin protected locomotor function throughout the assay to levels comparable to that of the non-transgenic control, with only a slight decline in function observed from day 6 onwards (Figure 4.4A). This indicates that while the glial expression of clusterin was unable to significantly extend the lifespan of flies, it is able to delay the degeneration of motor neurons at least in the early stages of the disease. As mentioned previously, the assay was only carried out for 10 days. This is because flies that express TDP-43 have a very rapid decline in longevity from day 10 onwards as seen in Figure 4.3, having a mean lifespan of only 12 days. It was thus difficult to obtain data over a longer time period.

The climbing assay was performed according to experimental procedures already established within the laboratory, in which pre-prepared pipette tubes were re-used for each repeat of the assay and a traditional negative geotaxis assay was performed to measure how many flies climbed above a pre-determined height after 30 seconds. An alternative "Rapid Iterative Negative Geotaxis or "RING" assay has recently been described (Nichols *et al*., 2012), in which 25 flies are transferred into 10 cm polystyrene tubes, and the average height climbed per vial over three seconds is recorded. The RING assay is advantageous as more replicates are able to be measured at one time, over a shorter time frame giving it a very high throughput. This assay is also more sensitive as the height climbed in a defined period is quantified, meaning that more subtle differences in climbing defects may be observed. The authors describing the RING assay also note that it is critical that vials must not be reused after each day of testing, as flies placed into used vials do not climb to the same extent as they would in fresh vials (Nichols *et al*., 2012). This may explain why results presented here show a decline in motor function in the control and clusterin groups during day 6 and 7, and in fact a gradual decline for all groups over the course of the assay. Ideally the experiment would be repeated using the RING assay with a larger number of flies, or at least repeated using fresh pipettes each day. Regardless, from the results obtained it does appear that the co-expression of clusterin in the dEAAT1 positive glial cells is able to prevent or at least delay the TDP-43 induced degeneration of motor

neurons in the *Drosophila* model, although the mechanisms by which it does this remain to be identified and will be discussed further in Chapter 5.

4.4.8 Expression of TDP-43 in a subset of glial cells in *Drosophila* results in rapid spread to other cell types

The brains of adult *Drosophila* were imaged at various time points to determine if TDP-43 was able to spread from the glial cells where it was expressed, to other cell types. The results reveal that from 24 hours onwards TDP-43 was observed in cells that did not express dEAAT1. This is similar to what was observed in the larvae (Chapter 3.3.1), and is consistent with TDP-43 moving out from dEAAT1 positive glial cells to other surrounding cell types. Prior to induction, there was no detectable expression of GFP or TDP-43, meaning that the proteins of interest were not being expressed before the flies were moved to 25 °C. In hind sight, it would have been ideal to have earlier image time points, for example 1, 5, and 10 h after protein expression was induced, as by 24 h most of the TDP-43 had already exited from the dEAAT1 cells. Images at earlier time points would allow confirmation of the initial localisation of TDP-43 in the desired cells, and also provide a time frame for how quickly the protein was able to spread. Regardless, combined with the results obtained using the larval expression system, it appears likely that TDP-43 is able to rapidly spread from the cells in which it was initially expressed, and that this movement was not prevented by co-expression of clusterin. It remains possible that clusterin can delay the spread of TDP-43 during the first 24 h of expression before becoming overwhelmed, but this would require further work to confirm this.

In summary, the results obtained in this study using *Drosophila* reveal that even when TDP-43 is expressed in a subset of glial cells, it results in a reduction of lifespan and decrease in locomotor ability, confirming the important role that non-neuronal cells play in the pathology of ALS. The results particularly highlight the potential importance of glutamate transporters, as the toxic phenotypes occurred when TDP-43 expression was restricted to the glial cells which contained them, despite the fact that they represent only 10% of total glial population. It was also demonstrated that co-expression of clusterin with TDP-43 did not significantly extend lifespan but did rescue the locomotor function of flies. This supports the possibility of chaperones being developed for use as therapeutics in the future, either by up-regulation of endogenous chaperones or the administration of synthetic alternatives.

CHAPTER 5: INTERACTIONS BETWEEN CLUSTERIN AND TDP-43 *IN VITRO* AND IN CELLS

5.1 INTRODUCTION

Experiments performed with *Drosophila* described in the previous two chapters suggest that when overexpressed in the glial cells, TDP-43 is able to rapidly spread to surrounding cell types including motor neurons. Results also show that glial TDP-43 expression leads to decreased lifespan and locomotor defects, with the co-expression of clusterin able to reduce these phenotypes to some extent. The experiments described in this chapter were designed to examine interactions between clusterin and TDP-43 beyond those observed in the *Drosophila* model, by addressing the following three aims;

1) To perform an assay to establish if clusterin is able to inhibit the *in vitro* aggregation of TDP-43, thus potentially accounting for the reduction in toxicity and locomotor defects observed in *Drosophila*.
2) Investigate whether interactions between clusterin and TDP-43 occur in humans by examining human spinal cord tissue from control and ALS patients, to detect co-localisation of TDP-43 and clusterin.
3) To develop a mammalian cultured cell system that could be used to examine cell-to-cell spread of TDP-43, with the eventual goal of determining any effects of clusterin.

It is well established that in the nervous system, clusterin is constitutively expressed by neurons and both resting and active astrocytes. Expression is upregulated in response to stress such as traumatic brain injury, nerve crush, inhibition of Hsp90 and glutamate induced excitotoxicity (Pasinetti *et al*., 1994; Charnay *et al*., 2008; Van Beek *et al*., 2000; Wiggins *et al*., 2003; May *et al*., 1992; Ohlsson *et al*., 2003; Zinkie *et al*., 2013). In addition it is also overexpressed in the brains of people affected by CNS disorders such as Alzheimer's disease, and increased clusterin mRNA levels have been detected in the spinal cord and motor cortex of SALS patients (Grewal *et al*., 1999). Neuronal loss has also been shown to result in a substantial increase in clusterin mRNA levels in neighbouring cells, for example following neuronal death in rats there is a rapid overexpression of clusterin mRNA in the remaining living neurons, and a delayed up-regulation in the glia (May *et al*., 1992; Strocchi *et al*., 1999). Under normal conditions clusterin is secreted into the extracellular space where it acts as an extracellular chaperone, however recently it has been demonstrated that under conditions of stress it can be retrotranslocated from the secretory system to the cytosol, where it may act as an intracellular chaperone (Nizard *et al*., 2007; Li *et al*., 2013). Intracellular accumulation of clusterin has also been observed in the dying neurons of rats (Dragunow *et al*., 1995). More recently, lumbar spinal cords from symptomatic transgenic $SOD1^{G93A}$ mice were found to contain neuronal inclusions that labelled

strongly for both clusterin and SOD1 (Zinkie *et al*., 2013). Clusterin is expressed by neurons and overexpressed during times of stress (including ALS) and is a constituent of SOD1 containing inclusions in rat motor neurons in the lumbar spinal cord. Thus it was of interest to examine whether in human cases of ALS, clusterin was found in the cytosol of motor neurons and if it was a constituent of TDP-43 containing inclusions. This would be the first case of intracellular clusterin protein being observed in human ALS tissue, and could provide evidence for interactions between clusterin and TDP-43.

To investigate whether clusterin might interact with TDP-43 inside the motor neurons of ALS patients, the white and grey matter of human spinal cord tissue was examined for the presence of intracellular clusterin and TDP-43. The spinal cord is separated into 31 segments; 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal. Thoracic sections were used in this study. When viewing a transverse section of spinal cord, such as the samples that were analysed, the white matter can be seen in the periphery surrounding the symmetrical H shaped grey matter in the centre. The grey matter contains the neurons and glia, and is divided into the dorsal, lateral, and ventral horns (Figure 5.1).

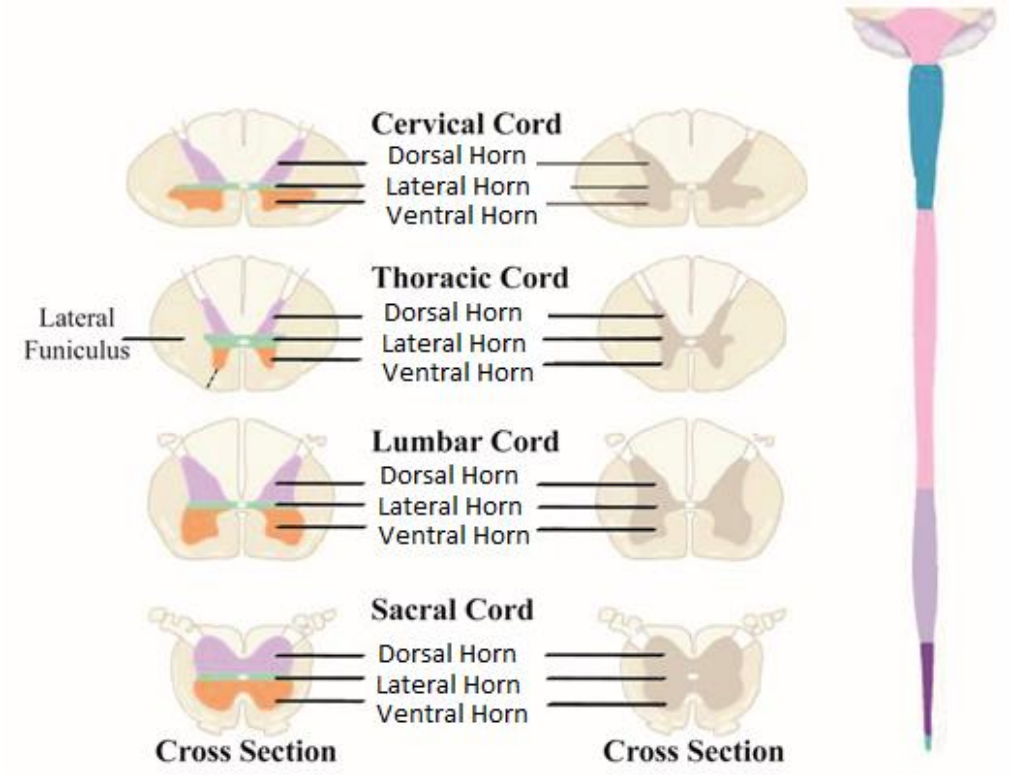

Figure 5.1: Spinal cord section showing the white and grey matter in four spinal cord levels. Sections of the H shaped grey matter are highlighted in purple, green and orange in the left images. The spinal cord image on the right shows the locations of the cervical, thoracic, lumbar and sacral levels (top to bottom; modified from Dafny, 2010).

When imaging the grey matter of spinal cord sections, it was the ventral horn that was imaged, as this is where the motor neurons that innervate skeletal muscles are found, as well as interneurons that help to fine tune motor output. The white matter contains bundles of myelinated axon tracts, which carry information up and down the spinal cord. Myelin is formed by oligodendrocytes in the central nervous system and is essential to the survival and function of neurons. Up to 99% of an axon's surface can be myelinated and thus only a small percent is exposed to the extracellular environment. The horns of the grey matter divide the white matter into posterior, lateral and anterior columns or funiculi. Images presented of the white matter are of the lateral funiculus, a name given to describe a large group of nerve fibres found in a certain area (Cho, 2015; Bican *et al*., 2013; Craven, 2005; Morrison et al., 2013).

The remaining aim of the work described in this chapter was to develop an experiment where the prion-like spread of TDP-43 between different cell types could be examined in cell culture. The aim was to test whether such a spreading process as observed in *Drosophila* occurred *in vitro*, with the major potential advantage being that results obtained using cell cultures would be easy to quantify and replicate. Chapter 3 outlined the difficulties associated with quantifying the spread of TDP-43 and effect of clusterin co-expression in the fly models, so a cell model utilizing flow cytometry was designed to hopefully circumvent these issues. Chapter 1 introduced the term prion-like used to describe a protein propagation mechanism that is proposed to be a common force driving neurodegeneration in a number of diseases, including ALS. For a protein to act in a prion-like way it must be able to propagate in a self-templating manner and transmit cell-to-cell. As outlined in section 1.7.1, it has been demonstrated that aggregated SOD1 can act as a seed to induce misfolding and aggregation of native SOD1 protein, as well as transmit between cultured cells. Similarly TDP-43 aggregates have been shown to drive the co-aggregation of native TDP-43 and are capable of propagating between cultured SH-SY5Y cells (refer to section 1.7.2). This chapter describes the development of a co-culture system using murine motor neuron like and astrocyte cell lines to examine whether spread from glia to motor neurons seen in *Drosophila* also occurred *in vitro*.

5.2 METHODS

5.2.1 Immunohistochemistry of human spinal cord sections

Three prepared sections from three different control spinal cord tissue samples (see section 2.3) were examined according to section 2.4.1 of materials and methods, with 25 images in total collected. Two prepared sections from two different sporadic ALS spinal cord tissue samples were similarly examined, with 24 images in total collected.

5.2.2 *In vitro* protein aggregation assay

Refer to section 2.7 of materials and methods.

5.2.3 Co-culture of cells

Murine Ast-1 (astrocyte-like) cells were transiently transfected with (i) M337V TDP-43 tagged with C-terminal GFP (MT TDP-43-GFP), or (ii) a C terminal region of TDP-43 (amino acids 216-414 only; CTF-TDP-GFP) tagged with C-terminal GFP. To ensure the optimal transfection efficiency 3 different transfection reagents were tested using 4 different ratios of transfection reagent:DNA. X-tremeGENE HP DNA Transfection Reagent (Roche), X-tremeGENE 9 DNA Transfection Reagent (Roche) and Lipofectamine® 2000 Transfection Reagent (ThermoFisher Scientific) were tested at ratios of 1:1, 3:1, 4:1 and 5:1. Using Lipofectamine® 2000 at a ratio of 4:1 yielded the highest transfection efficiency of ~40% (data not shown) and thus these conditions were used for all further transfection of Ast-1 cells. Cells transfected with MT TDP-43-GFP were stressed with MG-132 to induce aggregation, while cells transfected with the C terminal fragment of TDP-43 formed aggregates without additional treatment (section 2.8.5). Transfected cells were then mixed equally with murine NSC-34 (motor neuron-like) cells that were labelled with calcein orange (section 2.8.5) and grown in co-culture in DMEM supplemented with 5% (v/v) FCS. Flow cytometry was performed after 24 h to detect cell populations that were positive for both calcein orange and GFP, representing NSC-34 cells that were associated with TDP-43-GFP that had originated in the Ast-1 cells (Figure 5.2). Compensation was applied to the samples to remove any "spill over" fluorescence between emission collection windows for the two different fluors. A compensation matrix was computed by defining positive and negative populations for each fluorescence channel used, and then applying the matrix computed to the appropriate samples. Samples that had been compensated were then used for the remaining analysis.

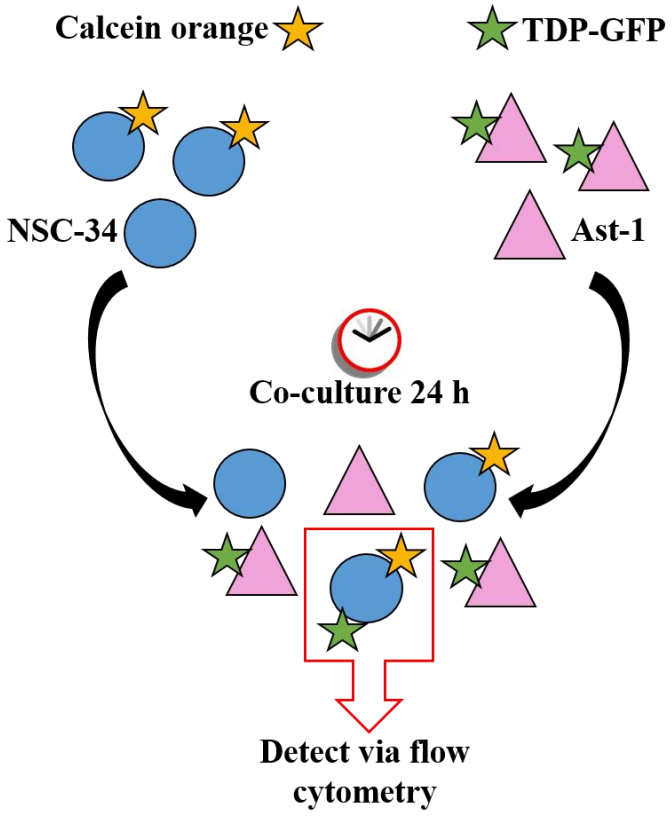

Figure 5.2: Experimental outline for co-culture experiment. NSC-34 cells labelled with calcein orange were mixed 1:1 with Ast-1 cells transfected with GFP labelled TDP-43 and grown in co-culture for 24 h. Flow cytometry was used to detect calcein labelled NSC-34 cells that contain TDP-GFP that originated from the Ast-1 cells.

5.2.4 Preparation and application of exogenous TDP-43 to cells

Murine N2a (neuroblastoma) cells were transiently transfected with MT TDP-43-GFP or CTF-TDP-GFP. The cell lysate (prepared according to methods outlined in section 2.8.4) from these cells was added to NSC-34 cells and incubated for 24-48 h in DMEM supplemented with 5% (v/v) FCS. Flow cytometry and confocal microscopy was performed after 24 or 48 h to detect (green fluorescing) NSC-34 cells that also had GFP fluorescence, which would represent NSC-34 cells that had taken up TDP-GFP originating from the N2a cells. Cell lysate from N2a cells was added to the NSC-34 cells at a ratio of 2:1 (for every 1 NSC-34 acceptor cell, the lysate from 2 transfected N2a cells was added) in an attempt to increase the extent of uptake of TDP-43.

5.3 RESULTS

5.3.1 Clusterin is able to potently inhibit the aggregation of a C-terminal fragment of TDP-43 *in vitro*

After observing both an increase in lifespan and rescue of motor activity in TDP-43 flies that co-expressed clusterin, as well as a decrease in the spread of TDP-43 in larvae, we next sought to confirm that clusterin is able to inhibit the aggregation of TDP-43 by performing an *in vitro* aggregation assay. Owing to its inherently high propensity to aggregate, full length TDP-43 is notoriously difficult to express and purify, limiting *in vitro* studies of its aggregation. The C-terminal domain of TDP-43 is the most aggregation prone region of the protein and a synthetic peptide corresponding to this region provides a practical option to examine features of TDP-43 aggregation *in vitro*. Under the conditions tested, the TDP-$43_{286-331}$ peptide began to aggregate after a lag phase of about 2 h (Figure 5.3) and clusterin was able to increase this lag phase and inhibit aggregation at ratios as low as 1:25000 (Clu:TDP-43). At a ratio of 1:1000 clusterin completely inhibited aggregation of TDP-$43_{286-331.}$ The aggregation of TDP-$43_{286-331}$ was not affected by the addition of SOD1 (a non-chaperone control protein) even when added at a 1:500 ratio of SOD1: TDP-$43_{286-331}$.

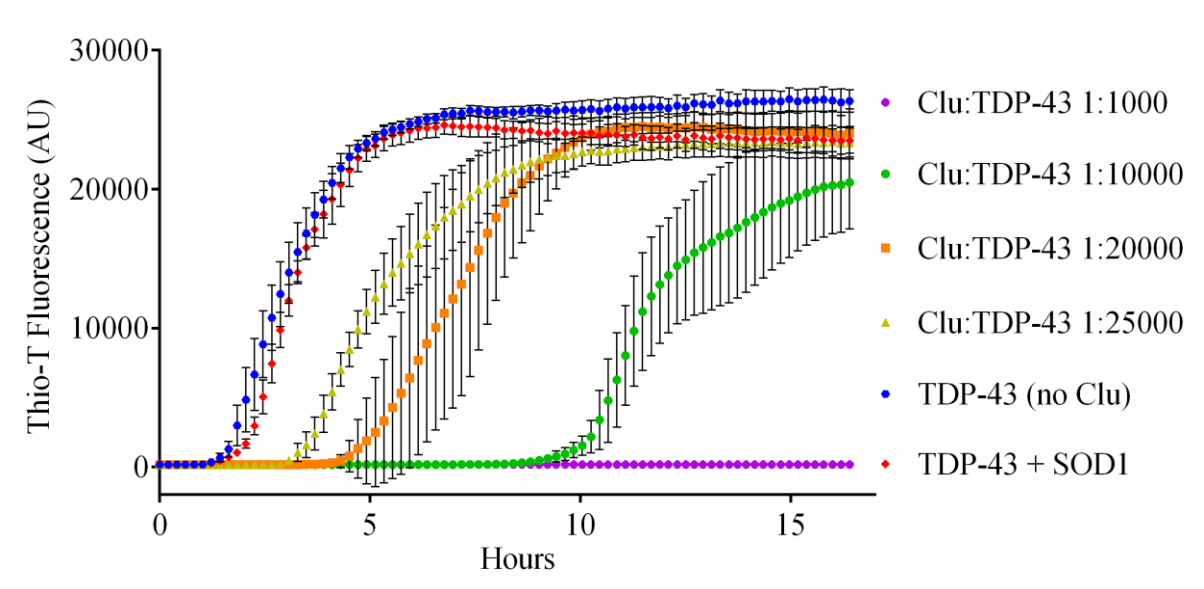

Figure 5.3: Clusterin inhibits the in vitro aggregation of a C-terminal TDP-43 peptide. TDP-$43_{286-331}$ (224 µM in HEPES, pH 7.4) was incubated at 37 °C with shaking, in the presence of various molar ratios of Clu:peptide (see key) or with the non-chaperone control protein bovine superoxide dismutase (SOD1) at a molar ratio of SOD1:TDP-43 = 1:500. Peptide aggregation was monitored by Thioflavin T fluorescence. Error bars represent the standard error of the mean (n=4). The result shown is representative of several independent experiments.

5.3.2 Intracellular clusterin is observed in ALS-patient post mortem spinal cord motor neurons and the myelin sheath of oligodendrocytes and was seen to co-localise with TDP-43.

In this chapter we sought to examine for the first time the location of clusterin in human ALS and control spinal cord tissue, and importantly whether it co-localised with TDP-43. We examined both the white and grey matter of the spinal cord in 3 control and 2 SALS samples. White matter consists almost exclusively of bundles of motor and sensory axons each coated with a myelin sheath created by oligodendrocytes, which have a dense and uniform longitudinal orientation in the spinal cord (Sparrey, 2009). In both SALS samples examined clusterin and TDP-43 were detected in "rings" of fluorescence, in a pattern which corresponds to previously published data showing specific cellular markers for Schwann cells, which are the equivalent of oligodendrocytes in the peripheral nervous system (Brown *et al*., 2012) and myelinated axons in the spinal cord of mice (Kang et al., 2013). It was thus concluded that the "ring" staining observed probably corresponds to myelinated axons. In the control sample TDP-43 also showed dense staining in (unidentified) areas outside oligodendrocytes. In the control sample designated 05/418, clusterin and TDP-43 staining patterns were detected that were very similar to those observed in the SALS tissue (Figure 5.4, left panel). To ensure that the secondary fluor-labelled antibodies were not binding non-specifically, white and grey matter of ALS tissue (09/066) that had been reacted with only the secondary antibodies was also examined. As expected no fluorescence was detected (appendix 3).

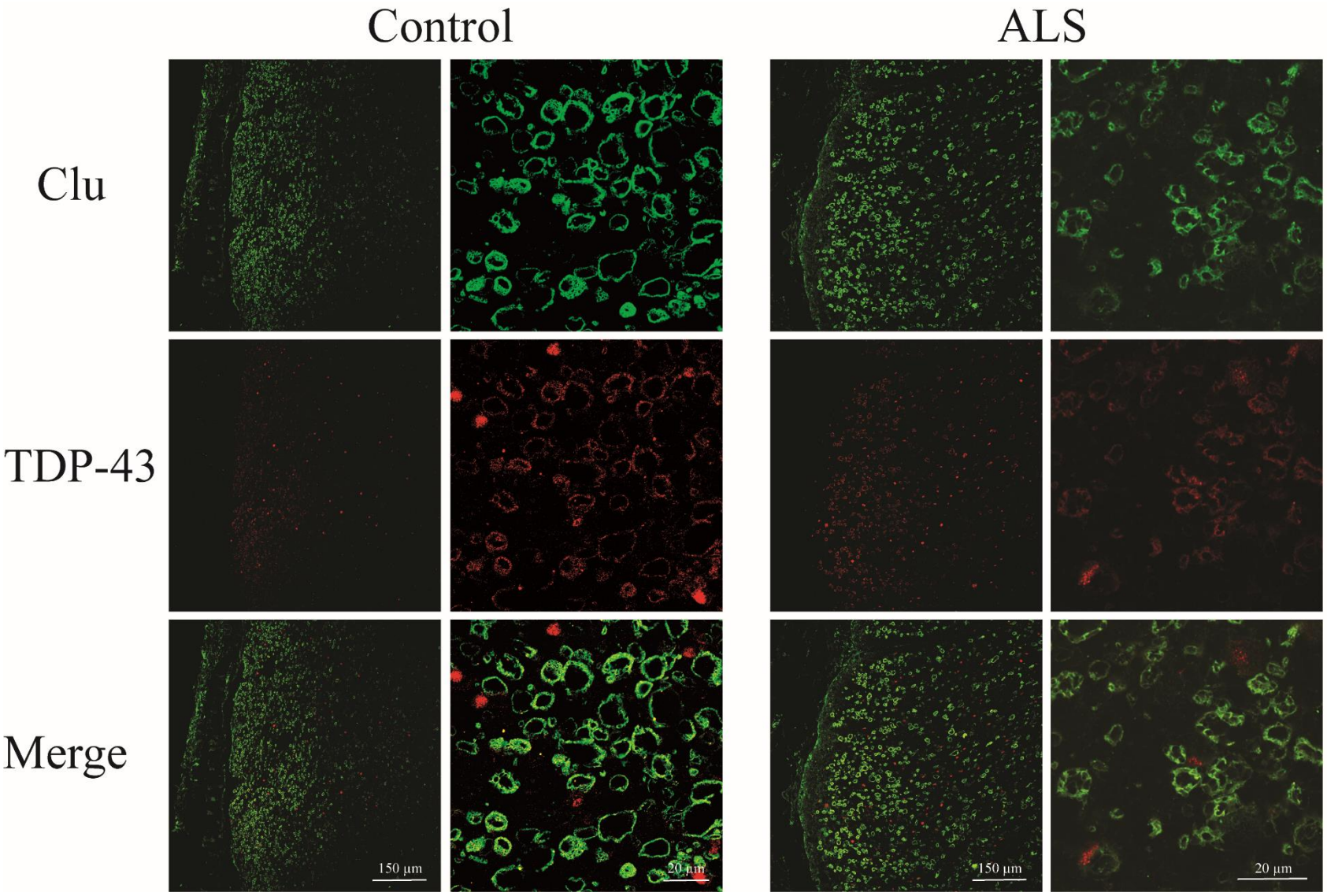
Control
ALS
Clu
TDP-43
Merge
150 μm
20 μm
150 μm
20 μm

Figure 5.4: Clusterin and TDP immunoreactivity in the white matter of control (05/418) and ALS (10/223) human spinal cord. Low (left) and high (right) power confocal microscopy images of thoracic spinal cord tissue sections. Clusterin is shown by green staining and TDP-43 is shown by red staining.

Clusterin levels and staining patterns in the white matter of each of the 3 control samples were then examined to see if there was any variation between them (Figure 5.5). In sample 07/743, there was a marked decrease in the level of clusterin observed and none of the characteristic "ring" patterns of clusterin staining seen in the other two control samples. Sample 07/743 had a post mortem interval (time elapsed since patient death) of 68 h, higher than both the other controls (50 h for 05/418 and 33 h for 04/273).

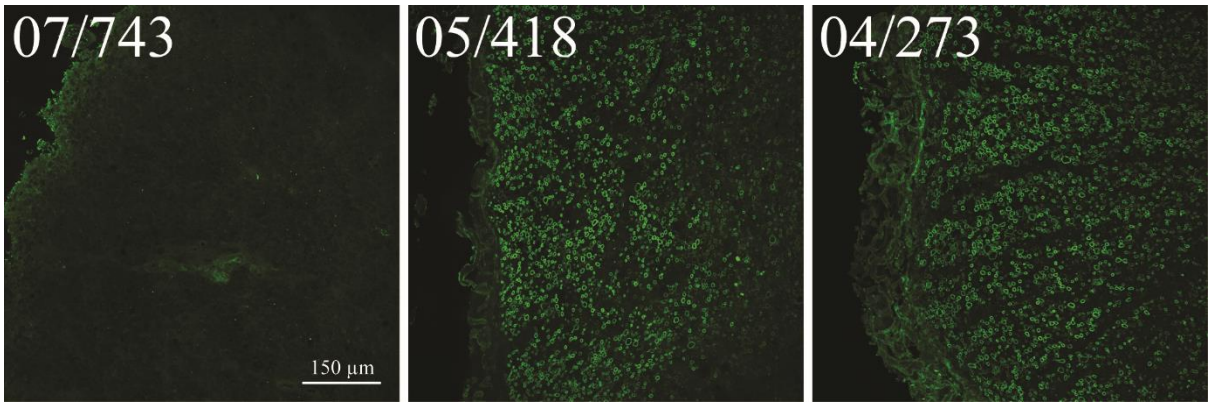

Figure 5.5: Clusterin immunoreactivity in the white matter of three different samples of control human spinal cord. Clusterin is shown by green staining.

Next, clusterin and TDP-43 were examined in the grey matter of human thoracic spinal cord sections, which contain neuronal cell bodies and glial cells (Figure 5.6). In all ALS and control samples high levels of cytoplasmic clusterin were detected within large motor neurons located in the ventral horn. In the control samples clusterin appeared as distinct puncta, whereas in the ALS tissue staining was more diffuse. In control tissue TDP-43 was observed in the nucleus of motor neurons, whereas cytoplasmic TDP-43 aggregates were seen in SALS tissue. Occasionally nuclear TDP-43 was also seen in SALS tissue. Importantly, even in this small sample size, we found an example of co-localisation between clusterin and TDP-43 in the tissue of a SALS patient, but not in control tissue, as shown by white arrow head.

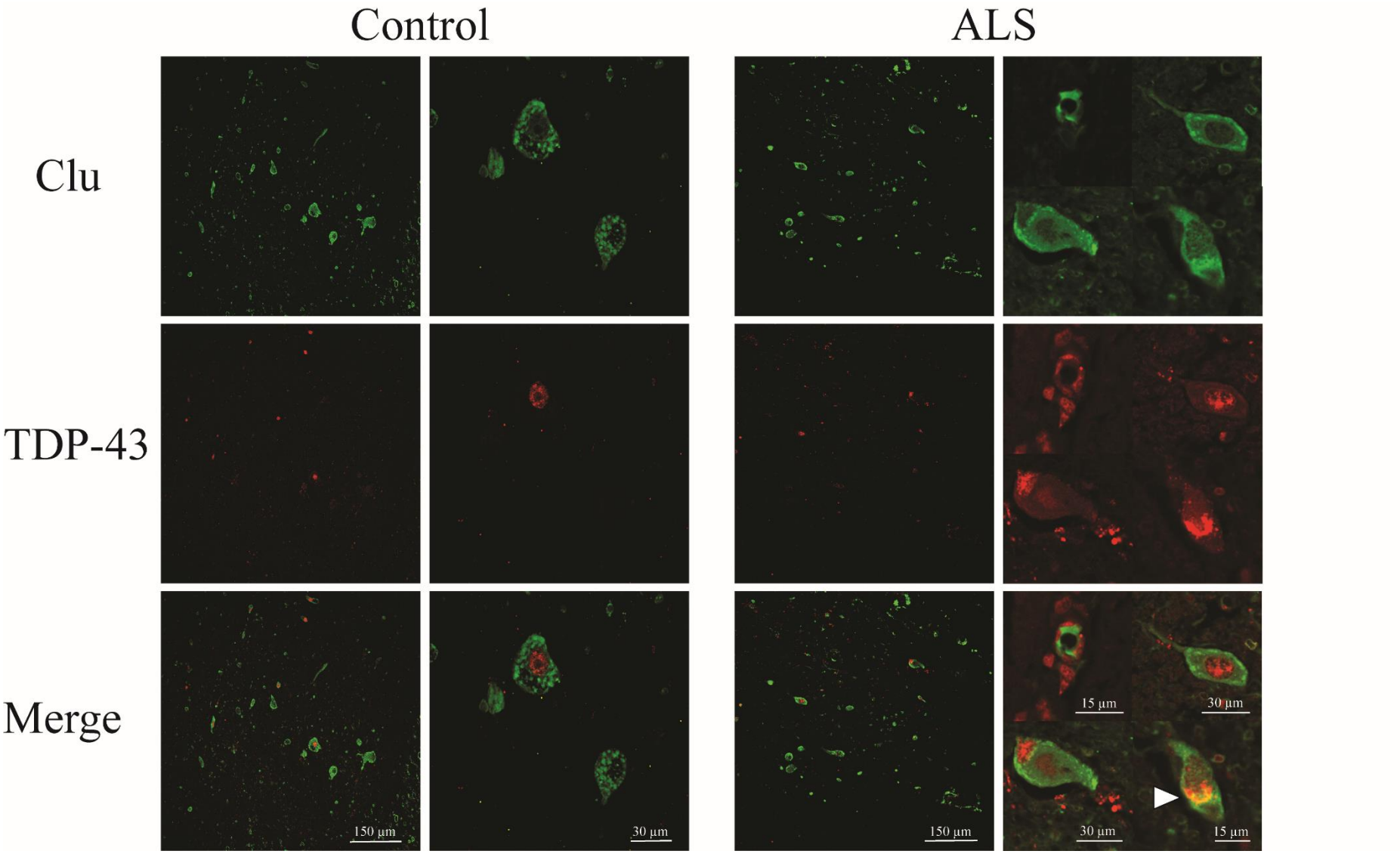
Control
ALS
Clu
TDP-43
Merge
150 μm
30 μm
150 μm
15 μm
30 μm
30 μm
15 μm

Figure 5.6: Clusterin and TDP-43 immunoreactivity in the grey matter of control (05/418) and ALS (10/223) human thoracic spinal cord sections. Low (left) and high (right) power confocal microscopy images of motor neurons in thoracic spinal cord tissue sections. The high power images (right) of the ALS tissue contains 4 separately acquired images, presented in the one panel, with a unique scale bar for each image. Cytoplasmic clusterin (green) and nuclear TDP-43 (red) staining was observed in the motor neurons of both control and ALS tissue. Cytoplasmic TDP-43 was observed in the motor neurons of ALS tissue, in one case co-localising with clusterin (white arrow head).

5.3.3 Establishing a co-culture system to examine the spread of TDP-43 between cells

The results presented in the previous two chapters obtained using transgenic *Drosophila* suggest that TDP-43 is able to spread between cell types *in vivo*, but the nature of the models used made it difficult to quantify spread. Thus a co-culture system was established in which spread between different cell types could be quantified by flow cytometry. The M377V mutant of TDP-43 was initially chosen to transfect the "donor" cells of this system (Ast-1), as when stressed with MG-132 these cells form aggregates visible by confocal microscopy. The dye calcein orange™ was chosen to label the "acceptor" cells (NSC-34). Calcein orange™ is a fluorescent probe used for labelling and monitoring cellular functions of live cells, and is spectrally distinct from GFP. It is also well retained within cells and thus will not spread to neighbouring cells in culture. To determine if calcein orange™ could be used to track cell populations over several days, NSC-34 cells were incubated with 10 or 20 µM calcein and analysed by flow cytometry after 24, 48 and 72 h. Cells incubated with 20 µM calcein could be easily differentiated from unlabelled cells at 24 h after labelling (Figure 5.7).

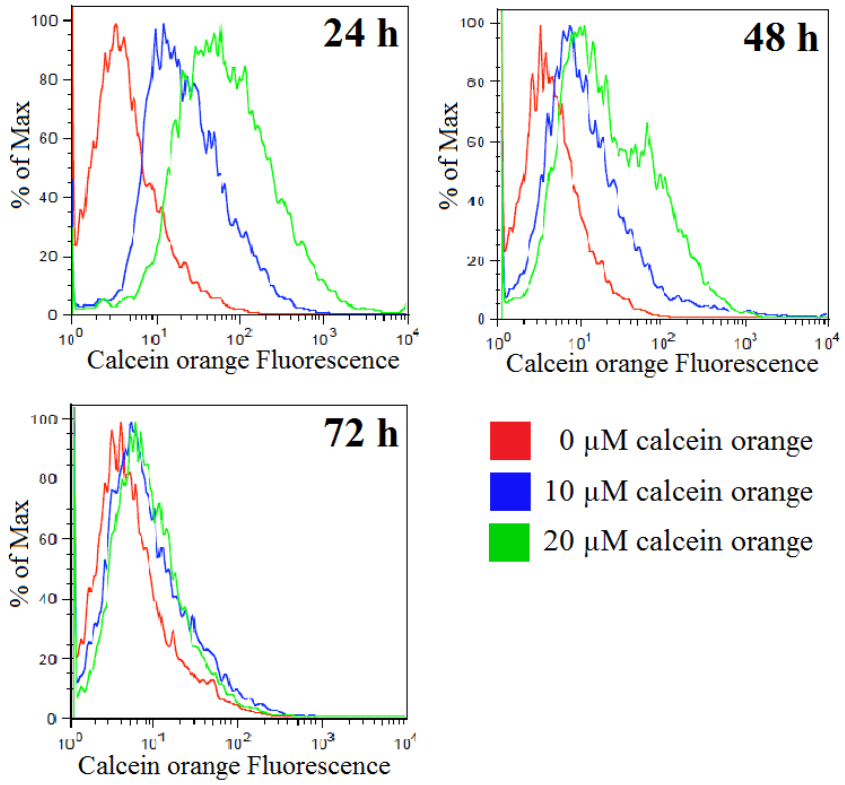

Figure 5.7: Calcein orange labelling of NSC-34 cells. Fluorescence histogram overlays for NSC-34 cells labelled with 0, 10 or 20 µM calcein orange and analysed after 24, 48 and 72 h by flow cytometry.

Ast-1 cells transiently transfected with MT-TDP-43-GFP were added to NSC-34 cells labelled with calcein orange. After 24 h cells were analysed by flow cytometry. Dead cells were identified by staining with RedDot2 and reduced FSC; live cells were gated and used for all further analysis (Figure 5.8).

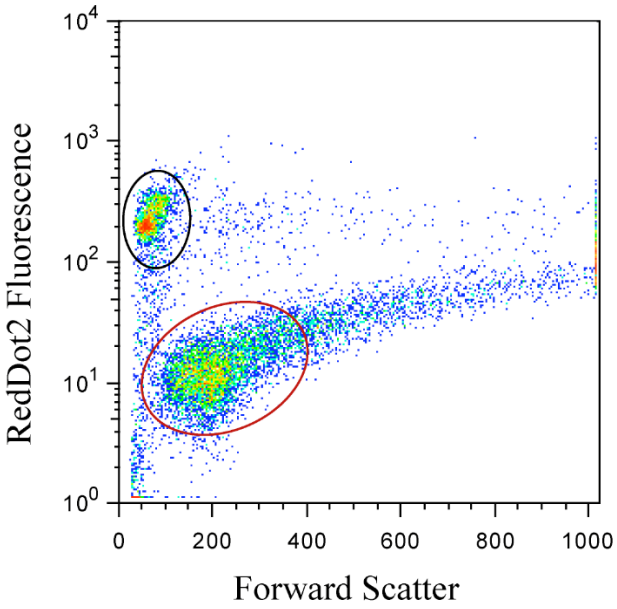

Figure 5.8: Pseudo colour plot of co-cultured Ast-1 and NSC-34 cells labelled with RedDot2. Plot shows forward scatter and RedDot2 fluorescence (695/40-A) of a mix of Ast-1 and NSC-34 cells. Live cells (red elipse) and dead cells (black elipse) were electronically gated and only the fluorescence of live cells collected.

To identify cells that were dual labelled for both calcein orange and GFP, unlabelled NSC-34 cells were analysed by flow cytometry and the data displayed as pseudo colour plots showing both calcein orange and GFP fluorescence. A gate was applied to the plot to separate events into four quadrants, with the lower left representing cells that did not contain either calcein orange or GFP, the lower right representing cells containing calcein orange only, the upper left containing GFP only, and the upper right containing both GFP and calcein orange (Figure 5.9). Gates were set so that no control cells ever appeared in the upper right quadrant.

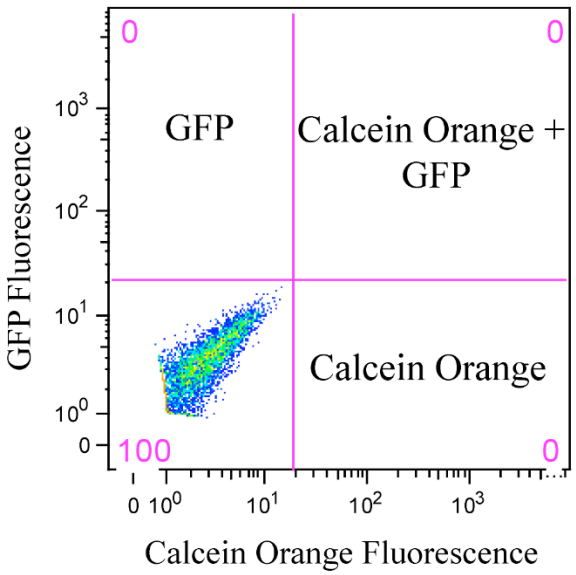

Figure 5.9: Pseudo colour plot showing calcein orange (575/26-A) and GFP (515/20-A) fluorescence of NSC-34 control cells. Gates have been placed to separate the cells into 4 quadrants representing cell populations that are positive for GFP, calcein orange, or calcein orange + GFP fluorescence. In this image the bottom left quadrant contains 100% of the cells and these do not contain either GFP or calcein orange.

The gating strategy was then applied to analyses of co-cultured cells, to reveal the % of cells that contained both GFP and calcein orange (Figure 5.10).

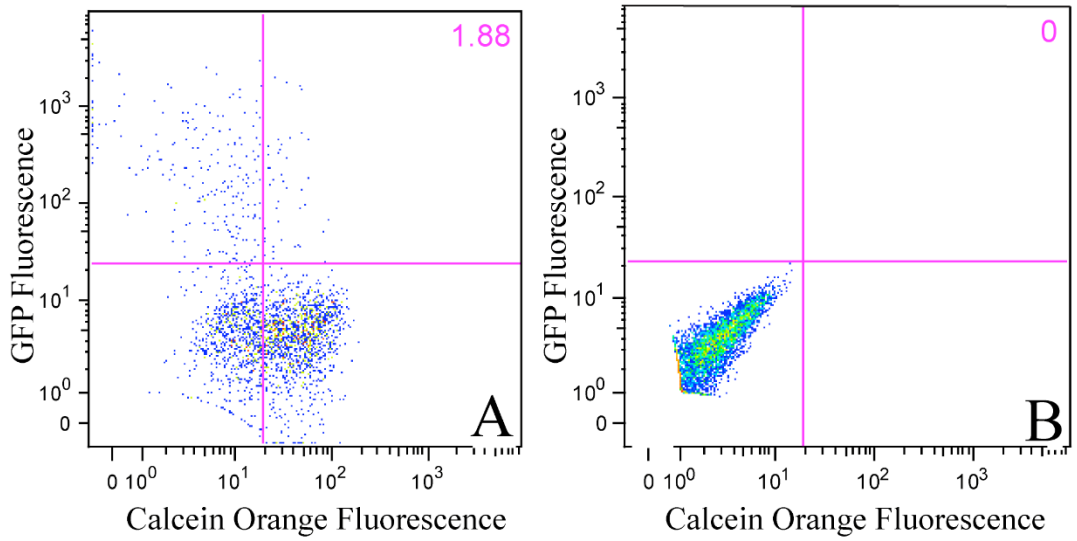

Figure 5.10: Pseudo colour plots of (A) co-cultured cells and (B) NSC-34 control cells. Plots show calcein orange (575/26-A) and GFP (515/20-A) fluorescence. Gates that were set using the control cells (Fig. 5.9 above) have been applied to the co-cultured cells. In panel A, 1.88% of live cells from the co-culture contain both GFP and calcein.

Results showed that in the co-culture 1.62% ± 0.27 (range, n=2) of cells contained both GFP and calcein orange fluorescence, meaning that these were NSC-34 cells containing GFP tagged TDP-43 that originated in Ast-1 cells. This experiment was then repeated using a GFP tagged C terminal fragment of TDP-43 (CTF-TDP-GFP), with the hope that as the C terminal fragment of TDP-43 is very aggregation prone, the transiently transfected cells would contain a higher number of aggregates resulting in more spread between cell types. Compensation and gating was performed as described above. Results showed that 1.29% ± 0.05 (range, n=2) of cells contained both GFP and calcein orange fluorescence meaning that these were NSC-34 cells containing CTF-TDP-GFP that originated in Ast-1 cells.

5.3.4 Establishing a cell culture system to examine the uptake of exogenous non-autonomous cell-generated TDP-43

In an attempt to increase the amount of cell to cell transfer of TDP-43 measured, a culture system was developed in which donor (N2a) cells were lysed after being transiently transfected with GFP tagged MT TDP-43 and the whole cell lysate was added to the receptor (NSC-34) cells. N2a cells were used as the donor cells this time as it is a suitable transfection host and generally yields a higher transfection efficiency than Ast-1 cells. To determine the % of acceptor cells that were associated with GFP, both unlabelled (control) NSC-43 cells and NSC-43 cells that had been co-cultured with

N2a cell lysate (containing MT TDP-43-GFP) were analysed by flow cytometry. Below is an example of the process undertaken to set appropriate gates in order to assess TDP-43 uptake. Cell size and granularity (forward scatter (FSC) and side scatter (SSC), respectively), for a population of unlabelled NSC-34 cells after 24 h are plotted in Figure 5.11A. Dead cells were identified by plotting RedDot2 fluorescence versus FSC; live cells were gated and used for all further analysis (Figure 5.11B). GFP fluorescence and FSC scatter for live cells only were then displayed as pseudo colour plots and a gate placed on unlabelled NSC-34 cells to separate the cell population into two sections representing the cut off where cells appearing in the top section count as GFP positive and those in the bottom section do not (Figure 5.11C). The gates defined using the unlabelled NSC-34 cells were then applied to the co-culture samples, and the % of GFP positive cells for both control and co-culture sample were plotted as bar graphs.

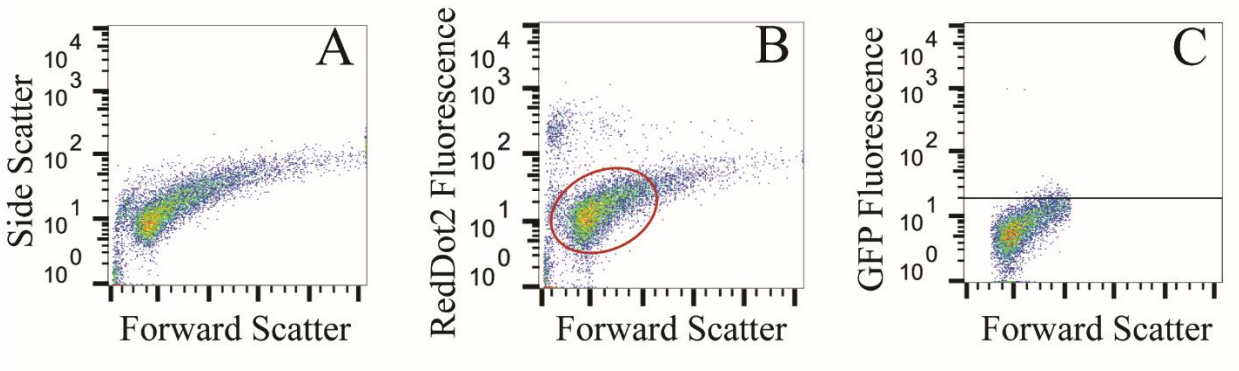

Figure 5.11: Plots of NSC-34 control cells labelled with RedDot2. Plots show A) forward and side scatter, B) RedDot2 fluorescence versus forward scatter; live cells have been selected by gating (red elipse), and C) GFP fluorescence of live cells. The horizontal line in C separates the plot into two sections, with the top section representing cells that are positive for GFP, and the bottom section cells that are negative for GFP.

The first experiment was performed according to the methods outlined in sections 2.8.4. Samples were analysed by flow cytometry after 24 and 48 h of culture. After 24 h, 0.9% of acceptor NSC-34 cells were positive for GFP fluorescence when compared to the control NSC-34 cells. After 48 hours in the presence of exogenous MT TDP-43-GFP, this number increased to 1.6% compared to control NSC-34 cells, a statistically significant difference (P=0.0204; Figure 5.12).

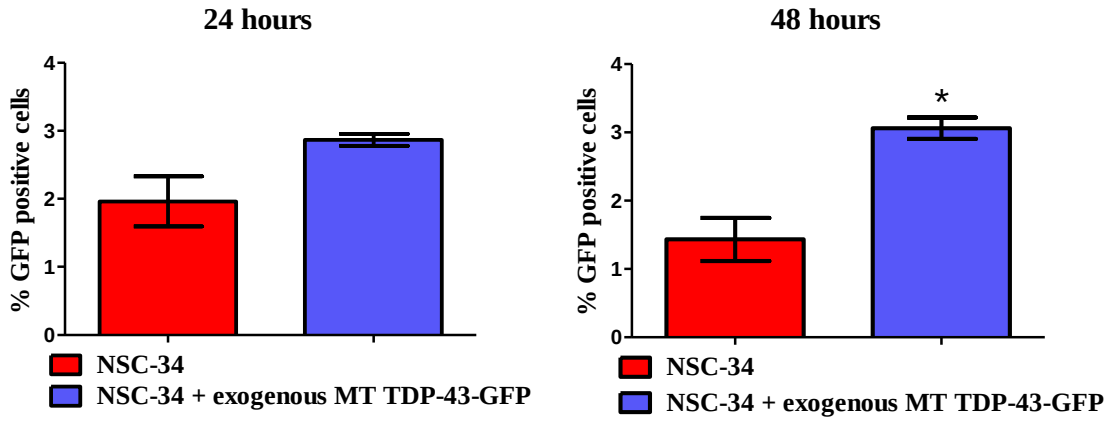

Figure 5.12: Percentages of NSC-34 cells gated as "GFP positive" following 24 or 48 h incubation with (or without) cell lysate containing MT TDP-43-GFP. * = significant difference (p=0.0204, n = 3). Error bars represent SEM.

This experiment was repeated with NSC-34 cells that were grown on coverslips, which 48 h after the addition of the cell lysate containing MT TDP-43-GFP were mounted on microscope slides and imaged on the confocal microscope. This was to determine if the TDP-43 found associated with cells via flow cytometry, was in fact inside the cells. Confocal microscopy revealed a small population of cells that contained MT TDP-43-GFP. Examples of both nuclear (Figure 5.13A and B) and cytoplasmic (Figure 5.13C and D) MT TDP-43-GFP were observed. The nuclear MT TDP-43-GFP had a diffuse pattern of fluorescence, while the cytoplasmic MT TDP-43-GFP appeared to be punctate and granular. In addition the cells that contained cytoplasmic MT TDP-43-GFP show signs of apoptosis, appearing rounded and condensed with signs of blebbing (Häcker, 2000).

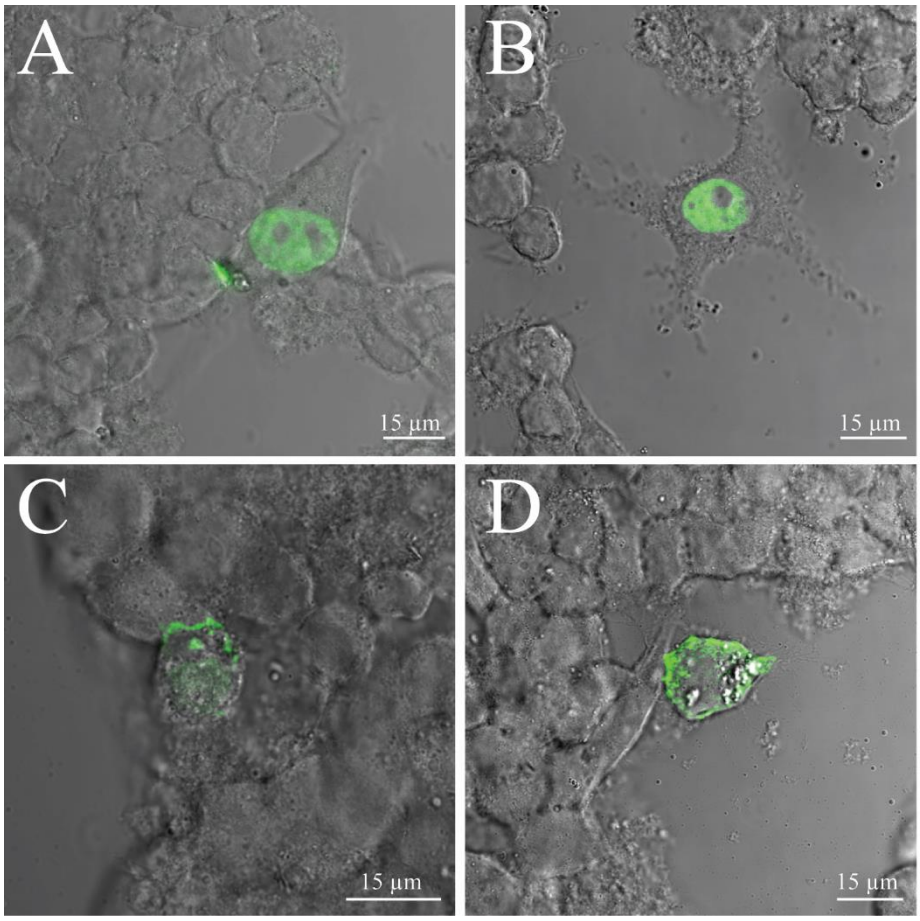

Figure 5.13: Confocal microscopy images showing NSC-34 cells that have taken up exogenous MT TDP-43-GFP originally expressed in transfected N2a cells. NSC-34 cells were cultured in the presence of cell lysate prepared from transfected N2a cells that had generated intracellular aggregates of MT TDP-43. TDP-43 is shown in green. 34 images were collected from cells grown on 2 individual coverslips. Results presented are representative of multiple images.

The experiment was then repeated, but instead of transfecting the donor cells with MT-TDP-43-GFP, they were transfected with a plasmid encoding CTF-TDP-GFP (see section 2.8.3). After 24 h in the presence of exogenous CTF-TDP-GFP, 0.95% of acceptor NSC-34 cells were positive for GFP fluorescence when compared to the control NSC-34 cells, a statistically significant difference (P=0.003). By 48 hours this number had dropped to 0.7% and was not significantly different to control cells (P=0.1784; Figure 5.14).

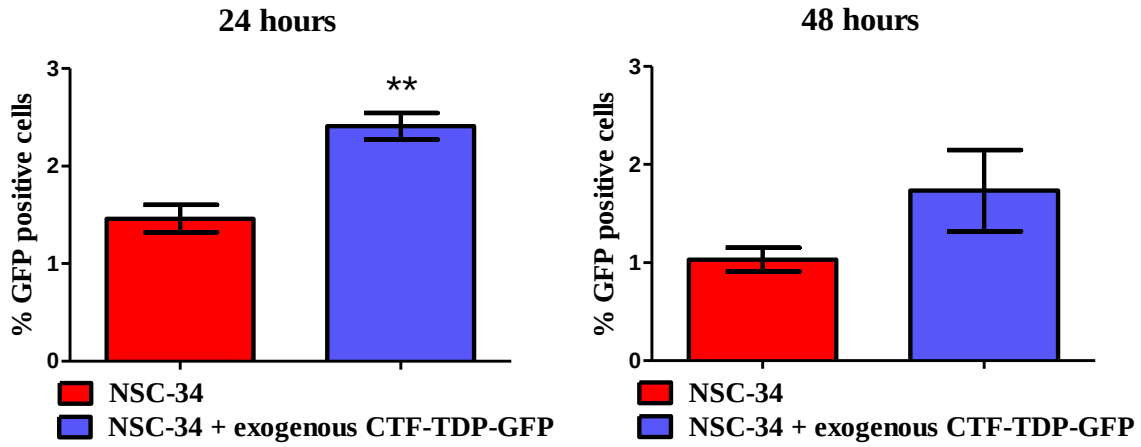

Figure 5.14: Percentages of NSC-34 cells gated as "GFP positive" following 24 or 48 h incubation with exogenous CTF-TDP-GFP. N = 3. ** = significantly different (P=0.003).

As was done previously with MT-TDP-43, cell lysate from cells containing CTF-TDP-GFP was added to NSC-34 cells that had been grown on coverslips. After 24 hours these cells were imaged on the confocal microscope to determine the location of CTF-TDP-GFP within the cells. CTF-TDP-GFP was seen in cells that appeared to be dead or dying, as well as those that were still alive (Figure 5.15). Panels A and B of Figure 5.15 show strong CTF-TDP-GFP fluorescence in cells that appear to be small and condensed suggesting they are dying. Interestingly CTF-TDP-GFP was observed in what appeared to be cellular debris in the areas around dying cells, or in the cytosol of nearby cells, as indicated by white arrows. Panels C and D show instances where CTF-TDP-GFP appeared to span the distance between two cells that were close to one another, either by a single unbroken process (C) or by what could be discreet aggregates in close proximity (D). Finally cytoplasmic CTF-TDP-GFP was also observed with staining appearing punctate and granular (E and F). No clear examples of CTF-TDP-GFP appearing in the nucleus of healthy looking cells was observed.

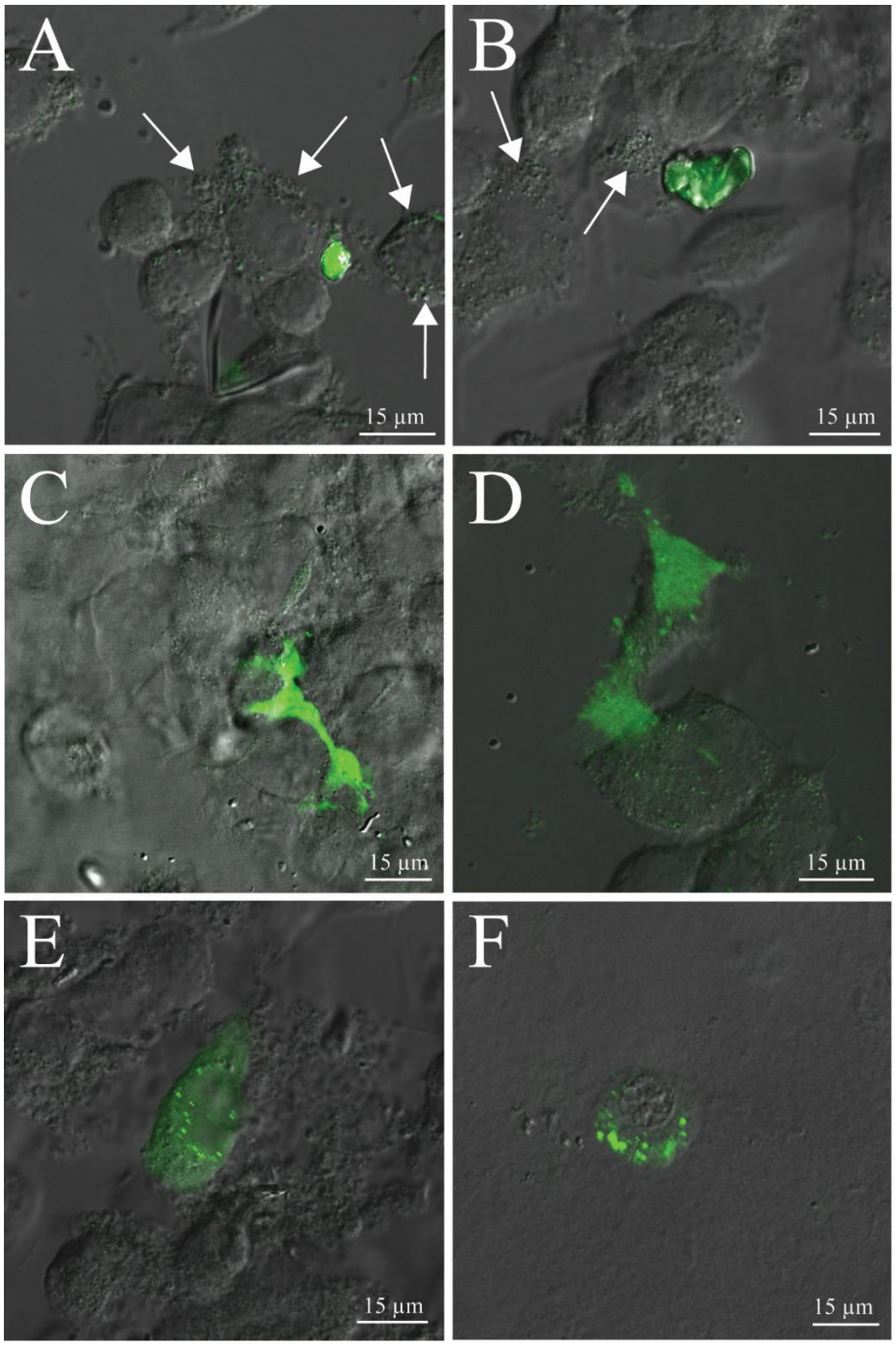

Figure 5.15: Confocal microscopy images showing NSC-34 cells that have taken up exogenous CTF-TDP-GFP, originally expressed in transfected N2a cells. NSC-34 cells were cultured in the presence of cell lysate prepared from transfected N2a cells that had generated intracellular aggregates of C-terminal TDP-43 fragments. CTF-TDP-GFP is shown in green and was observed in dead or dying cells (A, B), between two cells that were close to one another (C, D) and in cytosolic aggregates (E, F). White arrows indicate instances where CTF-TDP-GFP was observed in what appeared to be cellular debris. 42 images were collected from cells grown on 2 individual coverslips. Results presented are representative of multiple images.

5.4 DISCUSSION

5.4.1 Clusterin potently inhibits the aggregation of a C-terminal fragment of TDP-43 *in vitro*

Previous work reported in this thesis showed that *in vivo* clusterin was able to protect motor function when co-expressed with TDP-43 in the glial cells of *Drosophila*, and reduce the spread of TDP-43 to surrounding cells, however the mechanisms by which it was able to do so were unknown. An aggregation assay was performed to examine whether the protective effect observed in *Drosophila* might be due to clusterin preventing the aggregation of TDP-43. It is well established that clusterin is able to inhibit the amorphous aggregation of a broad range of proteins with varying structures at ratios of clusterin:substrate varying from 1:1.3 to 1:11 (Humphreys et al., 1999; Poon et al., 2000), but it has also been shown to inhibit amyloid fibril formation of certain proteins at much lower ratios, ranging from 1:100 to 1:500 (Yerbury et al., 2007). We tested the ability of clusterin to inhibit the aggregation of a peptide corresponding to the C-terminal domain of TDP-43 (CTF-TDP) at various clusterin:client protein ratios. We found that clusterin was surprisingly potent, as it was able to completely inhibit the aggregation of CTF-TDP at a ratio of 1:1000, and was able to increase the lag time prior to aggregation at a variety of ratios of clusterin: CTF-TDP (Figure 5.3). The ability of clusterin to inhibit aggregation even at very low concentrations is thought to be due to the species along the fibril forming pathway with which clusterin interacts. Briefly, this pathway begins with the formation of soluble prefibrillar oligomers or nuclei during the lag phase, which can sequester aggregation prone intermediates which leads to fibril growth during the elongation phase, finally followed by the formation of mature fibrils (Wilson et al., 2007). Clusterin is thought to bind to prefibrillar oligomers that are present during the lag phase of fibril formation. Oligomers are capable of initiating aggregation even when present in low abundance which could potentially explain how clusterin could be so efficient even at such low clusterin:substrate ratios (Yerbury et al., 2007; Wilson et al., 2007). In support of this clusterin has been shown to efficiently sequester oligomeric forms of the amyloid-β_{1-40} peptide formed during both aggregation of amyloid-β monomers and the disaggregation of amyloid-β fibrils (Narayan et al., 2011). While TDP-43 deposits in ALS are generally described as being amorphous or granular, studies have shown that synthetic peptides of regions of the C-terminal domain of TDP-43 are capable of forming fibrillar structures or β-sheet rich oligomers *in vitro.* For example the TDP-$43_{287-322}$ peptide fragment formed fibres and had an extremely high aggregation propensity, while TDP-$43_{366-431}$ could self-associate to form Thio T-reactive oligomers that may serve as a nuclei for aggregation (Chen et al., 2010; Mompean et al., 2014). Most importantly, the same peptide used in the current study has been identified as an amyloidogenic and neurotoxic region of the C-terminus, with sigmoidal Thio-T fluorescence and aggregation curves common to those of other amyloid forming proteins. Time lapse atomic force microscopy revealed that the aggregation process began with the presence of granular oligomers followed by mixtures of oligomers and short

protofibrils and finally mature fibrils (Weirui et al., 2011). Full length TDP-43 has also been shown to form toxic amyloid oligomers that have exposed hydrophobic surfaces in frontotemporal lobar dementia patients (Fang et al., 2014). Thus it is possible that in the *Drosophila* model used (Chapters 3 & 4), clusterin may be binding to toxic oligomers via exposed hydrophobic regions, to either prevent the aggregation of CTF-TDP or prevent damage caused by toxic oligomeric species. Due to the ability of clusterin to potently inhibit the aggregation of TDP-$43_{286\text{-}331,}$ future experiments utilising *Drosophila* models could also express a C-terminal fragment of TDP-43 in the glial cells to determine whether co-expression of clusterin results in an increased protective effect.

5.4.2 Intracellular clusterin is observed in ALS-patient post mortem spinal cord motor neurons and myelin sheaths and was seen to co-localise with TDP-43

The results in Chapter 4 indicate that in the *Drosophila* model used, clusterin co-expression reduces the loss of motor function that results from the expression of TDP-43, suggesting that clusterin and TDP-43 may be interacting in some way. In humans clusterin is widely expressed in the nervous system particularly by neurons and astrocytes in the spinal cord, and has been shown to retrotranslocate from the secretory pathway to the cytosol under conditions of ER stress, which is implicated in the pathology of ALS. Thus it is logical that clusterin may be found inside the motor neurons of ALS patients, potentially associated with TDP-43 containing aggregates. Human thoracic spinal cord tissue from both SALS and control individuals were examined via confocal microscopy to visualise the location of TDP-43 and clusterin in the white and grey matter.

The appearance of TDP-43 in the grey matter of both control and ALS patient tissue sections was as expected. In control patient tissue TDP-43 was localised to the nucleus of cells, while in ALS patient tissue it was found primarily in the cytosol in what appeared to be large inclusions (Figure 5.6). In the white matter TDP-43 was observed in both the control and ALS patient tissue samples. As outlined above, white matter contains myelinated axon tracts. Myelin is exclusively produced by oligodendrocytes which are among the most abundant cell types in the CNS, and so it was thought that in the samples examined TDP-43 was in the cytoplasmic extensions of oligodendrocytes that make up the myelin sheath. Cytoplasmic localisation of TDP-43 has previously been shown to occur in oligodendrocytes. Consistent with our results, the immunolabelling of ALS tissue in previous studies has revealed wide spread TDP-43 positive inclusions in oligodendrocytes in the grey and white matter of spinal cord samples (Philips *et al.*, 2013; Brettschneider *et al.*, 2014). However in contrast to our results, Philips *et al.* (2013) did not detect any TDP-43 in the spinal cords of control patients. Testing a greater number of control tissue samples for the presence of TDP-43 may reveal whether the results observed in this current study are atypical. Additionally co-staining for TDP-43 and markers of oligodendrocytes would confirm that TDP-43 was present in this cell type.

Abnormalities in oligodendrocytes have also been described in mouse models of ALS. In SOD1 transgenic mice oligodendrocytes were seen to undergo progressive and extensive degeneration (Kang *et al.,* 2013; Philips *et al.,* 2013) and also contained increased amounts of SOD1 in both the nuclei and cytoplasm (Yiwen *et al.,* 2014). It is thought that abnormalities in oligodendrocytes including the presence of aggregation prone proteins may affect their ability to provide adequate support to motor neurons. Oligodendrocyte dysfunction may contribute in a non-cell autonomous way to the death of motor neurons in ALS.

The presence of clusterin was also examined in both the grey and white matter. In the grey matter cytoplasmic clusterin was observed in the large ventral horn motor neurons of both control and ALS tissue samples (Figure 5.6). In the control tissue clusterin appeared as small distinct bright spots which is consistent with its expected presence inside the ER and Golgi apparatus where it is glycosylated and cleaved before being secreted into the extracellular space. However, in the motor neurons from ALS patients clusterin staining appeared to be more diffuse consistent with it being present throughout the cytosol. Clusterin was also observed in the white matter of 2/3 of the control tissue samples, in what may be myelin sheaths of oligodendrocytes (Figure 5.4). A larger sample size including control samples from younger individuals may help distinguish if clusterin in oligodendrocytes is a normal feature or age related. Chaperones including Hsp90, Hsp70, and α-B crystallin have previously been detected in cultured primary oligodendrocytes and myelin fractions from mouse brains. Multiple chaperones are involved in the correct synthesis of myelin proteins, and in cases of multiple sclerosis, a disease that involves de-myelination, Hsp70 has been shown to associate with myelin basic protein, possibly in an attempt to prevent degradation of the myelin sheath (Neri *et al.,* 1997; Aquino *et al.,* 1998; Cwiklinska *et al.,* 2003; Jung and Michalak, 2011). Additionally, in cultured oligodendrocytes HSPs have been found associated with tau-positive inclusion bodies, which are feature of a variety of neurodegenerative diseases such as Alzheimer's disease (Richter-Ladnsberg and Bauer, 2004). Clusterin has also been previously observed in the damaged oligodendrocytes of rats. During Wallerian degeneration (a process where part of a neuronal axon dies after being cut or crushed) clusterin appeared in white matter non-neuronal cells presumed to be oligodendrocytes, in large globules, accompanied by the breakdown of myelinated axons (Tornqvist *et al.,* 1996). Oligodendrocytes are very metabolically active cells responsible for the maintenance of the myelin sheath, a process requiring a great amount of energy, meaning they are particularly sensitive to stress. It has therefore been suggested that an effective protein quality control system is crucial for oligodendrocytes to both survive and adequately perform their function of supporting neuronal cells (Goldbaum and Richter-Landsberg, 2004). The role of astrocytes and microglia have been well studied recently and it is accepted that astrocytes are important contributors

to disease. The role of oligodendrocytes is less well studied but oligodendroglial dysfunction may be a contributor to motor neuron dysfunction in ALS (Philips *et al.*, 2013).

Finally it was found that in motor neurons of spinal cord grey matter, TDP-43 and clusterin were observed to both be in the cytoplasm in ALS patients, but not control patients (Figure 5.6). Notably in one case clusterin and TDP-43 were seen to co-localise within a motor neuron cytoplasm of an ALS patient, but not in controls. In the instances where TDP-43 and clusterin did not co-localise, clusterin was found in the immediate vicinity of the TDP-43 positive aggregate, often even surrounding it. These results suggest that in ALS clusterin might with TDP-43 *in vivo*. When viewed together with the ability of clusterin co-expression to prevent TDP-43 induced locomotor dysfunction and reduce the spread of TDP-43 in *Drosophila* (see Chapters 3 and 4), the results are consistent with clusterin interacting with TDP-43 to affect ALS pathology. Ideally a greater number of spinal cord sections should be examined to detect more instances of clusterin and TDP-43 co-localising within human motor neurons.

5.4.3 Co-culture systems demonstrate the spread of TDP-43 from astrocyte cells to motor neuron like cells

Results obtained using the *Drosophila* model (Chapters 3 & 4) suggested that *in vivo* TDP-43 is able to spread from the glial cells where it was initially expressed to surrounding cells including motor neurons. In this chapter an *in vitro* co-culture system was established in order to quantify the spread of TDP-43 from astrocytes to motor neurons using murine cells. The M337V mutant and 216-414 C-terminal fragment, both tagged with GFP were expressed in Ast-1 cells, which were then grown in co-culture with NSC-34 cells. Flow cytometry revealed that after 24 hours, 1.62% of NSC-34 cells contained MT-TDP-43, and 1.29% of NSC-34 cells contained CTF-TDP-GFP (section 5.3.3), both of which had originated in the Ast-1 cells, indicating that TDP-43 had spread from the astrocyte cells to the motor neuron like cells. This result is consistent with a previous study which demonstrated that phosphorylated TDP-43 aggregates could be propagated between cultured SH-SY5Y cells (Nonaka *et al.*, 2013). They found that 2.9% of acceptor SH-SY5Y cells contained TDP-43 originating from the donor SH-SY5Y cells, a slightly higher percentage than observed in this current study. The co-culture experiments performed in the Nonaka study had the following differences when compared to those described in this thesis; (i) cells were grown in co-culture for 3 days, which was not possible in this current study due to the dye used to label the acceptor cells, which after 3 days was no longer able to distinguish acceptor cells. Perhaps if an alternative method of labelling acceptor cells was established, allowing the co-culture experiment to proceed for 3 days, a greater level of spread might be observed. For example rather than using calcein orange to label the NSC-34 acceptor cells, antibodies specific to NSC-34 cells could be used instead. This would be advantageous as it would allow for the co-culture

experiment to proceed for 3 days or more, but would also increase the wash steps required throughout the experiment thus increasing amount of cells lost from the coverslip. (ii) SH-SY5Y cells were used as both the donor and acceptor cells. SH-SY5Y are a human neuroblastoma cell line which is particularly suitable for transfections, while the Ast-1 cells used in this thesis generally did not have a high transfection efficiency. It appears likely that there is a dose dependant relationship between expression levels of protein in donor cells and protein uptake in acceptor cells, which is why a second culture system, utilising cells with a higher transfection efficiency was generated. Regardless of the low percentage of acceptor cells observed to contain TDP-43, it appears that both MT-TDP-43-GFP and CTF-TDP-GFP are able to spread from astrocyte cells to motor neuron like cells, although the mechanisms by which it does so remain unclear. The ability of TDP-43 aggregates to spread from cell to cell in ALS patients may explain the progressive onset of symptoms, and lead to new therapeutic strategies involving stopping the propagation of aggregated proteins.

5.4.4 Motor neuron like cells take up exogenously applied TDP-43

After demonstrating that both MT TDP-43-GFP and CTF-TDP-GFP were capable of spreading from one cell type to another in co-culture, an attempt was made to increase the percentage of cells that took up TDP-43. It was decided to lyse cells expressing TDP-43, and apply the cell lysate containing aggregated TDP-43 to the acceptor cells. This would allow the cells to be incubated in the presence of TDP-43 for longer than 24 hours, as calcein orange would no longer be needed to track the acceptor cells. It was hoped that by allowing the cells to grow in the presence of TDP-43 for a longer period of time, a greater percentage would take up TDP-43. Additionally the donor cells were changed from Ast-1 to N2a cells, as N2a cells have a higher transfection efficiency than Ast-1 cells.

Cells were first transfected with MT TDP-43-GFP, lysed, and the lysate incubated for 24 or 48 h with NSC-34 cells which were subsequently analysed by flow cytometry. After 48 h a significant number of NSC-34 cells were positive for GFP fluorescence (1.6%, P=0.0204) compared to control cells, suggesting that they had at least associated with GFP-tagged MT TDP-43 (Figure 5.12). Cells were then imaged using a confocal microscope to determine the location of MT TDP-43-GFP and see whether the NSC-34 cells had indeed taken up the protein. MT TDP-43-GFP was observed either in the nucleus of intact, normal looking cells, or as aggregates in the cytoplasm of cells that looked to be dying (Figure 5.13). These results show that MT TDP-43-GFP is taken up by cells and can form cytoplasmic aggregates that appear to be toxic to the cells they are in. It has been suggested that one way TDP-43 may propagate between cells is via the release of aggregates from dying cells, which can be taken up by surrounding cells. The presence of non-aggregated nuclear MT TDP-43-GFP may be the result of native TDP-43 from the cell lysate being taken up by the acceptor NSC-34 cells. After transfecting the N2a cells with MT TDP-43-GFP, they are treated with MG-132 to induce

aggregation. This process does not ensure the aggregation of all the MT TDP-43-GFP present in cells, thus there will non-aggregated MT TDP-43-GFP that originated in the nucleus present in the cell lysate added to the NSC-34 cells.

This experiment was then repeated using a GFP tagged C-terminal TDP-43 fragment. As C-terminal fragments are extremely aggregation prone, transfected cells expressing these form cytoplasmic inclusions without any need to treat with MG-132. In fact when visually examined with a microscope, every cell that was transfected with the CTF-TDP-GFP contained inclusions. It was found that when MT TDP-43-GFP was applied to the cells, the fraction of live cells that contained GFP was greatest at the 48 h time point, however when CTF-TDP-GFP was applied, the fraction of live cells containing GFP was greatest at 24 h (Figure 5.14). This could mean that either the C-terminal fragment of TDP-43 is more toxic than the MT TDP-43-GFP, or that it is more readily degraded. Confocal microscopy showed no nuclear CTF-TDP-GFP in the NSC-34 acceptor cells. C-terminal TDP-43 fragments are particularly aggregation prone and form abnormal cytoplasmic inclusions, examples of which were observed (Figure 5.15E-F). CTF-TDP-GFP was also seen in cells that were very clearly dead or dying (Figure 5.15A-B), which is logical as the aggregates formed from C-terminal fragments are toxic, and can cause neuronal toxicity via toxic gain of functions for example by interfering with normal cellular functions (Zhang *et al.*, 2009; Chunxing *et al.*, 2010). Interestingly in the instances when a dying cell containing TDP-43 was observed, cellular debris containing CTF-TDP-GFP was also seen in close proximity. This may represent one possible way that TDP-43 is able to spread in culture, through the release of TDP-43 aggregates from dying cells, which are able to be taken up by surrounding cells. Future experiments should be performed to determine whether the uptake observed is specific to TDP-43-GFP, or if other GFP-labelled proteins would also be taken up to a similar extent.

These experiments were successful in demonstrating that both MT and C-terminal fragments of TDP-43 can spread from astrocytes to motor neuron like cells where they can form cytoplasmic aggregates which may be toxic to cells (toxicity assays would need to be performed to confirm this). However, the percentage of cells which had taken up TDP-43 was low. Various alterations to the culture systems were made in an attempt to increase the extent of uptake. For example, in addition to MT TDP-43, a C-terminal fragment of TDP-43 were used. This fragment is very aggregation prone and results in a higher number of inclusions in transfected cells. The establishment of a culture system which measured the uptake of exogenously added cell-generated TDP-43 allowed a greater amount of TDP-43 to be added to the motor neuron cells. As the donor cells are lysed in this system, non-astrocytic donor cells could be used. N2a cells were chosen as they are easily transfected and result in a greater number of cells expressing TDP-43. For every NSC-34 acceptor cell, lysate from 2 transfected N2a cells was added (outlined in section 5.2.4). Despite these various strategies aimed at

increasing TDP-43 uptake, the greatest percentage of cells containing TDP-43 measured was 1.62%. While this number is consistent with a previous study which found that 2.9% of cells took up TDP-43 in a co-culture system (Nonaka *et al.*, 2013), experiments measuring the effects of clusterin on uptake or the mechanisms of TDP-43 spread would be more reliable and easier to quantify if this fraction of cells were higher. A greater percentage of cells would be particularly beneficial for the flow cytometry analyses, which rely on setting gates to define GFP positive and negative cells; thus a larger population of cells containing GFP would result in less error. It is likely that in order to increase the percentage of cells that take up TDP-43, exogenous TDP-43 will need to be added to acceptor cells in greater quantities than were achievable in this study. One way to do this would be to centrifuge the lysate from a larger number of cells transfected with TDP-43 to concentrate the aggregated TDP-43 and remove cellular debris. Once this process has been optimised an array of further experiments could then be performed, including investigating the mechanisms of TDP-43 propagation between cells, and any effects that clusterin may have on this propagation. In addition a control experiment should be performed to ensure that any spread of TDP-43-GFP observed is specific to the presence of the TDP-43 protein, and not GFP. A simple way to test this would be to repeat the experiment as described in section 5.2.4, but rather than transfect the N2a cells with MT TDP-43-GFP or CTF-TDP-GFP, they should be transfected with pEGFP-N1.

It is thought that the mechanisms allowing TDP-43 to transfer between cells may be similar to those described for SOD1 (see section 1.7), and future experiments should be performed to test this. One simple method would be to inhibit pinocytosis by incubating cells at a low temperature, and determining if this affected the uptake of TDP-43. While this is an easy method, low temperatures also inhibit most other physiological processes in cells. An alternative method would be treating cells with a pharmacological inhibitor of micropinocytosis, such as 5-(N-ethyl-N-isopropyl)-amiloride which blocks the sodium/proton exchanger, and examining if this inhibits the uptake of aggregated TDP-43. The secretion of TDP-43 via exosomes could also be examined. Previous studies have isolated exosomal fractions by ultra-centrifuging culture supernatants to remove cellular debris and pellet membrane vesicles (Gomes *et al.*, 2007). The same could be done with conditioned medium collected from cultures of cells transfected to express TDP-43. The pellets could then be examined by electron microscopy to determine if exosomes were present as has been done previously for SOD1 (Grad *et al.*, 2014). This same study also used immunogold labelling with a SOD1 specific antibody to show surface localised SOD1 on exosomes, a technique which could be modified to detect TDP-43. Western blot analysis could also be used to confirm the presence of exosomal proteins such as LAMP-1 and flotillin-1 in the exosomal pellet fractions (Alvarez-Erviti *et al.*, 2011). Finally, further experiments could be performed to determine if clusterin is able to prevent the spread of TDP-43 between cells or the uptake of TDP-43. This could be done in one of three ways; i) siRNA could be

used to knock down the expression of clusterin in acceptor cells, followed by analysis via flow cytometry and confocal microscopy to determine whether this results in an increase in TDP-43 spread and uptake. ii) Clusterin could be overexpressed in the acceptor cells, followed by the same analyses. iii) Before the addition of exogenous TDP-43 to acceptor cells, TDP-43 could be incubated with purified clusterin to determine whether this decreases uptake or TDP-43 related toxicity.

The results in this chapter suggest a possible role for clusterin in preventing TDP-43 aggregation in ALS. The aggregation assay performed demonstrates that clusterin is able to potently inhibit the aggregation of a C-terminal fragment of TDP-43, and the human tissue data shows that clusterin and TDP-43 are both found in the same cellular compartments of motor neurons in human ALS patients, and may even co-localise within cells. The role of extracellular chaperones in neurodegenerative diseases has been studied very little, and the ability of clusterin to interact with TDP-43 extracellularly, possibly while TDP-43 is being transmitted between cells, or intracellularly after retrotranslocation to the cytosol, may provide new and exciting opportunities for the development of novel treatment strategies, some of which will be discussed in the next chapter.

CHAPTER 6: CONCLUSIONS

6.1 AIMS

This project aimed to investigate the three aspects of ALS pathogenesis. Firstly, the ability of TDP-43 to spread from one cell type to another was assessed *in vivo* using a *Drosophila* model of ALS (see chapters 3 & 4) and *in vitro* using neuronal and astrocyte cell lines (see Chapter 5). TDP-43 has not yet been shown to spread from cell-to-cell *in vivo*, and demonstrating that it is able to do so would provide further evidence that TDP-43 acts in a prion-like way in the pathogenesis of ALS and would help to explain the spread of symptoms that is observed in the disease. Secondly, the extent to which non-neuronal cells contribute to disease progression *in vivo* was investigated by expressing TDP-43 in a subset of glial cells of *Drosophila* to determine if this resulted in motor neuron defects and premature mortality (see section 4.3.1). While the death of motor neurons is a hallmark of ALS, understanding the role non-neuronal cells play in disease progression may ultimately help to design future therapeutic strategies possibly targeted at glial cells. Finally, interactions between the clusterin and TDP-43 were investigated *in vitro*, *in vivo* using the *Drosophila* model, and in post mortem human tissue from ALS patients. Clusterin is a potent chaperone with broad substrate specificity and is able to be retrotranslocated to the cytosol under conditions of ER stress (see section 1.3.3). If clusterin is shown to inhibit the aggregation of TDP-43 it opens the possibility of utilising extracellular chaperones as potential therapeutics. There are as of yet no published reports of clusterin being a constituent of TDP-43 inclusions in human ALS cases, but this observation would provide evidence that in ALS clusterin is interacting with TDP-43, possibly via retrotranslocation to the cytosol where it acts as an intracellular chaperone to inhibit TDP-43 aggregation, or perhaps via extracellular interactions with TDP-43 as it spreads from cell-to-cell.

6.2 PRION-LIKE SPREAD OF TDP-43

This study was successful in developing models in which the cell-to-cell spread of TDP-43 could be examined in cell-culture, and more importantly *in vivo* using a *Drosophila* model of ALS. When expressed in the brains of *Drosophila* either from the embryonic or adult stage of the fly's life cycle, TDP-43 was later observed in cell types that differed from the subset of glial cells in which it was originally expressed (Figure 3.4, Figure 4.5 and Figure 4.6). Immunohistochemistry revealed some of these cells to be motor neurons (Figure 3.7 and Figure 3.8). This was unique to TDP-43 as when the RFP tagged control protein mCD8 was expressed in its place, it was only observed in the cells where initially expressed (Figure 3.6). Similarly, when astrocyte cells (Ast-1) expressing MT or a C-terminal fragment of TDP-43 were grown in co-culture with motor neuron like cells (NSC-34), flow cytometry showed that after 24 hours up to 1.6% of the motor neuron cells had taken up TDP-43 (section 5.3.3).

It was known that TDP-43 was entering the NSC-34 cells as opposed to associating with the cell surface only, as confocal microscopy of cells which had been grown in the presence of exogenously applied MT TDP-43 or C-termainal fragments of TDP-43 revealed the protein to be present in cytoplasmic inclusions within cells (Figure 5.13 and Figure 5.15). From these results it was thus concluded that TDP-43 is capable of cell-to-cell spread, in this case from glial cells to motor neurons. The ability of TDP-43 to propagate between cells may provide a molecular basis for the clinically observed spread of symptoms in the disease. The results in this thesis are novel in that they represent the first time that cell-to-cell spread of TDP-43 has been demonstrated *in vivo* and leads to the conclusion that like SOD1, TDP-43 fulfils the two criteria of being a prion-like protein: it is capable of propagating in a self-templating manner and of cell-to-cell spread. While this thesis demonstrates that TDP-43 is able to propagate amongst cells, the mechanisms by which it does so remain to be elucidated. As TDP-43 is an intracellular protein in order for it to propagate between cells it would need to be released from the original cell and taken up by another. The ability of both MT and WT SOD1 aggregates to penetrate the cell membrane of cells via micropinocytosis has previously been demonstrated, and both can be secreted from cells in exosomes which can subsequently be taken up by surrounding cells (Munch *et al.*, 2011; Basso *et al.*, 2013; Grad *et al.*, 2014). It is thought that TDP-43 may propagate via similar mechanisms, with TDP-43 aggregates being taken up by cells where they can recruit native TDP-43 into aggregates, leading to a self-perpetuating system of aggregation within cells and spread between cells. Results throughout this thesis support this theory, and further experiments such as those described in section 5.4.4 should be conducted to confirm this. These experiments could help determine whether TDP-43 spreads via the release of exosomes, and if TDP-43 is taken up by cells via pinocytosis as theorised below in Figure 6.1

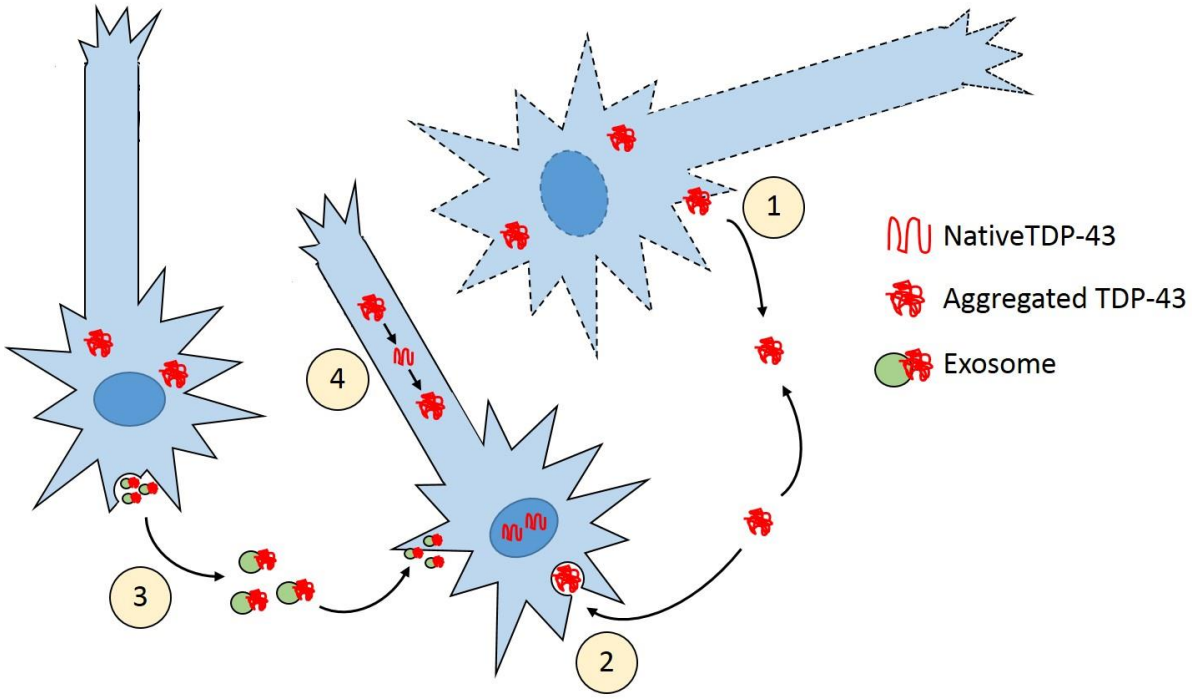

Figure 6.1: Putative mechanisms for the intracellular transmission of TDP-43. Large aggregates containing misfolded TDP-43 may be release from dead or dying cells (1) and subsequently taken up by neighbouring cells via pinocytosis (2). Alternatively misfolded TDP-43 may enter the ER-Golgi system and the vesicle-mediated secretory pathway and become incorporated into an exosome, where it exits the cell via secretion. These exosomes might then be taken up by neighbouring cells (3). Misfolded TDP-43 may also induce subsequent cycles of propagated protein misfolding which can convert native TDP-43 into non-native TDP-43 and ultimately aggregates (4).

6.3 THE ROLE OF NON-NEURONAL CELLS

The role of non-neuronal cells in ALS pathogenicity was examined using a *Drosophila* model of ALS, where the expression of TDP-43 was restricted to glial cells, specifically those that express the glutamate transporter dEAAT1. As discussed in sections 1.4.2.3 and 1.4.2.7, non-neuronal cells can contribute to neurodegeneration in various ways, for example via the secretion of various toxic factors from activated astrocytes or the loss of glutamate transporters in the astrocytes of ALS patients, resulting in glutamate induced excitotoxicity. The loss of glutamate transporters has been confirmed in both human ALS cases and animal models of the disease, but it is unknown whether loss of transporters is a primary cause of ALS or a secondary effect of the disease. Previous studies using *Drosophila* models have shown that glial expression of TDP-43 was both lethal to flies and hindered

motor function, and glutamate transporter knockdown was sufficient to shorten the lifespan of flies (Estes *et al*., 2013; Diaper *et al*., 2013a). In this study we found that the expression of TDP-43 only in glial cells which expressed the *Drosophila* glutamate transporter dEAAT1 was sufficient to cause significantly reduced lifespan (Figure 4.2) and locomotor defects (Figure 4.4), despite the fact that this cell type represents only 10% of the total glial cells. This combined with the links between TDP-43 and EAAT levels outlined in section 4.4.2 suggests that not only is TDP-43 capable of hindering the effectiveness of glutamate transporters, but this hindrance is enough to cause disease phenotypes even when the cells containing the transporters are surrounded by healthy astrocytes. This suggests that of the two ways non-neuronal cells can contribute to neurodegeneration in ALS, the loss of glutamate transporters may be the most critical, and thus the design of therapeutic strategies targeting non-neuronal cells should focus on preventing the loss of glutamate transport. Future experiments could examine whether the co-expression of clusterin could prevent the loss of glutamate transporters in the *Drosophila* model used in the current study. Previous studies have used reverse transcription polymerase chain reaction (RT-PCR) to examine alterations in mRNA levels of EAAT1 in *Drosophila* (Diaper *et al.,* 2013a) and such techniques could be utilised to examine the effect of clusterin and TDP-43 co-expression.

6.4 INTERACTIONS BETWEEN CLUSTERIN AND TDP-43

Interactions between TDP-43 and clusterin were examined *in vitro, in vivo,* and in post mortem thoracic spinal cord sections from ALS patients. Clusterin was co-expressed with TDP-43 in the glial cells of *Drosophila* to determine if the chaperone could (i) reduce the spread of TDP-43 from cell to cell, (ii) increase the longevity of flies expressing TDP-43 and (iii) protect flies against locomotor defects induced by the expression of TDP-43. While clusterin expression did not significantly extend the lifespan of flies expressing TDP-43, it did have a significant impact on the spread of TDP-43 and prevented the deterioration of motor neurons as flies expressing TDP-43 and clusterin had a climbing index at the assay end point comparable to control flies (Figure 4.4). As discussed in section 4.4.7 flies expressing TDP-43 had an average lifespan of 15 days, but the climbing assay was only carried out for 10 days to prevent bias towards healthier living flies. It is thus likely that while clusterin expression was able to protect locomotor function up to day 10 of the assay, it would be unable to do so throughout the entire lifespan of the flies. The experiments performed with *Drosophila* suggest that clusterin was able to prevent or at least delay the toxicity to motor neurons caused by the expression of TDP-43. What the experiments do not show are the mechanisms by which it is doing so, or if clusterin was acting in the extracellular or intracellular space.

To determine if the protective effects of clusterin observed in *Drosophila* were due to it acting as a chaperone with TDP-43, an *in vitro* aggregation assay was performed. It was found that clusterin is a

potent inhibitor of the aggregation of TDP-$43_{286-331}$ peptide, which represents a C-terminal fragment of the protein (Figure 5.3). Clusterin increased the lag phase of TDP-$43_{286-331}$ aggregation at ratios as low as 1:25000 (Clu:TDP-43) and completely inhibited aggregation at a ratio of 1:1000. While it would have been ideal to test the chaperone activity of clusterin on full length TDP-43, as mentioned in section 5.3.2 the protein is extremely aggregation prone and is thus very difficult to express and purify. However in ALS TDP-43 undergoes several pathological modifications (outlined in section 1.6.2) including cleavage to generate C-terminal fragments which contribute to ALS pathogenesis. The glycine rich C-terminal domain of TDP-43, referred to as the prion-like domain due to its similar amino acid composition to yeast prion forming domains, contains the majority of mutations associated with ALS. Thus *in vitro* experiments using peptides corresponding to the C-terminal domain of TDP-43 are relevant to ALS pathogenesis. The ability of clusterin to inhibit the aggregation of a C-terminal fragment of TDP-43 leads to the possibility that it may have a protective role in ALS, and suggests that in *Drosophila* clusterin may have protected motor neurons from TDP-43 mediated toxicity by inhibiting the aggregation of TDP-43.

Next, immunohistochemistry was performed on sections of thoracic spinal cord from ALS patients to visualise the location of TDP-43 within motor neurons, and to determine if cytoplasmic clusterin was present inside these cells and if clusterin co-localised with aggregates containing TDP-43. In the ALS samples clusterin was found diffusely in the cytosol of many cells as well as in distinct puncta, and one motor neuron was identified in which clusterin co-localised with TDP-43 (Figure 5.6). In the examples where clusterin and TDP-43 did not co-localise, clusterin was often found in close proximity to TDP-43. Co-localisation between clusterin and TDP-43 was not seen in the control samples. These observations suggest that clusterin may be involved in ALS and interacts with TDP-43 in an attempt to prevent protein aggregation. In the case where clusterin was found co-localised with TDP-43, it is possible that clusterin was interacting with TDP-43 in one of two ways. Clusterin could act as an extracellular chaperone, binding to the exposed hydrophobic regions of misfolded TDP-43 as it transmits between cells, which was shown to be possible using the *Drosophila* model. These chaperone-client complexes could then be internalised via receptor mediated endocytosis and directed to the lysosome to be degraded (summarised in Figure 1.2). Alternatively clusterin could be acting as an intracellular chaperone, after being retrotranslocated to the cytosol (see section 1.3.3). During either process, it is likely that in the end stages of disease clusterin may become overwhelmed and the chaperone becomes sequestered into aggregates, a theory supported by the identification of several intracellular chaperones present in inclusions from ALS tissue (see section 1.5.3). While the experiments performed in this thesis do not reveal if clusterin is acting extracellularly or intracellularly, the fact remains that clusterin can be present in both spaces and may in fact have a dual action in ALS. Clusterin can inhibit the aggregation of a broad range of proteins with differing

structures, and it is therefore possible that clusterin is also able to inhibit the aggregation of other proteins involved in ALS. Proteins such as SOD1, the RNA-binding protein fused in sarcoma (FUS) and optineurin are all present in protein aggregates in ALS. Perhaps staining human tissue for clusterin and the aforementioned proteins via immunohistochemistry could reveal instances of co-localisation between clusterin and other proteins involved in ALS.

6.5 IMPLICATIONS FOR THE DEVELOPMENT OF THERAPEUTIC STRATEGIES

As emphasised throughout this thesis, ALS is a disease with multiple pathological mechanisms that contribute to neurodegeneration, and thus a successful treatment will likely need to target multiple mechanisms simultaneously, or even target the one mechanism in multiple ways. Prion-like mechanisms of protein propagation is one mechanisms that contributes to neurodegeneration in ALS. It may also represent a molecular pathway that is common to several aggregating proteins involved in ALS. The development of therapeutic strategies designed to inhibit these processes could be ideal as they have the potential to be effective on multiple proteins. There are two main approaches that could be used if treatments aimed at disrupting prion-like mechanisms of protein propagation were developed. The first would be to try to stop proteins from undergoing self-propagated misfolding by stabilising proteins in their native state, and the second would be to disrupt the uptake or release of protein aggregates thus preventing transmission to surrounding cells. Several advances have already been made on the first approach. For example transthyretin (TTR) is a native tetrameric protein that can unfold and undergo amyloidosis *in vivo* resulting in heart and nervous system pathology. Small molecules that bind to and stabilise the tetramer have been developed. One such example is tafamidis, which is able to stop TTR amyloidosis and has been approved by the European Medicines Agency for use as a treatment in humans (Johnson *et al.,* 2012). Other molecules have been identified which act as chaperones by binding to and stabilizing MT SOD1 against aggregation and unfolding. Although these are not ideal for use in therapeutics in humans as they have the potential to bind other protein targets, they represent a starting point for drug development (Nowak *et al.,* 2010). Disrupting the uptake or release of protein aggregates could be achieved with the use of antibodies to "capture" protein seeds as they move between cells, or by targeting receptors needed for the uptake or release of proteins. The former approach has the advantage of specifically targeting protein aggregation, while the latter approach is disadvantageous as inhibiting endocytosis, micropinocytosis, or exosome release could have adverse effects by interfering with other normal processes (Brettschneider *et al.,* 2015). Additionally clearing protein aggregates from the extracellular space as they transmit between cells may also be effective. The prion-like mechanisms involved in ALS protein propagation present multiple opportunities for therapeutic interventions, and it is likely that if a successful treatment is developed in the future it would not involve just one of the above approaches, but multiple.

Glutamate induced excitotoxicity is another mechanism associated with ALS pathogenesis. Riluzole is the only drug approved for the treatment of ALS, and works by blocking the release of glutamate. However this drug only increases the lifespan of patients by a few months. The increase in knowledge regarding the loss of glutamate transporters in ALS suggests that strategies aimed at restoring the levels and function of said transporters may be successful in preventing excitotoxicity, especially if used in combination with glutamate blockers. Immunohistochemical analysis of the spinal cords of ALS patients revealed that the astroglial-type glutamate transporter EAAT2 is selectively diminished in the later stages of disease progression (Sasaki *et al.*, 2000). More recently the *Drosophila* glutamate transporter dEAAT1 was identified as a potential direct target of TDP-43 when it was demonstrated that glial specific overexpression of TDP-43 resulted in decreased dEAAT1 transcript levels (Diaper *et al.*, 2013a). These studies combined with the results described in this thesis suggest that TDP-43 dysfunction can affect glutamate transporter levels. As clusterin can inhibit the aggregation of TDP-43, perhaps it or other chaperones could be utilised in future therapeutic strategies aimed at preventing the dysfunction and aggregation of TDP-43, thus preventing the TDP-43 induced loss of glutamate transporters.

As discussed in section 1.9, many intracellular chaperones have been shown to prevent aggregation and protect against neurotoxicity at least in the early stages of disease progression, especially when used in combination. The discovery that clusterin, an extracellular chaperone, can potently inhibit the aggregation of TDP-43 means that it should be further investigated for use in future therapeutic strategies. The work described in this thesis showed that clusterin is able to inhibit the aggregation of TDP-43 and is found associated with TDP-43 in neuronal inclusions in ALS tissue, but it did not determine whether in these systems the two proteins are initially interacting in the extracellular or intracellular space. It may be possible that clusterin represents a unique chaperone that has the potential to do both. If clusterin and TDP-43 were indeed interacting in the extracellular environment, clusterin could potentially be utilised to interact with TDP-43 and SOD1 as they transmit between cells. If clusterin was acting in the intracellular environment it could be used to inhibit the aggregation of proteins within cells to help prevent the release of protein aggregates into the extracellular space. The use of small molecules that act as chaperones, or the increased expression of multiple chaperones including clusterin, could represent a successful strategy to treat ALS, especially if used in combination with other approaches such as the drug riluzole, which targets glutamate induced excitotoxicity.

6.6 FUTURE DIRECTIONS

TDP-43 has been shown to be capable of propagating in a self-templating manner and this thesis demonstrated that it is also capable of cell-to-cell transmission, but did not determine the mechanisms

by which it does so. These mechanisms may be similar to those described for SOD1 as TDP-43 has been found in exosomes isolated from the blood and CSF of ALS patients (Feneberg *et al*., 2014). The work described in this thesis could be expanded in the future as described in detail in section 5.4.4 to investigate how TDP-43 is propagating between cells and how it enters cells, and if clusterin is able to influence these processes. An *in vitro* aggregation assay demonstrated that clusterin is able to inhibit the aggregation of TDP-43, and in the *Drosophila* model used the co-expression of clusterin was able to reduce the locomotor defects associated with TDP-43 expression. However it was not determined if this was due to clusterin acting on TDP-43 in the extracellular or intracellular environment. Similarly, while the presence of TDP-43 and clusterin co-localised in the motor neurons of ALS patients suggested that in ALS these proteins may interact, where the proteins might interact remains unknown. Future experiments could focus on determining whether TDP-43 and clusterin interact in the intracellular or extracellular space. This could be done using the *Drosophila* model developed. The retrotranslocation of clusterin to the cytosol is dependent on ER stress and experiments could test *Drosophila* for markers of ER stress. Previously it has been shown that the expression of TDP-43 in the motor neurons of flies results in the increase of ER stress markers (Gregory, 2011), and it could be examined whether expression of TDP-43 in dEAAT1 expressing glial cells results in a similar increase in ER stress markers. The presence of such markers would indicate that expression of TDP-43 in the subset of glial cells was sufficient to induce ER stress thus enabling clusterin to relocate to the cytosol. Additionally it could be determined how clusterin is getting into the cytosol. Nizard *et al.* (2009) postulate that retrotranslocation occurs through a mechanism similar to the ER associated protein degradation pathway. Small interfering RNA (siRNA) can easily be introduced into cultured cells and is used to knock down gene expression. Using siRNA, known retrotranslocation channel proteins could be silenced in an attempt to determine whether clusterin translocates from the ER/Golgi to the cytoplasm via these channels.

6.7 SUMMARY

This study has established for the first time that TDP-43 is able to spread from cell-to-cell *in vivo*, indicating that in ALS TDP-43 may have prion-like modes of transmission similar to those of SOD1. However, this work should be expanded using the cell culture methods developed to determine what mechanisms are involved in TDP-43 transfer between cells. In addition it was established that expression of the extracellular chaperone clusterin in the glial cells of *Drosophila* was able to protect flies against motor neuron death induced by the expression of TDP-43. It was also shown that clusterin is able to potently inhibit the aggregation of TDP-$43_{286\text{-}331}$ *in vitro*, suggesting that in *Drosophila* clusterin may have prevented the death of motor neurons by inhibiting the cytoplasmic aggregation of TDP-43. Finally, this study identified for the first time the presence of clusterin in the cytosol of motor neurons from the spinal cords of ALS patients where it was found to be co-localised

with aggregates containing TDP-43. This suggests that clusterin may inhibit the cytoplasmic aggregation of proteins, including TDP-43, in ALS. Defining both the mechanisms involved in protein propagation and aggregation, as well as roles that chaperones play in inhibiting these processes, will lead to an increased understanding of the complex and multifactorial disease that is ALS, and could ultimately provide the basis for the development of new therapies.

CHAPTER 7: REFERENCES

Aguirre, T., Van Den Bosch, L., Goetschalckx, K., Tilkin, P., Mathijs, G., Cassiman, J. J. & Robberecht, W. (1998). "Increased sensitivity of fibroblasts from amyotrophic lateral sclerosis patients to oxidative stress". *Annals of Neurology,* **43,** 452-457.

Allen, Simon, Heath, Paul Roy, Kirby, Janine, Wharton, Stephen Barrie, Cookson, Mark Robert, Menzies, Fiona Mhairi, Banks, Rosamonde Elizabeth & Shaw, Pamela Jean (2003). "Analysis of the cytosolic proteome in a cell culture model of familial amyotrophic lateral sclerosis reveals alterations to the proteasome, antioxidant defenses, and nitric oxide synthetic pathways". *J Biol Chem,* **278,** 6371-6383.

Alvarez-Erviti, Lydia, Seow, Yiqi, Schapira, Anthony H., Gardiner, Chris, Sargent, Ian L., Wood, Matthew J. A. & Cooper, J. Mark (2011). "Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission". *Neurobiology of Disease,* **42,** 360-367.

Andreadou, Elisabeth, Kapaki, Elisabeth, Kokotis, Panagiotis, Paraskevas, George P., Katsaros, Nikolaos, Libitaki, Georgia, Petropoulou, Olga, Zis, Vassilios, Sfagos, Constantinos & Vassilopoulos, Demetrios (2008). "Plasma glutamate and glycine levels in patients with amyotrophic lateral sclerosis". *In Vivo (Athens, Greece),* **22,** 137-141.

Aquino, D. A., Peng, D., Lopez, C. & Farooq, M. (1998). "The constitutive heat shock protein-70 is required for optimal expression of myelin basic protein during differentiation of oligodendrocytes". *Neurochemical Research,* **23,** 413-420.

Arai, Tetsuaki, Hasegawa, Masato, Akiyama, Haruhiko, Ikeda, Kenji, Nonaka, Takashi, Mori, Hiroshi, Mann, David, Tsuchiya, Kuniaki, Yoshida, Mari, Hashizume, Yoshio & Oda, Tatsuro (2006). "TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis". *Biochemical and Biophysical Research Communications,* **351,** 602-611.

Arawaka, Shigeki, Machiya, Youhei & Kato, Takeo (2010). "Heat shock proteins as suppressors of accumulation of toxic prefibrillar intermediates and misfolded proteins in neurodegenerative diseases". *Current Pharmaceutical Biotechnology,* **11,** 158-166.

Arvan, P., Rossmann, M.G., Grau, U.M., Zuber, H., Frank, G. & Tratschin, J.D. (2002). "Secretory pathway quality control operating in golgi, plasmalemmal, and endosomal systems.". *Traffic,* **3,** 771-780.

Atkin, J. D., Farg, M. A., Turner, B. J., Tomas, D., Lysaght, J. A., Nunan, J., Rembach, A., Nagley, P., Beart, P. M., Cheema, S. S. & Horne, M. K. (2006). "Induction of the unfolded protein response in familial amyotrophic lateral sclerosis and association of protein-disulfide isomerase with superoxide dismutase 1". *J Biol Chem,* **281,** 30152-65.

Australia, Mnd. 2013. *MND death statistics* [Online]. Available: https://www.mndaust.asn.au/Discover-our-research/grants-and-research-meetings/Statistics.aspx#Top.

Awasaki, Takeshi, Lai, Sen-Lin, Ito, Kei & Lee, Tzumin (2008). "Organization and postembryonic development of glial cells in the adult central brain of Drosophila". *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience,* **28,** 13742-13753.

Ayala, Youhna M., Misteli, Tom & Baralle, Francisco E. 2008. TDP-43 Regulates Retinoblastoma Protein Phosphorylation through the Repression of Cyclin-Dependent Kinase 6 Expression. National Academy of Sciences.

Ayala, Youhna M., Pantano, Sergio, D'ambrogio, Andrea, Buratti, Emanuele, Brindisi, Antonia, Marchetti, Caterina, Romano, Maurizio & Baralle, Francisco E. (2005). "Human, Drosophila, and C.elegans TDP43: Nucleic Acid Binding Properties and Splicing Regulatory Function". *Journal of Molecular Biology,* **348,** 575-588.

Ayers, Jacob I., Fromholt, Susan, Koch, Morgan, Debosier, Adam, Mcmahon, Ben, Xu, Guilian & Borchelt, David R. (2014). "Experimental transmissibility of mutant SOD1 motor neuron disease". *Acta Neuropathol,* **128,** 791-803.

Bailey, R. W., Keith Dunker, A., Brown, C. J., Garner, E. C. & Griswold, M. D. (2001). "Clusterin, a Binding Protein with a Molten Globule-like Region". *Biochemistry,* **40**.

Banks, Gareth T., Kuta, Anna, Isaacs, Adrian M. & Fisher, Elizabeth M. C. (2008). "TDP-43 is a culprit in human neurodegeneration, and not just an innocent bystander". *Mammalian Genome: Official Journal Of The International Mammalian Genome Society,* **19,** 299-305.

Basso, M., Pozzi, S., Tortarolo, M., Fiordaliso, F., Bisighini, C., Pasetto, L., Spaltro, G., Lidonnici, D., Gensano, F., Battaglia, E., Bendotti, C. & Bonetto, V. (2013). "Mutant copper-zinc superoxide dismutase (SOD1) induces protein secretion pathway alterations and exosome release in astrocytes: implications for disease spreading and motor neuron pathology in amyotrophic lateral sclerosis". *J Biol Chem,* **288,** 15699-711.

Basso, Manuela, Samengo, Giuseppina, Nardo, Giovanni, Massignan, Tania, D'alessandro, Giuseppina, Tartari, Silvia, Cantoni, Lavinia, Marino, Marianna, Cheroni, Cristina, De Biasi, Silvia, Giordana, Maria Teresa, Strong, Michael J., Estevez, Alvaro G., Salmona, Mario, Bendotti, Caterina & Bonetto, Valentina (2009). "Characterization of Detergent-Insoluble Proteins in ALS Indicates a Causal Link between Nitrative Stress and Aggregation in Pathogenesis". *Plos One,* **4,** 1-15.

Batulan, Zarah, Taylor, David M., Aarons, Rebecca J., Minotti, Sandra, Doroudchi, Mohammad M., Nalbantoglu, Josephine & Durham, Heather D. (2006). "Induction of multiple heat shock proteins and neuroprotection in a primary culture model of familial amyotrophic lateral sclerosis". *Neurobiology of Disease,* **24,** 213-225.

Benatar, Michael, Polak, Meraida, Kaplan, Samantha & Glass, Jonathan (2006). "Preventing familial amyotrophic lateral sclerosis: Is a clinical trial feasible?". *Journal of the Neurological Sciences,* **251,** 3-9.

Bendotti, Caterina, Tortarolo, Massimo, Suchak, Sachin K., Calvaresi, Novella, Carvelli, Lucia, Bastone, Antonio, Rizzi, Massimo, Rattray, Marcus & Mennini, Tiziana (2001). "Transgenic SOD1 G93A mice develop reduced GLT-1 in spinal cord without alterations in cerebrospinal fluid glutamate levels". *J Neurochem,* **79,** 737-746.

Berg, Ina, Nilsson, K. Peter R., Thor, Stefan & Hammarström, Per (2010). "Efficient imaging of amyloid deposits in Drosophila models of human amyloidoses". *Nature Protocols,* **5,** 935-944.

Bergemalm, D., Forsberg, K., Srivastava, V., Graffmo, K. S., Andersen, P. M., Brannstrom, T., Wingsle, G. & Marklund, S. L. (2010). "Superoxide dismutase-1 and other proteins in inclusions from transgenic amyotrophic lateral sclerosis model mice". *J Neurochem,* **114,** 408-18.

Besson, M. T., Soustelle, L. & Birman, S. (1999). "Identification and structural characterization of two genes encoding glutamate transporter homologues differently expressed in the nervous system of Drosophila melanogaster". *FEBS Lett,* **443,** 97-104.

Bican, Orhan, Minagar, Alireza & Pruitt, Amy A. (2013). "The Spinal Cord. A Review of Functional Neuroanatomy". *Neurologic Clinics,* **31,** 1-18.

Bilsland, L. G., Sahai, E., Kelly, G., Golding, M., Greensmith, L. & Schiavo, G. (2010). "Deficits in axonal transport precede ALS symptoms in vivo". *Proc Natl Acad Sci U S A,* **107,** 20523-8.

Blizzard, Catherine A., Southam, Katherine A., Dawkins, Edgar, Lewis, Katherine E., King, Anna E., Clark, Jayden A. & Dickson, Tracey C. (2015). "Identifying the primary site of pathogenesis in amyotrophic lateral sclerosis - vulnerability of lower motor neurons to proximal excitotoxicity". *Disease Models & Mechanisms,* **8,** 215-224.

Blokhuis, Anna M., Groen, Ewout J. N., Koppers, Max, Van Den Berg, Leonard H. & Pasterkamp, R. Jeroen (2013). "Protein aggregation in amyotrophic lateral sclerosis". *Acta Neuropathol,* **125,** 777-794.

Booth, G. E., Kinrade, E. F. & Hidalgo, A. (2000). "Glia maintain follower neuron survival during Drosophila CNS development". *Development (Cambridge, England),* **127,** 237-244.

Bowen, Samanthaateh Davidson D. Deinhardt Katrinbird Margaret M. Price Karen M. Baker Cathy S. Robson Joanna C. Swash Michaelshamsuddin Wassimkawar Shaliniel-Tawil Tariqroos Jesperhoyle Andrewnickols Carole D. Knowles Charles H. Pullen Anthony H. Luthert Phillip

J. Weller Roy O. Hafezparast Majidfranklin Robin J. M. (2007). "The phagocytic capacity of neurones". *European Journal of Neuroscience,* **25,** 2947-2955.

Brand, A. H. & Perrimon, N. (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes". *Development (Cambridge, England),* **118,** 401-415.

Brettschneider, Johannes, Arai, Kimihito, Del Tredici, Kelly, Toledo, Jon B., Robinson, John L., Lee, Edward B., Kuwabara, Satoshi, Shibuya, Kazumoto, Irwin, David J., Fang, Lubin, Van Deerlin, Vivianna M., Elman, Lauren, Mccluskey, Leo, Ludolph, Albert C., Lee, Virginia M. Y., Braak, Heiko & Trojanowski, John Q. (2014). "TDP-43 pathology and neuronal loss in amyotrophic lateral sclerosis spinal cord". *Acta Neuropathol,* **128,** 423-437.

Brettschneider, Johannes, Del Tredici, Kelly, Lee, Virginia M. Y. & Trojanowski, John Q. (2015). "Spreading of pathology in neurodegenerative diseases: a focus on human studies". *Nature Reviews. Neuroscience,* **16,** 109-120.

Brodsky, J. L. & Mc Cracken, A. A. (1999). "ER protein quality control and proteasome-mediated protein degradation". *Cell and Developmental Biology,* **10,** 507-513.

Brown, Angus M., Evans, Richard D., Black, Joel & Ransom, Bruce R. (2012). "Schwann cell glycogen selectively supports myelinated axon function". *Annals of Neurology,* **72,** 406-418.

Bruening, W., Roy, J., Giasson, B., Figlewicz, D. A., Mushynski, W. E. & Durham, H. D. (1999). "Up-regulation of protein chaperones preserves viability of cells expressing toxic Cu/Zn-superoxide dismutase mutants associated with amyotrophic lateral sclerosis". *J Neurochem,* **72,** 693-699.

Bruijn, L. I., Becher, M. W., Lee, M. K., Anderson, K. L., Jenkins, N. A., Copeland, N. G., Sisodia, S. S., Rothstein, J. D., Borchelt, D. R., Price, D. L. & Cleveland, D. W. (1997). "ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions". *Neuron,* **18,** 327-338.

Buchner, J. (1996). "Supervising the fold: functional principles of molecular chaperones". *FASEB Journal: Official Publication Of The Federation Of American Societies For Experimental Biology,* **10,** 10-19.

Bunina, T. L. (1962). "[On intracellular inclusions in familial amyotrophic lateral sclerosis]". *Zh Nevropatol Psikhiatr Im S S Korsakova,* **62,** 1293-9.

Buratti, E. & Baralle, F. E. (2001). "Characterization and functional implications of the RNA binding properties of nuclear factor TDP-43, a novel splicing regulator of CFTR exon 9". *J Biol Chem,* **276,** 36337-36343.

Buratti, Emanuele, Brindisi, Antonia, Giombi, Maurizio, Tisminetzky, Sergio, Ayala, Youhna M. & Baralle, Francisco E. (2005). "TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing". *J Biol Chem,* **280,** 37572-37584.

Burkey, B.F., De Silva, H.V & Harmony, J.A.K (1991). "Intracellular processing of apolipoprotein J precursor to the mature heterodimer.". *Journal of Lipid Research,* **32,** 1039-1048.

Calero, M., Rostagno, A., Matsubara, E., Zlokovic, B., Frangione, B. & Ghiso, J. (2000). "Apolipoprotein J (clusterin) and Alzheimer's disease". *Microsc Res Tech,* **50,** 305-15.

Canton, T., Pratt, J., Stutzmann, J. M., Imperato, A. & Boireau, A. (1998). "Glutamate uptake is decreased tardively in the spinal cord of FALS mice". *Neuroreport,* **9,** 775-8.

Cascarina, Sean M. & Ross, Eric D. (2014). "Yeast prions and human prion-like proteins: sequence features and prediction methods". *Cellular And Molecular Life Sciences: CMLS,* **71,** 2047-2063.

Charcot, Jean-Martin & Joffroy, Alix 1869. *Deux cas d'atrophie musculaire progressive avec lésions de la substance grise et des faisceaux antérolatéraux de la moelle épinière,* Paris, Masson.

Charnay, Y., Imhof, A., Vallet, P. G., Hakkoum, D., Lathuiliere, A., Poku, N., Aronow, B., Kovari, E., Bouras, C. & Giannakopoulos, P. (2008). "Clusterin expression during fetal and postnatal CNS development in mouse". *Neuroscience,* **155,** 714-724.

Chen, Allan K. H., Lin, Ryan Y. Y., Hsieh, Eva Z. J., Tu, Pang-Hsien, Chen, Rita P. Y., Liao, Tai-Yan, Chen, Wenlung, Wang, Chih-Hsien & Huang, Joseph J. T. (2010). "Induction of

amyloid fibrils by the C-terminal fragments of TDP-43 in amyotrophic lateral sclerosis". *Journal Of The American Chemical Society,* **132,** 1186-1187.

Cheung, Margaret S., Garcia, Angel E. & Onuchic, José N. 2002. Protein Folding Mediated by Solvation: Water Expulsion and Formation of the Hydrophobic Core Occur after the Structural Collapse. National Academy of Sciences.

Chia, Ruth, Tattum, M. Howard, Jones, Samantha, Collinge, John, Fisher, Elizabeth M. C. & Jackson, Graham S. (2010). "Superoxide Dismutase 1 and tgSOD1G93A Mouse Spinal Cord Seed Fibrils, Suggesting a Propagative Cell Death Mechanism in Amyotrophic Lateral Sclerosis". *Plos One,* **5,** 1-10.

Chiti, F. & Dobson, C. M (2006). "Protein misfolding, functional amyloid, and human disease". *Ann Rev Biochem,* **75,** 333-366.

Cho, T. A. (2015). "Spinal cord functional anatomy". *Continuum (Minneap Minn),* **21,** 13-35.

Chunxing, Yang, Weijia, Tan, Whittle, Catheryne, Linghua, Qiu, Lucheng, Cao, Akbarian, Schahram & Zuoshang, Xu (2010). "The C-Terminal TDP-43 Fragments Have a High Aggregation Propensity and Harm Neurons by a Dominant-Negative Mechanism". *Plos One,* **5,** 1-15.

Clement, A. M., Nguyen, M. D., Roberts, E. A., Garcia, M. L., Boillée, S., Rule, M., Mcmahon, A. P., Doucette, W., Siwek, D., Ferrante, R. J., Brown, R. H., Julien, J. P., Goldstein, L. S. B. & Cleveland, D. W. 2003. Wild-Type Nonneuronal Cells Extend Survival of SOD1 Mutant Motor Neurons in ALS Mice. American Association for the Advancement of Science.

Cleveland, D. W. & Rothstein, J. D. (2001). "From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS". *Nature Reviews. Neuroscience,* **2,** 806-819.

Colombrita, Claudia, Zennaro, Eleonora, Fallini, Claudia, Weber, Markus, Sommacal, Andreas, Buratti, Emanuele, Silani, Vincenzo & Ratti, Antonia (2009). "TDP-43 is recruited to stress granules in conditions of oxidative insult". *J Neurochem,* **111,** 1051-1061.

Corcia, P. & Gordon, P. H. (2012). "Amyotrophic lateral sclerosis and the clinical potential of dexpramipexole". *Ther Clin Risk Manag,* **8,** 359-66.

Corrado, Lucia, Ratti, A., Gellera, C., Buratti, E., Castellotti, B., Carlomagno, Y., Ticozzi, N., Mazzini, L., Testa, L., Taroni, F., Baralle, F. E., Silani, V. & D'alfonso, S. (2009). "High frequency of TARDBP gene mutations in Italian patients with amyotrophic lateral sclerosis". *Human Mutation,* **30,** 688-694.

Costigan, M., Mannion, R. J., Kendall, G., Lewis, S. E., Campagna, J. A., Coggeshall, R. E., Meridith-Middleton, J., Tate, S. & Woolf, C. J. (1998). "Heat shock protein 27: developmental regulation and expression after peripheral nerve injury". *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience,* **18,** 5891-5900.

Cozzolino, Mauro, Ferri, Alberto & Carrì, Maria Teresa (2008). "Amyotrophic lateral sclerosis: from current developments in the laboratory to clinical implications". *Antioxid Redox Signal,* **10,** 405-443.

Craven, John (2005). "Major ascending and descending tracts in the spinal cord". *Anaesthesia & Intensive Care Medicine,* **6,** 1.

Cruts, Marc, Gijselinck, Ilse, Van Langenhove, Tim, Van Der Zee, Julie & Van Broeckhoven, Christine (2013). "Review: Current insights into the C9orf72 repeat expansion diseases of the FTLD/ALS spectrum". *Trends in Neurosciences,* **36,** 450-459.

Csermely, Peter, Sőti, Csaba, Kalmar, Eva, Papp, Eszter, Pato, Balint, Vermes, Akos & Sreedhar, Amere S. (2003). "Molecular chaperones, evolution and medicine". *Journal of Molecular Structure: THEOCHEM,* **666,** 373-380.

Cwiklinska, Hanna, Mycko, Marcin P., Luvsannorov, Otgonbajar, Walkowiak, Bogdan, Brosnan, Celia F., Raine, Cedric S. & Selmaj, Krzysztof W. (2003). "Heat shock protein 70 associations with myelin basic protein and proteolipid protein in multiple sclerosis brains". *International Immunology,* **15,** 241-249.

Da Cruz, S. & Cleveland, D. W. (2011). "Understanding the role of TDP-43 and FUS/TLS in ALS and beyond". *Curr Opin Neurobiol,* **21,** 904-19.

Dafny, Nachum. 2015. *Chapter 3: Anatomy of the Spinal Cord* [Online]. Available: http://nba.uth.tmc.edu/neuroscience/s2/chapter03.html 2015].

Daoud, H., Valdmanis, P. N., Kabashi, E., Dion, P., Dupré, N., Camu, W., Meininger, V. & Rouleau, G. A. (2009). "Contribution of TARDBP mutations to sporadic amyotrophic lateral sclerosis". *Journal Of Medical Genetics,* **46,** 112-114.

Davies, K.J.A. & Delsignore, M.E. (1987). "Protein damage and degradation by oxygen radicals.". *J Biol Chem,* **262,** 9908-9913.

Davis, Daniel M. & Sowinski, Stefanie (2008). "Membrane nanotubes: dynamic long-distance connections between animal cells". *Nature Reviews Molecular Cell Biology,* **9,** 431-436.

De Silva, H.V , Harmony, J.A.K , Stuart, W.D , Gil, C.M & Robbins, J. (1990). "Apolipoprotein J: Structure and tissue distribution.". *Biochemistry (Washington),* **29,** 5380-5389.

De Vos, Kurt J., Chapman, Anna L., Tennant, Maria E., Manser, Catherine, Tudor, Elizabeth L., Lau, Kwok-Fai, Brownlees, Janet, Ackerley, Steven, Shaw, Pamela J., Mcloughlin, Declan M., Shaw, Christopher E., Leigh, P. Nigel, Miller, Christopher C. J. & Grierson, Andrew J. (2007). "Familial amyotrophic lateral sclerosis-linked SOD1 mutants perturb fast axonal transport to reduce axonal mitochondria content". *Human Molecular Genetics,* **16,** 2720-2728.

Debure, L., Vayssiere, J.L., Rincheval, V., Loison, F., Le Drean, Y. & Michel, D. (2003). "Intracellular clusterin causes juxtanuclear aggregate formation and mitochondrial alteration.". *Journal of Cell Science,* **116,** 3109-3121.

Dejesus-Hernandez, Mariely, Mackenzie, Ian r, Boeve, Bradley f, Boxer, Adam l, Baker, Matt, Rutherford, Nicola j, Nicholson, Alexandra m, Finch, Nicole a, Flynn, Heather, Adamson, Jennifer, Kouri, Naomi, Wojtas, Aleksandra, Sengdy, Pheth, Hsiung, Ging-Yuek r, Karydas, Anna, Seeley, William w, Josephs, Keith a, Coppola, Giovanni, Geschwind, Daniel h, Wszolek, Zbigniew k, Feldman, Howard, Knopman, David s, Petersen, Ronald c, Miller, Bruce l, Dickson, Dennis w, Boylan, Kevin b, Graff-Radford, Neill r & Rademakers, Rosa (2011). "Article: Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS". *Neuron,* **72,** 245-256.

Del Valle Rodríguez, Alberto, Didiano, Dominic & Desplan, Claude (2012). "Power tools for gene expression and clonal analysis in Drosophila". *Nature Methods,* **9,** 47-55.

Demandolx, D. & Davoust, J. (1997). "Multicolour analysis and local image correlation in confocal microscopy". *Journal of Microscopy,* **185,** 21-36.

Deng, H. X., Chen, W., Hong, S. T., Boycott, K. M., Gorrie, G. H., Siddique, N., Yang, Y., Fecto, F., Shi, Y., Zhai, H., Jiang, H., Hirano, M., Rampersaud, E., Jansen, G. H., Donkervoort, S., Bigio, E. H., Brooks, B. R., Ajroud, K., Sufit, R. L., Haines, J. L., Mugnaini, E., Pericak-Vance, M. A. & Siddique, T. (2011). "Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia". *Nature,* **477,** 211-5.

Deng, H. X., Zhai, H., Bigio, E. H., Yan, J., Fecto, F., Ajroud, K., Mishra, M., Ajroud-Driss, S., Heller, S., Sufit, R., Siddique, N., Mugnaini, E. & Siddique, T. (2010). "FUS-immunoreactive inclusions are a common feature in sporadic and non-SOD1 familial amyotrophic lateral sclerosis". *Annals of Neurology,* **67,** 739-748.

Diaper, Danielle C., Adachi, Yoshitsugu, Lazarou, Luke, Greenstein, Max, Simoes, Fabio A., Di Domenico, Angelique, Solomon, Daniel A., Lowe, Simon, Alsubaie, Rawan, Cheng, Daryl, Buckley, Stephen, Humphrey, Dickon M., Shaw, Christopher E. & Hirth, Frank (2013a). "Drosophila TDP-43 dysfunction in glia and muscle cells cause cytological and behavioural phenotypes that characterize ALS and FTLD". *Human Molecular Genetics,* **22,** 3883.

Diaper, Danielle C., Adachi, Yoshitsugu, Sutcliffe, Ben, Humphrey, Dickon M., Elliott, Christopher J. H., Stepto, Alan, Ludlow, Zoe N., Vanden Broeck, Lies, Callaerts, Patrick, Dermaut, Bart, Al-Chalabi, Ammar, Shaw, Christopher E., Robinson, Iain M. & Hirth, Frank (2013b). "Loss and gain of Drosophila TDP-43 impair synaptic efficacy and motor control leading to age-

related neurodegeneration by loss-of-function phenotypes". *Human Molecular Genetics*, **22,** 1539-1557.

Dimos, John T., Rodolfa, Kit T., Niakan, Kathy K., Weisenthal, Laurin M., Mitsumoto, Hiroshi, Chung, Wendy, Croft, Gist F., Saphier, Genevieve, Leibel, Rudy, Goland, Robin, Wichterle, Hynek, Henderson, Christopher E. & Eggan, Kevin 2008. Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons. American Association for the Advancement of Science.

Dobson, C. M (2003). "Protein folding and disease: a view from the first Horizon Symposium". *Nature Reviews Drug Discovery*, **2,** 154-160.

Dobson, Christopher M. & Karplus, Martin (1999). "The fundamentals of protein folding: bringing together theory and experiment". *Current Opinion in Structural Biology*, **9,** 92-101.

Doherty, Johnna, Logan, Mary A., Taşdemir, Ozge E. & Freeman, Marc R. (2009). "Ensheathing glia function as phagocytes in the adult Drosophila brain". *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience*, **29,** 4768-4781.

Dong, Aling, Shen, Jikui, Krause, Melissa, Akiyama, Hideo, Hackett, Sean F., Lai, Hong & Campochiaro, Peter A. (2006). "Superoxide dismutase 1 protects retinal cells from oxidative damage". *Journal Of Cellular Physiology*, **208,** 516-526.

Dormann, Dorothee, Capell, Anja, Carlson, Aaron M., Shankaran, Sunita S., Rodde, Ramona, Neumann, Manuela, Kremmer, Elisabeth, Matsuwaki, Takashi, Yamanouchi, Keitaro, Nishihara, Masugi & Haass, Christian (2009). "Proteolytic processing of TAR DNA binding protein-43 by caspases produces C-terminal fragments with disease defining properties independent of progranulin". *J Neurochem*, **110,** 1082-1094.

Dragunow, M., Preston, K., Dodd, J., Young, D., Lawlor, P. & Christie, D. (1995). "Clusterin accumulates in dying neurons following status epilepticus". *Brain Research. Molecular Brain Research*, **32,** 279-290.

Dunn, Kenneth W., Kamocka, Malgorzata M. & Mcdonald, John H. (2011). "A practical guide to evaluating colocalization in biological microscopy". *American Journal Of Physiology. Cell Physiology*, **300,** C723-C742.

Estes, Patricia S., Boehringer, Ashley, Zwick, Rebecca, Tang, Jonathan E., Grigsby, Brianna & Zarnescu, Daniela C. (2011). "Wild-type and A315T mutant TDP-43 exert differential neurotoxicity in a Drosophila model of ALS". *Human Molecular Genetics*, **20,** 2308-2321.

Estes, Patricia S., Daniel, Scott G., Mccallum, Abigail P., Boehringer, Ashley V., Sukhina, Alona S., Zwick, Rebecca A. & Zarnescu, Daniela C. (2013). "Motor neurons and glia exhibit specific individualized responses to TDP-43 expression in a Drosophila model of amyotrophic lateral sclerosis". *Disease Models & Mechanisms*, **6,** 721-733.

Fallini, Claudia, Bassell, Gary J. & Rossoll, Wilfried (2012). "The ALS disease protein TDP-43 is actively transported in motor neuron axons and regulates axon outgrowth". *Human Molecular Genetics*, **21,** 3703-3718.

Feiguin, Fabian, Godena, Vinay K., Romano, Giulia, D'ambrogio, Andrea, Klima, Raffaella & Baralle, Francisco E. (2009). "Depletion of TDP-43 affects Drosophila motoneurons terminal synapsis and locomotive behavior". *FEBS Letters*, **583,** 1586-1592.

Feneberg, Emily, Steinacker, Petra, Lehnert, Stefan, Schneider, Anja, Walther, Paul, Thal, Dietmar R., Linsenmeier, Miriam, Ludolph, Albert C. & Otto, Markus (2014). "Limited role of free TDP-43 as a diagnostic tool in neurodegenerative diseases". *Amyotrophic Lateral Sclerosis & Frontotemporal Degeneration*, **15,** 351-356.

Fernández-Borges, Natalia, Eraña, Hasier, Elezgarai, Saioa R., Harrathi, Chafik, Gayosso, Mayela & Castilla, Joaquín (2013). "Infectivity versus Seeding in Neurodegenerative Diseases Sharing a Prion-Like Mechanism". *International Journal of Cell Biology***,** 1-9.

Ferrante, R. J., Browne, S. E., Shinobu, L. A., Bowling, A. C., Baik, M. J., Macgarvey, U., Kowall, N. W., Brown, R. H., Jr. & Beal, M. F. (1997). "Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis". *J Neurochem*, **69,** 2064-2074.

Fersht, Alan R. 2000. Transition-State Structure as a Unifying Basis in Protein-Folding Mechanisms: Contact Order, Chain Topology, Stability, and the Extended Nucleus Mechanism. National Academy of Sciences of the United States of America.

Fink, Anthony L. (1999). "Chaperone-Mediated Protein Folding". *Physiological Reviews,* **79,** 425-449.

Foran, Emily & Trotti, Davide (2009). "Glutamate transporters and the excitotoxic path to motor neuron degeneration in amyotrophic lateral sclerosis". *Antioxid Redox Signal,* **11,** 1587-1602.

French, Katie, Yerbury, Justin J. & Wilson, Mark R. (2008). "Protease Activation of α2-Macroglobulin Modulates a Chaperone-like Action with Broad Specificity". *Biochemistry,* **47,** 1176-1185.

Fuentealba, R. A., Udan, M., Bell, S., Wegorzewska, I., Shao, J., Diamond, M. I., Weihl, C. C. & Baloh, R. H. (2010). "Interaction with polyglutamine aggregates reveals a Q/N-rich domain in TDP-43". *J Biol Chem,* **285,** 26304-14.

Fujimura-Kiyono, C., Kimura, F., Ishida, S., Nakajima, H., Hosokawa, T., Sugino, M. & Hanafusa, T. (2011). "Onset and spreading patterns of lower motor neuron involvements predict survival in sporadic amyotrophic lateral sclerosis". *Journal of Neurology, Neurosurgery & Psychiatry,* **82,** 1244-1249.

Furukawa, Yoshiaki, Kaneko, Kumi, Watanabe, Shoji, Yamanaka, Koji & Nukina, Nobuyuki (2011). "A seeding reaction recapitulates intracellular formation of Sarkosyl-insoluble transactivation response element (TAR) DNA-binding protein-43 inclusions". *J Biol Chem,* **286,** 18664-18672.

Gadoth, Natan & Göbel, Hans Hilmar 2011. *Oxidative Stress and Free Radical Damage in Neurology,* Humana Press.

Gargiulo-Monachelli, G. M. Janota F. Bettini M. Shoesmith C. L. Strong M. J. Sica R. E. P. (2012). "Regional spread pattern predicts survival in patients with sporadic amyotrophic lateral sclerosis". *European Journal of Neurology,* **19,** 834-841.

Gendron, T. F., Josephs, K. A. & Petrucelli, L. (2010). "Review: transactive response DNA-binding protein 43 (TDP-43): mechanisms of neurodegeneration". *Neuropathology And Applied Neurobiology,* **36,** 97-112.

Gifondorwa, D. J., Jimenz-Moreno, R., Hayes, C. D., Rouhani, H., Robinson, M. B., Strupe, J. L., Caress, J. & Milligan, C. (2012). "Administration of Recombinant Heat Shock Protein 70 Delays Peripheral Muscle Denervation in the SOD1(G93A) Mouse Model of Amyotrophic Lateral Sclerosis". *Neurol Res Int,* **2012,** 170426.

Gifondorwa, David J., Robinson, Mac B., Hayes, Crystal D., Taylor, Anna R., Prevette, David M., Oppenheim, Ronald W., Caress, James & Milligan, Carolanne E. (2007). "Exogenous delivery of heat shock protein 70 increases lifespan in a mouse model of amyotrophic lateral sclerosis". *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience,* **27,** 13173-13180.

Gitcho, M. A., Baloh, R. H., Chakraverty, S., Mayo, K., Norton, J. B., Levitch, D., Hatanpaa, K. J., White, C. L., Iii, Bigio, E. H., Caselli, R., Baker, M., Al-Lozi, M. T., Morris, J. C., Pestronk, A., Rademakers, R., Goate, A. M. & Cairns, N. J. (2008). "TDP-43 A315T mutation in familial motor neuron disease". *Annals of Neurology,* **63,** 535-538.

Goedert, Michel, Clavaguera, Florence & Tolnay, Markus (2010). "The propagation of prion-like protein inclusions in neurodegenerative diseases". *Trends in Neurosciences,* **33,** 317-325.

Gomes, Catarina, Keller, Sascha, Altevogt, Peter & Costa, Júlia (2007). "Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis". *Neurosci Lett,* **428,** 43-46.

Gousset, Karine, Schiff, Edwin, Langevin, Christelle, Marijanovic, Zrinka, Caputo, Anna, Browman, Duncan T., Chenouard, Nicolas, De Chaumont, Fabrice, Martino, Angelo, Enninga, Jost, Olivo-Marin, Jean-Christophe, Männel, Daniela & Zurzolo, Chiara (2009). "Prions hijack tunnelling nanotubes for intercellular spread". *Nature Cell Biology,* **11,** 328-336.

Grad, Leslie I., Fernando, Sarah M. & Cashman, Neil R. (2015). "From molecule to molecule and cell to cell: Prion-like mechanisms in amyotrophic lateral sclerosis". *Neurobiology of Disease,* **77,** 257-265.

Grad, Leslie I., Guest, Will C., Yanai, Anat, Pokrishevsky, Edward, O'neill, Megan A., Gibbs, Ebrima, Semenchenko, Valentyna, Yousefi, Masoud, Wishart, David S., Plotkin, Steven S. & Cashman, Neil R. 2011. Intermolecular transmission of Superoxide dismutase 1 misfolding in living cells. National Academy of Sciences.

Grad, Leslie I., Yerbury, Justin J., Turner, Bradley J., Guest, William C., Pokrishevsky, Edward, O'neill, Megan A., Yanai, Anat, Silverman, Judith M., Zeineddine, Rafaa, Corcoran, Lisa, Kumita, Janet R., Luheshi, Leila M., Yousefi, Masoud, Coleman, Bradley M., Hill, Andrew F., Plotkin, Steven S., Mackenzie, Ian R. & Cashman, Neil R. (2014). "Intercellular propagated misfolding of wild-type Cu/Zn superoxide dismutase occurs via exosome-dependent and -independent mechanisms". *Proc Natl Acad Sci U S A,* **111,** 3620-3625.

Graversen, J. H., Madsen, M. & Moestrup, S.K. (2002). "CD163: a signal receptor scavenging haptoglobin-hemoglobin complexes from plasma.". *International Journal of Biochemistry and Cell Biology,* **34,** 309-314.

Greek, Ray & Hansen, Lawrence A. (2013). "Questions regarding the predictive value of one evolved complex adaptive system for a second: exemplified by the SOD1 mouse". *Progress In Biophysics And Molecular Biology,* **113,** 231-253.

Greenway, Matthew J., Andersen, Peter M., Russ, Carsten, Ennis, Sean, Cashman, Susan, Donaghy, Colette, Patterson, Victor, Swingler, Robert, Kieran, Dairin, Prehn, Jochen, Morrison, Karen E., Green, Andrew, Acharya, K. Ravi, Brown, Robert H., Jr. & Hardiman, Orla (2006). "ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis". *Nature Genetics,* **38,** 411-413.

Gregory, Jenna M. (2011). "Investigating the Role of TDP-43 Aggregation in the Pathogenesis of ALD and FTD-U", PhD thesis, University of Cambridge, Cambridge.

Gregory, Jenna M., Barros, Teresa P., Meehan, Sarah, Dobson, Christopher M. & Luheshi, Leila M. (2012). "The Aggregation and Neurotoxicity of TDP-43 and Its ALS-Associated 25 kDa Fragment Are Differentially Affected by Molecular Chaperones in Drosophila". *Plos One,* **7,** 1-9.

Grewal, R. P., Morgan, T. E. & Finch, C. E. (1999). "C1qB and clusterin mRNA increase in association with neurodegeneration in sporadic amyotrophic lateral sclerosis". *Neurosci Lett,* **271,** 65-67.

Grueber, Wesley B., Ye, Bing, Yang, Chung-Hui, Younger, Susan, Borden, Kelly, Jan, Lily Y. & Jan, Yuh-Nung (2007). "Projections of Drosophila multidendritic neurons in the central nervous system: links with peripheral dendrite morphology". *Development (Cambridge, England),* **134,** 55-64.

Gu, H., Wei, X., Chen, S., Kurz, A., Muller, U., Gasser, T., Dodel, R. C., Farlow, M. R. & Du, Y. (2011). "Association of clusterin gene polymorphisms with late-onset Alzheimer's disease". *Dement Geriatr Cogn Disord,* **32,** 198-201.

Guo, H., Lai, L., Butchbach, M. E., Stockinger, M. P., Shan, X., Bishop, G. A. & Lin, C. L. (2003). "Increased expression of the glial glutamate transporter EAAT2 modulates excitotoxicity and delays the onset but not the outcome of ALS in mice". *Hum Mol Genet,* **12,** 2519-32.

Gurney, Mark E., Pu, Haifeng, Chiu, Arlene Y., Mauro, C. Dal Canto, Polchow, Cynthia Y., Alexander, Denise D., Caliendo, Jan, Hentati, Afif, Kwon, Young W., Deng, Han-Xiang, Chen, Wenje, Zhai, Ping, Sufit, Robert L. & Siddique, Teepu 1994. Motor Neuron Degeneration in Mice That Express a Human Cu,Zn Superoxide Dismutase Mutation. American Society for the Advancement of Science.

Hacker, G. (2000). "The morphology of apoptosis". *Cell Tissue Res,* **301,** 5-17.

Hall, E. D., Andrus, P. K., Oostveen, J. A., Fleck, T. J. & Gurney, M. E. (1998). "Relationship of oxygen radical-induced lipid peroxidative damage to disease onset and progression in a transgenic model of familial ALS". *Journal Of Neuroscience Research,* **53,** 66-77.

Hammond, Jennetta W., Griffin, Kelly, Jih, Gloria T., Stuckey, Jeanne & Verhey, Kristen J. (2008). "Co-operative versus independent transport of different cargoes by Kinesin-1". *Traffic (Copenhagen, Denmark),* **9,** 725-741.

Han, S., Choi, J. R., Soon Shin, K. & Kang, S. J. (2012). "Resveratrol upregulated heat shock proteins and extended the survival of G93A-SOD1 mice". *Brain Res,* **1483,** 112-7.

Hanson, Keith A., Kim, Sang Hwa, Wassarman, David A. & Tibbetts, Randal S. (2010). "Ubiquilin modifies TDP-43 toxicity in a Drosophila model of amyotrophic lateral sclerosis (ALS)". *J Biol Chem,* **285,** 11068-11072.

Hartl, F. U. & Hayer-Hartl, M. (2002). "Molecular chaperones in the cytosol: from nascent chain to folded protein. (Review: protein folding)". *Science,* **295**.

Harwood, Ceryl A., Mcdermott, Christopher J. & Shaw, Pamela J. (2009). "Physical activity as an exogenous risk factor in motor neuron disease (MND): a review of the evidence". *Amyotrophic Lateral Sclerosis: Official Publication Of The World Federation Of Neurology Research Group On Motor Neuron Diseases,* **10,** 191-204.

Henkel, Jenny S., Engelhardt, Joseph I., Siklós, László, Simpson, Ericka P., Kim, Seung H., Pan, Tianhong, Goodman, J. Clay, Siddique, Teepu, Beers, David R. & Appel, Stanley H. (2004). "Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue". *Annals of Neurology,* **55,** 221-235.

Higgins, Cynthia M. J., Jung, Cheolwha & Xu, Zuoshang (2003). "ALS-associated mutant SOD1G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes". *BMC Neuroscience,* **4,** 16-16.

Hirano, A., Nakano, I., Kurland, L. T., Mulder, D. W., Holley, P. W. & Saccomanno, G. (1984). "Fine structural study of neurofibrillary changes in a family with amyotrophic lateral sclerosis". *Journal Of Neuropathology And Experimental Neurology,* **43,** 471-480.

Hong, Elizabeth, Davidson, Alan R. & Kaiser, Chris A. 1996. A Pathway for Targeting Soluble Misfolded Proteins to the Yeast Vacuole. Rockefeller University Press.

Hough, C. D., Woods, D. F., Park, S. & Bryant, P. J. (1997). "Organizing a functional junctional complex requires specific domains of the Drosophila MAGUK Discs large". *Genes & Development,* **11,** 3242-3253.

Howland, David S., Liu, Jian, She, Yijin, Goad, Beth, Maragakis, Nicholas J., Kim, Benjamin, Erickson, Jamie, Kulik, John, Devito, Lisa, Psaltis, George, Degennaro, Louis J., Cleveland, Don W. & Rothstein, Jeffrey D. 2002. Focal Loss of the Glutamate Transporter EAAT2 in a Transgenic Rat Model of SOD1 Mutant-Mediated Amyotrophic Lateral Sclerosis (ALS). National Academy of Sciences.

Humphreys, D. T., Carver, J. A., Easterbrook-Smith, S. B. & Wilson, M. R. (1999). "Clusterin has chaperone-like activity similar to that of small heat shock proteins.". *J Biol Chem,* **274,** 6875-6881.

Iguchi, Yohei, Katsuno, Masahisa, Niwa, Jun-Ichi, Yamada, Shin-Ichi, Sone, Jun, Waza, Masahiro, Adachi, Hiroaki, Tanaka, Fumiaki, Nagata, Koh-Ichi, Arimura, Nariko, Watanabe, Takashi, Kaibuchi, Kozo & Sobue, Gen (2009). "TDP-43 depletion induces neuronal cell damage through dysregulation of Rho family GTPases". *J Biol Chem,* **284,** 22059-22066.

Ikenaka, Kensuke, Katsuno, Masahisa, Kawai, Kaori, Ishigaki, Shinsuke, Tanaka, Fumiaki & Sobue, Gen (2012). "Disruption of Axonal Transport in Motor Neuron Diseases". *International Journal of Molecular Sciences,* **13,** 1225-1238.

Ito, Kei, Urban, Joachim & Technau, Gerhard (1995). "Distribution, classification, and development of Drosophila glial cells in the late embryonic and early larval ventral nerve cord". *Roux's Archives of Developmental Biology,* **204,** 284.

Jain, Mohit Raja, Ge, Wei-Wen, Elkabes, Stella & Li, Hong (2008). "Amyotrophic lateral sclerosis: Protein chaperone dysfunction revealed by proteomic studies of animal models". *Proteomics. Clinical Applications,* **2,** 670-684.

Jenne, D. E. & Tschopp, J. (1989). "Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulphated

glycoprotein 2, a constituent of rat testis fluid.". *Proceedings of the National Academy of Science USA,* **86,** 7123-7127.

Johnson, Brian S., Mccaffery, J. Michael, Lindquist, Susan & Gitler, Aaron D. 2008. A Yeast TDP-43 Proteinopathy Model: Exploring the Molecular Determinants of TDP-43 Aggregation and Cellular Toxicity. National Academy of Sciences.

Johnson, Brian S., Snead, David, Lee, Jonathan J., Mccaffery, J. Michael, Shorter, James & Gitler, Aaron D. (2009). "TDP-43 is intrinsically aggregation-prone, and amyotrophic lateral sclerosis-linked mutations accelerate aggregation and increase toxicity". *J Biol Chem,* **284,** 20329-20339.

Johnson, Steven M., Connelly, Stephen, Fearns, Colleen, Powers, Evan T. & Kelly, Jeffery W. (2012). "The transthyretin amyloidoses: from delineating the molecular mechanism of aggregation linked to pathology to a regulatory-agency-approved drug". *Journal of Molecular Biology,* **421,** 185-203.

Johnston, J.A., Ward, C.L. & Kopito, R.R. (1998). "Aggresomes: A cellular response to misfolded proteins.". *The Journal Of Cell Biology,* **142,** 1883-1898.

Jones, S. E. & Jomary, C. (2002). "Clusterin". *Int J Biochem Cell Biol,* **34,** 427-31.

Jucker, Mathias & Walker, Lary C. (2013). "Self-propagation of pathogenic protein aggregates in neurodegenerative diseases". *Nature,* **501,** 45-51.

Jun, Gyungah, Naj, Adam C., Beecham, Gary W., Wang, Li-San, Buros, Jacqueline, Gallins, Paul J., Buxbaum, Joseph D., Ertekin-Taner, Nilufer, Fallin, M. Daniele, Friedland, Robert, Inzelberg, Rivka, Kramer, Patricia, Rogaeva, Ekaterina, St George-Hyslop, Peter, Cantwell, Laura B., Dombroski, Beth A., Saykin, Andrew J., Reiman, Eric M., Bennett, David A., Morris, John C., Lunetta, Kathryn L., Martin, Eden R., Montine, Thomas J., Goate, Alison M., Blacker, Deborah, Tsuang, Debby W., Beekly, Duane, Cupples, L. Adrienne, Hakonarson, Hakon, Kukull, Walter, Foroud, Tatiana M., Haines, Jonathan, Mayeux, Richard, Farrer, Lindsay A., Pericak-Vance, Margaret A. & Schellenberg, Gerard D. (2010). "Meta-analysis confirms CR1, CLU, and PICALM as alzheimer disease risk loci and reveals interactions with APOE genotypes". *Archives Of Neurology,* **67,** 1473-1484.

Jung, Joanna & Michalak, Marek (2011). "Cell surface targeting of myelin oligodendrocyte glycoprotein (MOG) in the absence of endoplasmic reticulum molecular chaperones". *Biochimica Et Biophysica Acta,* **1813,** 1105-1110.

Kabashi, Edor, Lin, Li, Tradewell, Miranda L., Dion, Patrick A., Bercier, Valérie, Bourgouin, Patrick, Rochefort, Daniel, Bel Hadj, Samar, Durham, Heather D., Vande Velde, Christine, Rouleau, Guy A. & Drapeau, Pierre (2010). "Gain and loss of function of ALS-related mutations of TARDBP (TDP-43) cause motor deficits in vivo". *Human Molecular Genetics,* **19,** 671-683.

Kabashi, Edor, Valdmanis, Paul N., Dion, Patrick, Spiegelman, Dan, Mcconkey, Brendan J., Vande Velde, Christine, Bouchard, Jean-Pierre, Lacomblez, Lucette, Pochigaeva, Ksenia, Salachas, Francois, Pradat, Pierre-Francois, Camu, William, Meininger, Vincent, Dupre, Nicolas & Rouleau, Guy A. (2008). "TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis". *Nature Genetics,* **40,** 572-574.

Kametani, Fuyuki, Nonaka, Takashi, Suzuki, Takehiro, Arai, Tetsuaki, Dohmae, Naoshi, Akiyama, Haruhiko & Hasegawa, Masato (2009). "Identification of casein kinase-1 phosphorylation sites on TDP-43". *Biochemical and Biophysical Research Communications,* **382,** 405-409.

Kang, Shin H., Li, Ying, Fukaya, Masahiro, Lorenzini, Ileana, Cleveland, Don W., Ostrow, Lyle W., Rothstein, Jeffrey D. & Bergles, Dwight E. (2013). "Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis". *Nature Neuroscience,* **16,** 571-579.

Kanouchi, T., Ohkubo, T. & Yokota, T. (2012). "Can regional spreading of amyotrophic lateral sclerosis motor symptoms be explained by prion-like propagation?". *Journal of Neurology, Neurosurgery & Psychiatry,* **83,** 739-745.

Karch, C. M. & Borchelt, D. R. (2010). "An examination of alpha B-crystallin as a modifier of SOD1 aggregate pathology and toxicity in models of familial amyotrophic lateral sclerosis". *J Neurochem,* **113,** 1092-100.

Kaufman, Randal J., Scheuner, Donalyn, Schröder, Martin, Shen, Xiaohua, Lee, Kyungho, Liu, Chuan Yin & Arnold, Stacey M. (2002). "The unfolded protein response in nutrient sensing and differentiation". *Nature Reviews. Molecular Cell Biology,* **3,** 411-421.

Kawamata, T., Akiyama, H., Yamada, T. & Mcgeer, P. L. (1992). "Immunologic reactions in amyotrophic lateral sclerosis brain and spinal cord tissue". *The American Journal Of Pathology,* **140,** 691-707.

Kieran, Dairin, Hafezparast, Majid, Bohnert, Stephanie, James, R. T. Dick, Martin, Joanne, Schiavo, Giampietro, Elizabeth, M. C. Fisher & Greensmith, Linda 2005. A Mutation in Dynein Rescues Axonal Transport Defects and Extends the Life Span of ALS Mice. Rockefeller University Press.

Kieran, Dairin, Kalmar, Bernadett, Dick, James R. T., Riddoch-Contreras, Joanna, Burnstock, Geoffrey & Greensmith, Linda (2004). "Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice". *Nature Medicine,* **10,** 402-405.

Kiernan, M. C., Vucic, S., Cheah, B. C., Turner, M. R., Eisen, A., Hardiman, O., Burrell, J. R. & Zoing, M. C. (2011). "Amyotrophic lateral sclerosis". *Lancet,* **377,** 942-55.

Kikuchi, Hitoshi, Almer, Gabriele, Yamashita, Satoshi, Guégan, Christelle, Nagai, Makiko, Xu, Zuoshang, Sosunov, Alexander A., Mckhann, Guy M. & Przedborski, Serge 2006. Spinal Cord Endoplasmic Reticulum Stress Associated with a Microsomal Accumulation of Mutant Superoxide Dismutase-1 in an ALS Model. National Academy of Sciences.

Koistinaho, Milla, Lin, Suizhen, Wu, Xin, Esterman, Michail, Koger, Deanna, Hanson, Jeffrey, Higgs, Richard, Liu, Feng, Malkani, Seema, Bales, Kelly R. & Paul, Steven M. (2004). "Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-beta peptides". *Nature Medicine,* **10,** 719-726.

Kong, J. & Xu, Z. (1998). "Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1". *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience,* **18,** 3241-3250.

Kong, Qiongman, Chang, Ling-Chu, Takahashi, Kou, Liu, Qibing, Schulte, Delanie A., Lai, Liching, Ibabao, Brian, Lin, Yuchen, Stouffer, Nathan, Das Mukhopadhyay, Chitra, Xing, Xuechao, Seyb, Kathleen I., Cuny, Gregory D., Glicksman, Marcie A. & Lin, Chien-Liang Glenn (2014). "Small-molecule activator of glutamate transporter EAAT2 translation provides neuroprotection". *The Journal Of Clinical Investigation,* **124,** 1255-1267.

Konsolaki, Mary (2013). "Fruitful research: drug target discovery for neurodegenerative diseases in Drosophila". *Expert Opinion On Drug Discovery,* **8,** 1503-1513.

Kopito, R. R. (2000). "Aggresomes, inclusion bodies and protein aggregation.". *Trends In Cell Biology,* **10,** 524-531.

Körner, Sonja, Kollewe, Katja, Fahlbusch, Marion, Zapf, Antonia, Dengler, Reinhard, Krampfl, Klaus & Petri, Susanne (2011). "Onset and spreading patterns of upper and lower motor neuron symptoms in amyotrophic lateral sclerosis". *Muscle & Nerve,* **43,** 636-642.

Koyama, Shingo, Arawaka, Shigeki, Chang-Hong, Ren, Wada, Manabu, Kawanami, Toru, Kurita, Keiji, Kato, Masaaki, Nagai, Makiko, Aoki, Masashi, Itoyama, Yasuto, Sobue, Gen, Chan, Pak H. & Kato, Takeo (2006). "Alteration of familial ALS-linked mutant SOD1 solubility with disease progression: Its modulation by the proteasome and Hsp70". *Biochemical and Biophysical Research Communications,* **343,** 719-730.

Kraemer, Brian C., Schuck, Theresa, Wheeler, Jeanna M., Robinson, Linda C., Trojanowski, John Q., Lee, Virginia M. Y. & Schellenberg, Gerard D. (2010). "Loss of murine TDP-43 disrupts motor function and plays an essential role in embryogenesis". *Acta Neuropathol,* **119,** 409-419.

Krauss, Sybille & Vorberg, Ina (2013). "Prions Ex Vivo: What Cell Culture Models Tell Us about Infectious Proteins". *International Journal of Cell Biology***,** 1-14.

Krishnan, J., Lemmens, R., Robberecht, W. & Van Den Bosch, L. (2006). "Role of heat shock response and Hsp27 in mutant SOD1-dependent cell death". *Experimental Neurology,* **200,** 301-310.

Kumimoto, Emily L., Fore, Taylor R. & Zhang, Bing (2013). "Transcriptome Profiling Following Neuronal and Glial Expression of ALS-Linked SOD1 in Drosophila". *G3 (Bethesda, Md.).*

Kumita, J.R., Poon, S., Caddy, G.L., Hagan, C.L., Dumoulin, M., Yerbury, J.J., Stewart, E.M., Robinson, C.V., Wilson, M. R. & Dobson, C. M (2007). "The extracellular chaperone clusterin potentially inhibits amyloid formation by interacting with prefibrillar species.". *Journal of Molecular Biology,* **369,** 157-167.

Kwiatkowski, T. J., Bosco, D. A., Leclerc, A. L., Tamrazian, E., Vanderburg, C. R., Russ, C., Davis, A., Gilchrist, J., Kasarskis, E. J., Munsat, T., Valdmanis, P., Rouleau, G. A., Hosler, B. A., Cortelli, P., De Jong, P. J., Yoshinaga, Y., Haines, J. L., Pericak-Vance, M. A., Yan, J., Ticozzi, N., Siddique, T., Mckenna-Yasek, D., Sapp, P. C., Horvitz, H. R., Landers, J. E. & Brown, R. H. 2009. Mutations in the FUS/TLS Gene on Chromosome 16 Cause Familial Amyotrophic Lateral Sclerosis. American Association for the Advancement of Science.

Lagier-Tourenne, C., Polymenidou, M. & Cleveland, D. W. (2010). "TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration". *Hum Mol Genet,* **19,** R46-64.

Lagier-Tourenne, Clotilde & Cleveland, Don W. (2009). "Rethinking ALS: the FUS about TDP-43". *Cell,* **136,** 1001-1004.

Lee, Bruce P. & Jones, Bradley W. (2005). "Transcriptional regulation of the Drosophila glial gene repo". *Mechanisms of Development,* **122,** 849-862.

Lee, T. & Luo, L. (1999). "Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis". *Neuron,* **22,** 451-461.

Lee, Young-Nam, Shim, Young-Jun, Kang, Byeong-Ho, Park, Joong-Jean & Min, Bon-Hong (2012). "Over-expression of human clusterin increases stress resistance and extends lifespan in Drosophila melanogaster". *Biochemical and Biophysical Research Communications,* **420,** 851-856.

Leskov, K.S., Klokov, D.Y., Li, J., Kinsella, T.J. & Boothman, D.A. (2003). "Synthesis and functional analyses of nuclear clusterin, a cell death protein.". *J Biol Chem,* **278,** 11590-11600.

Li, Yan, Ray, Payal, Rao, Elizabeth J., Shi, Chen, Guo, Weirui, Chen, Xiaoping, Woodruff, Elvin A., Fushimi, Kazuo, Wu, Jane Y. & Wang, Xiaodong 2010. A Drosophila Model for TDP-43 Proteinopathy. National Academy of Sciences.

Li, Yun R., King, Oliver D., Shorter, James & Gitler, Aaron D. (2013). "Stress granules as crucibles of ALS pathogenesis". *The Journal Of Cell Biology,* **201,** 361-372.

Lin, C. L., Bristol, L. A., Jin, L., Dykes-Hoberg, M., Crawford, T., Clawson, L. & Rothstein, J. D. (1998). "Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis". *Neuron,* **20,** 589-602.

Lin, C. L., Kong, Q., Cuny, G. D. & Glicksman, M. A. (2012). "Glutamate transporter EAAT2: a new target for the treatment of neurodegenerative diseases". *Future Med Chem,* **4,** 1689-700.

Liu-Yesucevitz, Liqun, Bilgutay, Aylin, Yong-Jie, Zhang, Vanderwyde, Tara, Citro, Allison, Mehta, Tapan, Zaarur, Nava, Mckee, Ann, Bowser, Robert, Sherman, Michael, Petrucelli, Leonard & Wolozin, Benjamin (2010). "Tar DNA Binding Protein-43 (TDP-43) Associates with Stress Granules: Analysis of Cultured Cells and Pathological Brain Tissue". *Plos One,* **5,** 1-15.

Liu, J., Shinobu, L. A., Ward, C. M., Young, D. & Cleveland, D. W. (2005). "Elevation of the Hsp70 chaperone does not effect toxicity in mouse models of familial amyotrophic lateral sclerosis". *J Neurochem,* **93,** 875-82.

Liu, Jian, Lillo, Concepción, Jonsson, P. Andreas, Velde, Christine Vande, Ward, Christopher M., Miller, Timothy M., Subramaniam, Jamuna R., Rothstein, Jeffery D., Marklund, Stefan, Andersen, Peter M., Brännström, Thomas, Gredal, Ole, Wong, Philip C., Williams, David S. & Cleveland, Don W. (2004). "Article: Toxicity of Familial ALS-Linked SOD1 Mutants from Selective Recruitment to Spinal Mitochondria". *Neuron,* **43,** 5-17.

Logroscino, G., Traynor, B. J., Hardiman, O., Chio, A., Couratier, P., Mitchell, J. D., Swingler, R. J. & Beghi, E. (2008). "Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues". *Journal of Neurology, Neurosurgery & Psychiatry,* **79,** 6-11.

Lu, Yubing, Ferris, Jacob & Gao, Fen-Biao (2009). "Frontotemporal dementia and amyotrophic lateral sclerosis-associated disease protein TDP-43 promotes dendritic branching". *Molecular Brain,* **2,** 30-30.

Maatkamp, Arjen, Vlug, Angela, Haasdijk, Elize, Troost, Dirk, French, Pim J. & Jaarsma, Dick (2004). "Decrease of Hsp25 protein expression precedes degeneration of motoneurons in ALS-SOD1 mice". *The European Journal Of Neuroscience,* **20,** 14-28.

Mackenzie, I. R., Bigio, E. H., Ince, P. G., Geser, F., Neumann, M., Cairns, N. J., Kwong, L. K., Forman, M. S., Ravits, J., Stewart, H., Eisen, A., Mcclusky, L., Kretzschmar, H. A., Monoranu, C. M., Highley, J. R., Kirby, J., Siddique, T., Shaw, P. J., Lee, V. M. & Trojanowski, J. Q. (2007). "Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations". *Annals of Neurology,* **61,** 427-434.

Magrané, Jordi & Manfredi, Giovanni (2009). "Mitochondrial function, morphology, and axonal transport in amyotrophic lateral sclerosis". *Antioxid Redox Signal,* **11,** 1615-1626.

Mahoney, Douglas J., Kaczor, Jan J., Bourgeois, Jacqueline, Yasuda, Nobuo & Tarnopolsky, Mark A. (2006). "Oxidative stress and antioxidant enzyme upregulation in SOD1-G93A mouse skeletal muscle". *Muscle & Nerve,* **33,** 809-816.

Manders, E. M. M., Verbeek, F. J. & Aten, J. A. (1993). "Measurement of co-localization of objects in dual-colour confocal images". *Journal of Microscopy,* **169,** 375.

Mannen, T., Iwata, M., Toyokura, Y. & Nagashima, K. (1977). "Preservation of a certain motoneurone group of the sacral cord in amyotrophic lateral sclerosis: its clinical significance". *Journal of Neurology, Neurosurgery & Psychiatry,* **40,** 464.

Marchetto, Maria C. N., Muotri, Alysson R., Mu, Yangling, Smith, Alan M., Cezar, Gabriela G. & Gage, Fred H. (2008). "Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells". *Cell Stem Cell,* **3,** 649-657.

Margineanu, I. & Ghetie, V. (1981). "A selective model of plasma protein catabolism.". *Journal of Theoretical Biology,* **90,** 101-110.

Martín, Iker, Ruiz, María F. & Sánchez, Lucas (2011). "The gene transformer-2 of Sciara (Diptera, Nematocera) and its effect on Drosophila sexual development". *BMC Developmental Biology,* **11,** 19-19.

Maruyama, Hirofumi, Morino, Hiroyuki, Ito, Hidefumi, Izumi, Yuishin, Kato, Hidemasa, Watanabe, Yasuhito, Kinoshita, Yoshimi, Kamada, Masaki, Nodera, Hiroyuki, Suzuki, Hidenori, Komure, Osamu, Matsuura, Shinya, Kobatake, Keitaro, Morimoto, Nobutoshi, Abe, Koji, Suzuki, Naoki, Aoki, Masashi, Kawata, Akihiro, Hirai, Takeshi, Kato, Takeo, Ogasawara, Kazumasa, Hirano, Asao, Takumi, Toru, Kusaka, Hirofumi, Hagiwara, Koichi, Kaji, Ryuji & Kawakami, Hideshi (2010). "Mutations of optineurin in amyotrophic lateral sclerosis". *Nature,* **465,** 223-226.

May, P. C., Robison, P., Fuson, K., Smalstig, B., Stephenson, D. & Clemens, J. A. (1992). "Sulfated glycoprotein-2 expression increases in rodent brain after transient global ischemia". *Brain Research. Molecular Brain Research,* **15,** 33-39.

Mendonça, D. M. F., Chimelli, L. & Martinez, A. M. B. (2006). "Expression of ubiquitin and proteasome in motorneurons and astrocytes of spinal cords from patients with amyotrophic lateral sclerosis". *Neurosci Lett,* **404,** 315-319.

Mercado, Pablo Arrisi, Ayala, Youhna M., Romano, Maurizio, Buratti, Emanuele & Baralle, Francisco E. (2005). "Depletion of TDP 43 overrides the need for exonic and intronic splicing enhancers in the human apoA-II gene". *Nucleic Acids Research,* **33,** 6000-6010.

Michel, D., Chatelain, G., North, S. & Brun, G. (1997). "Stress-induced transcription of the clusterin/apoJ gene". *The Biochemical Journal,* **328,** 45-50.

Minton, A.P. (2000). "Implications of macromolecular crowding for protein assembly.". *Current Opinion in Structural Biology*, **10,** 34-40.

Mitchell, J. D. & Borasio, G. D. (2007). "Amyotrophic lateral sclerosis". *Lancet*, **369,** 2031-41.

Mompeán, Miguel, Buratti, Emanuele, Guarnaccia, Corrado, Brito, Rui M. M., Chakrabartty, Avijit, Baralle, Francisco E. & Laurents, Douglas V. (2014). ""“Structural characterization of the minimal segment of TDP-43 competent for aggregation”". *Archives of Biochemistry and Biophysics*, **545,** 53-62.

Mondola, P., Annella, T., Serù, R., Santangelo, F., Iossa, S., Gioielli, A. & Santillo, M. (1998). "Secretion and increase of intracellular CuZn superoxide dismutase content in human neuroblastoma SK-N-BE cells subjected to oxidative stress". *Brain Research Bulletin*, **45,** 517-520.

Morrison, Brett M., Lee, Youngjin & Rothstein, Jeffrey D. (2013). "Oligodendroglia: metabolic supporters of axons". *Trends In Cell Biology*, **23,** 644-651.

Morrow, Geneviève, Samson, Mélanie, Michaud, Sébastien & Tanguay, Robert M. (2004). "Overexpression of the small mitochondrial Hsp22 extends Drosophila life span and increases resistance to oxidative stress". *FASEB Journal: Official Publication Of The Federation Of American Societies For Experimental Biology*, **18,** 598-599.

Morrow, Genevieve & Tanguay, Robert M (2015). "Drosophila melanogaster Hsp22: a mitochondrial small heat shock protein influencing the aging process". *Frontiers in Genetics*, **6**.

Moser, Jakob Maximilian, Bigini, Paolo & Schmitt-John, Thomas (2013). "The wobbler mouse, an ALS animal model". *Molecular Genetics And Genomics: MGG*, **288,** 207-229.

Münch, Christian, O'brien, John, Bertolotti, Anne & Henderson, Richard 2011. Prion-like propagation of mutant Superoxide dismutase-1 misfolding in neuronal cells. National Academy of Sciences.

Nagai, R., Mera, K., Nakajou, K., Fujiwara, Y., Iwao, Y., Imai, H., Murata, T. & Otagiri, M. (2007). "The ligand activity of AGE-proteins to scavenger receptors is dependent on their rate of modification by AGEs.". *Biochim. Biophys. Acta*, **1772,** 1192-1198.

Narayan, Priyanka, Orte, Angel, Clarke, Richard W., Bolognesi, Benedetta, Hook, Sharon, Ganzinger, Kristina A., Meehan, Sarah, Wilson, Mark R., Dobson, Christopher M. & Klenerman, David (2011). "The extracellular chaperone clusterin sequesters oligomeric forms of the amyloid-β(1-40) peptide". *Nature Structural & Molecular Biology*, **19,** 79-83.

Neri, C. L., Duchala, C. S. & Macklin, W. B. (1997). "Expression of molecular chaperones and vesicle transport proteins in differentiating oligodendrocytes". *Journal Of Neuroscience Research*, **50,** 769-780.

Neumann, Manuela, Sampathu, Deepak M., Kwong, Linda K., Truax, Adam C., Micsenyi, Matthew C., Chou, Thomas T., Bruce, Jennifer, Schuck, Theresa, Grossman, Murray, Clark, Christopher M., Mccluskey, Leo F., Miller, Bruce L., Masliah, Eliezer, Mackenzie, Ian R., Feldman, Howard, Feiden, Wolfgang, Kretzschmar, Hans A., Trojanowski, John Q. & Lee, Virginia M. Y. 2006. Ubiquitinated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis. American Association for the Advancement of Science.

Nichols, Charles D. (2006). "Drosophila melanogaster neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery". *Pharmacology & Therapeutics*, **112,** 677-700.

Nichols, Charles D., Becnel, Jaime & Pandey, Udai B. (2012). "Methods to assay Drosophila behavior". *Journal Of Visualized Experiments: Jove*.

Nizard, P., Tetley, S., Le Drean, Y., Watrin, T., Le Goff, P., Wilson, M. R. & Michel, D. (2007). "Stress-induced retrotranslocation of clusterin/ApoJ into the cytosol.". *Traffic*, **8,** 554-565.

Nonaka, Takashi, Arai, Tetsuaki, Buratti, Emanuele, Baralle, Francisco E., Akiyama, Haruhiko & Hasegawa, Masato (2009). "Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells". *FEBS Letters*, **583,** 394-400.

Nonaka, Takashi, Masuda-Suzukake, Masami, Arai, Tetsuaki, Hasegawa, Yoko, Akatsu, Hiroyasu, Obi, Tomokazu, Yoshida, Mari, Murayama, Shigeo, Mann, David M. A., Akiyama, Haruhiko & Hasegawa, Masato (2013). "Prion-like properties of pathological TDP-43 aggregates from diseased brains". *Cell Reports,* **4,** 124-134.

Norton, J. A., Stein, T. P. & Brennan, M. F. (1981). "Whole body protein synthesis and turnover in normal man and malnourished patients with and without known cancer". *Annals Of Surgery,* **194,** 123-128.

Nowak, Richard J., Cuny, Gregory D., Choi, Sungwoon, Lansbury, Peter T. & Ray, Soumya S. (2010). "Improving binding specificity of pharmacological chaperones that target mutant superoxide dismutase-1 linked to familial amyotrophic lateral sclerosis using computational methods". *Journal Of Medicinal Chemistry,* **53,** 2709-2718.

O'sullivan, J., Whyte, L., Drake, J. & Tenniswood, M. (2003). "Alterations in the post-translational modification and intracellular trafficking of clusterin in MCF-7 cells during apoptosis". *Cell Death Differ,* **10,** 914-27.

Ohlsson, Marcus, Bellander, Bo-Michael, Langmoen, Iver A. & Svensson, Mikael (2003). "Complement activation following optic nerve crush in the adult rat". *Journal Of Neurotrauma,* **20,** 895-904.

Okamoto, Koichi, Mizuno, Yuji & Fujita, Yukio (2008). "Bunina bodies in amyotrophic lateral sclerosis". *Neuropathology,* **28,** 109-115.

Ong, C., Yung, L. Y. L., Cai, Y., Bay, B. H. & Baeg, G. H. (2015). "Drosophila melanogaster as a model organism to study nanotoxicity". *Nanotoxicology,* **9,** 396-403.

Ou, S. H., Wu, F., Harrich, D., García-Martínez, L. F. & Gaynor, R. B. (1995). "Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs". *Journal Of Virology,* **69,** 3584-3596.

Papadeas, Sophia T., Kraig, Sarah E., O'banion, Colin, Lepore, Angelo C. & Maragakis, Nicholas J. (2011). "Astrocytes carrying the superoxide dismutase 1 (SOD1G93A) mutation induce wild-type motor neuron degeneration in vivo". *Proc Natl Acad Sci U S A,* **108,** 17803-17808.

Pasinelli, Piera & Brown, Robert H. (2006). "Molecular biology of amyotrophic lateral sclerosis: insights from genetics". *Nature Reviews. Neuroscience,* **7,** 710-723.

Pasinetti, G. M., Johnson, S. A., Oda, T., Rozovsky, I. & Finch, C. E. (1994). "Clusterin (SGP-2): a multifunctional glycoprotein with regional expression in astrocytes and neurons of the adult rat brain". *The Journal Of Comparative Neurology,* **339,** 387-400.

Patel, Yogesh J. K., Payne Smith, Martin D., De Belleroche, Jacqueline & Latchman, David S. (2005). "Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant-induced cell death in mammalian neuronal cells". *Brain Research. Molecular Brain Research,* **134,** 256-274.

Pereanu, Wayne, Shy, Diana & Hartenstein, Volker (2005). "Morphogenesis and proliferation of the larval brain glia in Drosophila". *Developmental Biology,* **283,** 191-203.

Philips, Thomas, Bento-Abreu, Andre, Nonneman, Annelies, Haeck, Wanda, Staats, Kim, Geelen, Veerle, Hersmus, Nicole, Küsters, Benno, Van Den Bosch, Ludo, Van Damme, Philip, Richardson, William D. & Robberecht, Wim (2013). "Oligodendrocyte dysfunction in the pathogenesis of amyotrophic lateral sclerosis". *Brain: A Journal Of Neurology,* **136,** 471-482.

Piao, Yue-Shan, Wakabayashi, Koichi, Kakita, Akiyoshi, Yamada, Mitsunori, Hayashi, Shintaro, Morita, Takashi, Ikuta, Fusahiro, Oyanagi, Kiyomitsu & Takahashi, Hitoshi (2003). "Neuropathology with clinical correlations of sporadic amyotrophic lateral sclerosis: 102 autopsy cases examined between 1962 and 2000". *Brain Pathology (Zurich, Switzerland),* **13,** 10-22.

Plumier, J. C., Hopkins, D. A., Robertson, H. A. & Currie, R. W. (1997). "Constitutive expression of the 27-kDa heat shock protein (Hsp27) in sensory and motor neurons of the rat nervous system". *The Journal Of Comparative Neurology,* **384,** 409-428.

Poon, S., Rybchyn, M.S., Easterbrook-Smith, S.B., Carver, J.A. & Wilson, M.R. (2000). "Clusterin is an ATP-independent chaperone with a very broad substrate specificity that stabilizes stressed proteins in a folding-competent state.". *Biochemistry,* **39,** 15953-15960.

Potter, S. Z. & Valentine, J. S. (2003). "The perplexing role of copper-zinc superoxide dismutase in amyotrophic lateral sclerosis (Lou Gehrig's disease)". *J Biol Inorg Chem,* **8,** 373-80.

Pramatarova, A., Laganiere, J., Roussel, J., Brisebois, K. & Rouleau, G. A. (2001). "Neuron-specific expression of mutant superoxide dismutase 1 in transgenic mice does not lead to motor impairment". *J Neurosci,* **21,** 3369-74.

Prochnow, H., Gollan, R., Rohne, P., Hassemer, M., Koch-Brandt, C. & Baiersdorfer, M. (2013). "Non-secreted clusterin isoforms are translated in rare amounts from distinct human mRNA variants and do not affect Bax-mediated apoptosis or the NF-kappaB signaling pathway". *Plos One,* **8,** e75303.

Project, Bpcc/Nsu Title Iii Cooperative. 2011. *Nervous System* [Online]. Available: https://my.bpcc.edu/content/blgy224/NervousSystem/NervousSystem9.html.

Prusiner, S, B. (1982). "Novel Proteinaceous Infectious Particles Cause Scrapie". *Science,* **216,** 136-44.

Ragonese, Paolo, Cellura, Eleonora, Aridon, Paolo, D'amelio, Marco, Spataro, Rossella, Taiello, Alfonsa Claudia, Maimone, Davide, La Bella, Vincenzo & Savettieri, Giovanni (2012). "Incidence of amyotrophic lateral sclerosis in Sicily: A population based study". *Amyotrophic Lateral Sclerosis: Official Publication Of The World Federation Of Neurology Research Group On Motor Neuron Diseases,* **13,** 284-287.

Rajendran, L., Honsho, M., Zahn, T. R., Keller, P., Geiger, K. D., Verkade, P. & Simons, K. (2006). "Alzheimer's disease beta-amyloid peptides are released in association with exosomes". *Proc Natl Acad Sci U S A,* **103,** 11172-7.

Ravits, J. M. & La Spada, A. R. (2009). "ALS motor phenotype heterogeneity, focality, and spread: deconstructing motor neuron degeneration". *Neurology,* **73,** 805-811.

Ravits, J., Paul, P. & Jorg, C. (2007). "Focality of upper and lower motor neuron degeneration at the clinical onset of ALS". *Neurology,* **68,** 1571-1575.

Reaume, A. G., Elliott, J. L., Hoffman, E. K., Kowall, N. W., Ferrante, R. J., Siwek, D. F., Wilcox, H. M., Flood, D. G., Beal, M. F., Brown, R. H., Jr., Scott, R. W. & Snider, W. D. (1996). "Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury". *Nature Genetics,* **13,** 43-47.

Reddy, K. B., Jin, G., Karode, M. C., Harmony, J. A. & Howe, P. H. (1996). "Transforming growth factor beta (TGF beta)-induced nuclear localization of apolipoprotein J/clusterin in epithelial cells". *Biochemistry,* **35,** 6157-6163.

Renton, Alan e, Majounie, Elisa, Waite, Adrian, Simón-Sánchez, Javier, Rollinson, Sara, Gibbs, J. raphael, Schymick, Jennifer c, Laaksovirta, Hannu, Van swieten, John c, Myllykangas, Liisa, Kalimo, Hannu, Paetau, Anders, Abramzon, Yevgeniya, Remes, Anne m, Kaganovich, Alice, Scholz, Sonja w, Duckworth, Jamie, Ding, Jinhui, Harmer, Daniel w, Hernandez, Dena g, Johnson, Janel o, Mok, Kin, Ryten, Mina, Trabzuni, Danyah, Guerreiro, Rita j, Orrell, Richard w, Neal, James, Murray, Alex, Pearson, Justin, Jansen, Iris e, Sondervan, David, Seelaar, Harro, Blake, Derek, Young, Kate, Halliwell, Nicola, Callister, Janis bennion, Toulson, Greg, Richardson, Anna, Gerhard, Alex, Snowden, Julie, Mann, David, Neary, David, Nalls, Michael a, Peuralinna, Terhi, Jansson, Lilja, Isoviita, Veli-Matti, Kaivorinne, Anna-Lotta, Hölttä-Vuori, Maarit, Ikonen, Elina, Sulkava, Raimo, Benatar, Michael, Wuu, Joanne, Chiò, Adriano, Restagno, Gabriella, Borghero, Giuseppe, Sabatelli, Mario, Heckerman, David, Rogaeva, Ekaterina, Zinman, Lorne, Rothstein, Jeffrey d, Sendtner, Michael, Drepper, Carsten, Eichler, Evan e, Alkan, Can, Abdullaev, Ziedulla, Pack, Svetlana d, Dutra, Amalia, Pak, Evgenia, Hardy, John, Singleton, Andrew, Williams, Nigel m, Heutink, Peter, Pickering-Brown, Stuart, Morris, Huw r, Tienari, Pentti j & Traynor, Bryan j (2011). "Article: A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of Chromosome 9p21-Linked ALS-FTD". *Neuron,* **72,** 257-268.

Richter-Landsberg, C. & Bauer, N. G. (2004). "Tau-inclusion body formation in oligodendroglia: the role of stress proteins and proteasome inhibition". *Int J Dev Neurosci,* **22,** 443-51.

Rival, Thomas, Soustelle, Laurent, Strambi, Colette, Besson, Marie-Thérèse, Iché, Magali & Birman, Serge (2004). "Decreasing glutamate buffering capacity triggers oxidative stress and neuropil degeneration in the Drosophila brain". *Current Biology: CB,* **14,** 599-605.

Robinow, S. & White, K. (1991). "Characterization and spatial distribution of the ELAV protein during Drosophila melanogaster development". *Journal Of Neurobiology,* **22,** 443-461.

Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'regan, J. P., Deng, H. X. & Et Al. (1993). "Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis". *Nature,* **362,** 59-62.

Rothstein, J. D., Dykes-Hoberg, M., Pardo, C. A., Bristol, L. A., Jin, L., Kuncl, R. W., Kanai, Y., Hediger, M. A., Wang, Y., Schielke, J. P. & Welty, D. F. (1996). "Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate". *Neuron,* **16,** 675-686.

Rothstein, J. D., Martin, L. J. & Kuncl, R. W. (1992). "Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis". *The New England Journal Of Medicine,* **326,** 1464-1468.

Rothstein, J. D., Tsai, G., Kuncl, R. W., Clawson, L., Cornblath, D. R., Drachman, D. B., Pestronk, A., Stauch, B. L. & Coyle, J. T. (1990). "Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis". *Ann Neurol,* **28,** 18-25.

Rothstein, J. D., Van Kammen, M., Levey, A. I., Martin, L. J. & Kuncl, R. W. (1995). "Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis". *Annals of Neurology,* **38,** 73-84.

Rutherford, Nicola J., Yong-Jie, Zhang, Baker, Matt, Gass, Jennifer M., Finch, Nicole A., Ya-Fei, Xu, Stewart, Heather, Kelley, Brendan J., Kuntz, Karen, Crook, Richard J. P., Sreedharan, Jemeen, Vance, Caroline, Sorenson, Eric, Lippa, Carol, Bigio, Eileen H., Geschwind, Daniel H., Knopman, David S., Mitsumoto, Hiroshi, Petersen, Ronald C. & Cashman, Neil R. (2008). "Novel Mutations in TARDBP (TDP-43) in Patients with Familial Amyotrophic Lateral Sclerosis". *PLoS Genetics,* **4,** 1-8.

Santilli, G., Aronow, B.J. & Sala, A. (2003). "Essential requirement of apolipoprotein J (clusterin) signalling for Ikappa B expression and regulation of NF-kappaB activity.". *Journal of Biolgical Chemistry,* **278,** 38214-38219.

Sasaki, S. (2010). "Endoplasmic Reticulum Stress in Motor Neurons of the Spinal Cord in Sporadic Amyotrophic Lateral Sclerosis.". *Journal of neuropathology and experimental neurology,* **69,** 346-355.

Sasaki, S. & Iwata, M. (1996). "Impairment of fast axonal transport in the proximal axons of anterior horn neurons in amyotrophic lateral sclerosis". *Neurology,* **47,** 535-540.

Sasaki, S., Komori, T. & Iwata, M. (2000). "Excitatory amino acid transporter 1 and 2 immunoreactivity in the spinal cord in amyotrophic lateral sclerosis". *Acta Neuropathol,* **100,** 138-144.

Sasaki, Shoichi (2011). "Autophagy in spinal cord motor neurons in sporadic amyotrophic lateral sclerosis". *Journal Of Neuropathology And Experimental Neurology,* **70,** 349-359.

Sasaki, Shoichi & Iwata, Makoto (2007). "Mitochondrial alterations in the spinal cord of patients with sporadic amyotrophic lateral sclerosis". *Journal Of Neuropathology And Experimental Neurology,* **66,** 10-16.

Saxena, Smita, Cabuy, Erik & Caroni, Pico (2009). "A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice". *Nature Neuroscience,* **12,** 627-636.

Schlachetzki, Johannes C. M., Saliba, Soraya Wilke & Pinheiro De Oliveira, Antonio Carlos (2013). "Studying neurodegenerative diseases in culture models". *Revista Brasileira de Psiquiatria,* **35,** S92-S100.

Schneider, Anja & Simons, Mikael (2013). "Exosomes: vesicular carriers for intercellular communication in neurodegenerative disorders". *Cell And Tissue Research,* **352,** 33-47.

Schubert, Ulrichanton Luis C. (2000). "Rapid degradation of a large fraction of newly synthesized proteins by proteasomes". *Nature,* **404,** 770.

Sekizawa, T., Openshaw, H., Ohbo, K., Sugamura, K., Itoyama, Y. & Niland, J. C. (1998). "Cerebrospinal fluid interleukin 6 in amyotrophic lateral sclerosis: immunological parameter and comparison with inflammatory and non-inflammatory central nervous system diseases". *Journal of the Neurological Sciences,* **154,** 194-199.

Senior, J., Delgado, C., Fisher, D., Tilcock, C. & Gregoriadis, G. (1991). "Influence of surface hydrophobicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles.". *Biochimica Biophys Acta,* **1062,** 77-82.

Serio, Andrea, Bilican, Bilada, Barmada, Sami J., Ando, Dale Michael, Zhao, Chen, Siller, Rick, Burr, Karen, Haghi, Ghazal, Story, David, Nishimura, Agnes Lumi, Carrasco, Monica A., Phatnani, Hemali P., Shum, Carole, Wilmut, Ian, Maniatis, Tom, Shaw, Christopher E., Finkbeiner, Steven & Chandran, Siddharthan (2013). "Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy". *Proc Natl Acad Sci U S A,* **110,** 4697-4702.

Shamu, C. E. (1998). "Splicing: HACking into the unfolded-protein response". *Current Biology: CB,* **8,** R121-R123.

Shan, Xiu, Chiang, Po-Min, Price, Donald L. & Wong, Philip C. 2010. Altered distributions of Gemini of coiled bodies and mitochondria in motor neurons of TDP-43 transgenic mice. National Academy of Sciences.

Sharp, Paul S., Akbar, Mohammed T., Bouri, Sonia, Senda, Atsushi, Joshi, Kieran, Chen, Han-Jou, Latchman, David S., Wells, Dominic J. & De Belleroche, Jacqueline (2008). "Protective effects of heat shock protein 27 in a model of ALS occur in the early stages of disease progression". *Neurobiology of Disease,* **30,** 42-55.

Shaw, P. J. (2005). "Molecular and cellular pathways of neurodegeneration in motor neurone disease". *Journal Of Neurology, Neurosurgery, And Psychiatry,* **76,** 1046-1057.

Shaw, P. J., Ince, P. G., Falkous, G. & Mantle, D. (1995). "Oxidative damage to protein in sporadic motor neuron disease spinal cord". *Annals of Neurology,* **38,** 691-695.

Shin, Ji-Yeon, Fang, Zhi-Hui, Yu, Zhao-Xue, Wang, Chuan-En, Li, Shi-Hua & Li, Xiao-Jiang 2005. Expression of Mutant Huntingtin in Glial Cells Contributes to Neuronal Excitotoxicity. Rockefeller University Press.

Siklós, László, Engelhardt, József, Harati, Yadollah, Smith, R. Glenn, Joó, Ferenc & Appel, Stanley H. (1996). "Ultrastructural evidence for altered calcium in motor nerve terminals in amyotrophc lateral sclerosis". *Annals of Neurology,* **39,** 203.

Smith, R. G., Henry, Y. K., Mattson, M. P. & Appel, S. H. (1998). "Presence of 4-hydroxynonenal in cerebrospinal fluid of patients with sporadic amyotrophic lateral sclerosis". *Annals of Neurology,* **44,** 696-699.

Sottrup-Jensen, L. (1989). "Alpha-macroglobulins: structure shape and mechanism of proteinase complex formation.". *J Biol Chem,* **264,** 11539-11542.

Soustelle, Laurent, Besson, Marie-Thérèse, Rival, Thomas & Birman, Serge (2002). "Terminal glial differentiation involves regulated expression of the excitatory amino acid transporters in the Drosophila embryonic CNS". *Developmental Biology,* **248,** 294-306.

Sparrey, Carolyn. 2008. *The role of constituent materials in spinal cord biomechanics* Doctor of Philosophy University of California

Sreedharan, Jemeen, Blair, Ian P., Tripathi, Vineeta B., Hu, Xun, Vance, Caroline, Rogelj, Boris, Ackerley, Steven, Durnall, Jennifer C., Williams, Kelly L., Buratti, Emanuele, Baralle, Francisco, De Belleroche, Jacqueline, Mitchell, J. Douglas, Leigh, P. Nigel, Al-Chalabi, Ammar, Miller, Christopher C., Nicholson, Garth & Shaw, Christopher E. 2008. TDP-43

Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis. American Association for the Advancement of Science.

Stirling, Peter C., Lundin, Victor F. & Leroux, Michel R. (2003). "Getting a grip on non-native proteins". *EMBO Reports,* **4,** 565-570.

Strocchi, P., Rauzi, F. & Cevolani, D. (1999). "Neuronal loss up-regulates clusterin mRNA in living neurons and glial cells in the rat brain". *Neuroreport,* **10,** 1789-1792.

Sturtz, L. A., Diekert, K., Jensen, L. T., Lill, R. & Culotta, V. C. (2001). "A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage". *J Biol Chem,* **276,** 38084-38089.

Sun, Zhihui, Diaz, Zamia, Fang, Xiaodong, Hart, Michael P., Chesi, Alessandra, Shorter, James & Gitler, Aaron D. (2011). "Molecular determinants and genetic modifiers of aggregation and toxicity for the ALS disease protein FUS/TLS". *Plos Biology,* **9,** e1000614-e1000614.

Swarup, Vivek, Phaneuf, Daniel, Dupré, Nicolas, Petri, Susanne, Strong, Michael, Kriz, Jasna & Julien, Jean-Pierre (2011). "Deregulation of TDP-43 in amyotrophic lateral sclerosis triggers nuclear factor κB-mediated pathogenic pathways". *The Journal Of Experimental Medicine,* **208,** 2429-2447.

Swash, Michael (2013). "How does ALS spread between neurones in the CNS?". *Journal Of Neurology, Neurosurgery, And Psychiatry,* **84,** 116-117.

Swingler, R. J., Fraser, H. & Warlow, C. P. (1992). "Motor neuron disease and polio in Scotland". *Journal Of Neurology, Neurosurgery, And Psychiatry,* **55,** 1116-1120.

Takahashi, Hitoshi, Ohama, Eisaku & Ikuta, Fusahiro (1991). "Are Bunina Bodies of Endoplasmic Reticulum Origin? An Ultrastructural Study of Subthalamic Eosinophilic Inclusions in a Case of Atypical Motor Neuron Disease". *Pathology International,* **41,** 889-894.

Takahashi, Kazutoshi, Tanabe, Koji, Ohnuki, Mari, Narita, Megumi, Ichisaka, Tomoko, Tomoda, Kiichiro & Yamanaka, Shinya (2007). "Article: Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors". *Cell,* **131,** 861-872.

Takeuchi, H., Kobayashi, Y., Yoshihara, T., Niwa, J., Doyu, M., Ohtsuka, K. & Sobue, G. (2002). "Hsp70 and Hsp40 improve neurite outgrowth and suppress intracytoplasmic aggregate formation in cultured neuronal cells expressing mutant SOD1". *Brain Res,* **949,** 11-22.

Tan, J. M., Wong, E. S. & Lim, K. L. (2009). "Protein misfolding and aggregation in Parkinson's disease". *Antioxid Redox Signal,* **11,** 2119-34.

Tandan, R. & Bradley, W. G. (1985). "Amyotrophic lateral sclerosis: Part 2. Etiopathogenesis". *Ann Neurol,* **18,** 419-31.

Tatom, J. B., Wang, D. B., Dayton, R. D., Skalli, O., Hutton, M. L., Dickson, D. W. & Klein, R. L. (2009). "Mimicking aspects of frontotemporal lobar degeneration and Lou Gehrig's disease in rats via TDP-43 overexpression". *Mol Ther,* **17,** 607-13.

Taylor, J. Paul, Hardy, John & Fischbeck, Kenneth H. 2002. Toxic Proteins in Neurodegenerative Disease. American Society for the Advancement of Science.

Tikka, Tiina M., Vartiainen, Nina E., Goldsteins, Gundars, Oja, Simo S., Andersen, Peter M., Marklund, Stefan L. & Koistinaho, Jari (2002). "Minocycline prevents neurotoxicity induced by cerebrospinal fluid from patients with motor neurone disease". *Brain: A Journal Of Neurology,* **125,** 722-731.

Tobisawa, Shinsuke, Hozumi, Yasukazu, Arawaka, Shigeki, Koyama, Shingo, Wada, Manabu, Nagai, Makiko, Aoki, Masashi, Itoyama, Yasuto, Goto, Kaoru & Kato, Takeo (2003). "Mutant SOD1 linked to familial amyotrophic lateral sclerosis, but not wild-type SOD1, induces ER stress in COS7 cells and transgenic mice". *Biochemical and Biophysical Research Communications,* **303,** 496-503.

Tohgi, H., Abe, T., Yamazaki, K., Murata, T., Ishizaki, E. & Isobe, C. (1999). "Increase in oxidized NO products and reduction in oxidized glutathione in cerebrospinal fluid from patients with sporadic form of amyotrophic lateral sclerosis". *Neurosci Lett,* **260,** 204-6.

Tollervey, James R., Curk, Tomaž, Rogelj, Boris, Briese, Michael, Cereda, Matteo, Kayikci, Melis, König, Julian, Hortobágyi, Tibor, Nishimura, Agnes L., Zupunski, Vera, Patani, Rickie, Chandran, Siddharthan, Rot, Gregor, Zupan, Blaž, Shaw, Christopher E. & Ule, Jernej (2011). "Characterizing the RNA targets and position-dependent splicing regulation by TDP-43". *Nature Neuroscience*, **14,** 452-458.

Tomonaga, M., Saito, M., Yoshimura, M., Shimada, H. & Tohgi, H. (1978). "Ultrastructure of the Bunina bodies in anterior horn cells of amyotrophic lateral sclerosis". *Acta Neuropathol*, **42,** 81-6.

Tong, Jianbin, Huang, Cao, Bi, Fangfang, Wu, Qinxue, Huang, Bo, Liu, Xionghao, Li, Fang, Zhou, Hongxia & Xia, Xu-Gang (2013). "Expression of ALS-linked TDP-43 mutant in astrocytes causes non-cell-autonomous motor neuron death in rats". *The EMBO Journal*, **32,** 1917-1926.

Törnqvist, E., Liu, L., Aldskogius, H., Holst, H. V. & Svensson, M. (1996). "Complement and clusterin in the injured nervous system". *Neurobiology of Aging*, **17,** 695-705.

Traynor, B. J., Codd, M. B., Corr, B., Forde, C., Frost, E. & Hardiman, O. (1999). "Incidence and prevalence of ALS in Ireland, 1995-1997: a population-based study". *Neurology*, **52,** 504-509.

Tripathi, Vineeta Bhasker, Baskaran, Pranetha, Shaw, Christopher E. & Guthrie, Sarah (2014). "Tar DNA-binding protein-43 (TDP-43) regulates axon growth in vitro and in vivo". *Neurobiology of Disease*, **65,** 25-34.

Trougakos, Ioannis P. (2013). "The molecular chaperone apolipoprotein j/clusterin as a sensor of oxidative stress: implications in therapeutic approaches - a mini-review". *Gerontology*, **59,** 514-523.

Turner, B. J. & Atkin, J. D. (2006). "ER stress and UPR in familial amyotrophic lateral sclerosis". *Curr Mol Med*, **6,** 79-86.

Udan-Johns, M., Bengoechea, R., Bell, S., Shao, J., Diamond, M. I., True, H. L., Weihl, C. C. & Baloh, R. H. (2014). "Prion-like nuclear aggregation of TDP-43 during heat shock is regulated by HSP40/70 chaperones". *Hum Mol Genet*, **23,** 157-70.

Urushitani, Makotosik Attilasakurai Takashinukina Nobuyukitakahashi Ryosukejulien Jean-Pierre (2006). "Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis". *Nature Neuroscience*, **9,** 108-118.

Valori, Chiara F., Brambilla, Liliana, Martorana, Francesca & Rossi, Daniela (2014). "The multifaceted role of glial cells in amyotrophic lateral sclerosis". *Cellular And Molecular Life Sciences: CMLS*, **71,** 287-297.

Van Beek, J., Chan, P., Bernaudin, M., Petit, E., Mackenzie, E. T. & Fontaine, M. (2000). "Glial responses, clusterin, and complement in permanent focal cerebral ischemia in the mouse". *Glia*, **31,** 39-50.

Van Blitterswijk, Marka, Dejesus-Hernandez, Mariely & Rademakers, Rosa (2012). "How do C9ORF72 repeat expansions cause amyotrophic lateral sclerosis and frontotemporal dementia: can we learn from other noncoding repeat expansion disorders?". *Current Opinion in Neurology*, **25,** 689-700.

Van Deerlin, Vivianna M., Leverenz, James B., Bekris, Lynn M., Bird, Thomas D., Yuan, Wuxing, Elman, Lauren B., Clay, Dana, Wood, Elisabeth Mccarty, Chen-Plotkin, Alice S., Martinez-Lage, Maria, Steinbart, Ellen, Mccluskey, Leo, Grossman, Murray, Neumann, Manuela, Wu, I. Lin, Yang, Wei-Shiung, Kalb, Robert, Galasko, Douglas R., Montine, Thomas J., Trojanowski, John Q., Lee, Virginia M. Y., Schellenberg, Gerard D. & Yu, Chang-En (2008). "TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis". *The Lancet. Neurology*, **7,** 409-416.

Vance, Caroline, Rogelj, Boris, Hortobágyi, Tibor, De Vos, Kurt J., Nishimura, Agnes Lumi, Sreedharan, Jemeen, Hu, Xun, Smith, Bradley, Ruddy, Deborah, Wright, Paul, Ganesalingam, Jeban, Williams, Kelly L., Tripathi, Vineeta, Al-Saraj, Safa, Al-Chalabi, Ammar, Leigh, P. Nigel, Blair, Ian P., Nicholson, Garth, De Belleroche, Jackie, Gallo, Jean-Marc, Miller, Christopher C. & Shaw, Christopher E. 2009. Mutations in FUS, an RNA Processing Protein,

Cause Familial Amyotrophic Lateral Sclerosis Type 6. American Association for the Advancement of Science.

Veyrat-Durebex, C., Corcia, P., Dangoumau, A., Laumonnier, F., Piver, E., Gordon, P. H., Andres, C. R., Vourc'h, P. & Blasco, H. (2014). "Advances in cellular models to explore the pathophysiology of amyotrophic lateral sclerosis". *Molecular Neurobiology,* **49,** 966-983.

Voigt, Aaron, Herholz, David, Fiesel, Fabienne C., Kaur, Kavita, Müller, Daniel, Karsten, Peter, Weber, Stephanie S., Kahle, Philipp J., Marquardt, Till & Schulz, Jörg B. (2010). "TDP-43-Mediated Neuron Loss In Vivo Requires RNA-Binding Activity". *Plos One,* **5,** 1-12.

Walker, G. A. & Lithgow, G. J. (2003). "Lifespan extension in C. elegans by a molecular chaperone dependent upon insulin-like signals". *Aging Cell,* **2,** 131-9.

Wang, Hurng-Yi, Wang, I. Fan, Bose, Jayaramakrishnan & Shen, C. K. James (2004). "Structural diversity and functional implications of the eukaryotic TDP gene family". *Genomics,* **83,** 130-139.

Wang, I. Fan, Reddy, Narsa M. & Shen, C. K. James (2002). "Higher order arrangement of the eukaryotic nuclear bodies". *Proc Natl Acad Sci U S A,* **99,** 13583-13588.

Wang, Jiou, Farr, George W., Zeiss, Caroline J., Rodriguez-Gil, Diego J., Wilson, Jean H., Furtak, Krystyna, Rutkowski, D. Thomas, Kaufman, Randal J., Ruse, Cristian I., Yates, John R., Perrin, Steve, Feany, Mel B. & Horwich, Arthur L. 2009. Progressive Aggregation despite Chaperone Associations of a Mutant SOD1-YFP in Transgenic Mice That Develop ALS. National Academy of Sciences.

Wang, Jiou, Martin, Elizabeth, Gonzales, Victoria, Borchelt, David R. & Lee, Michael K. (2008). "Differential regulation of small heat shock proteins in transgenic mouse models of neurodegenerative diseases". *Neurobiology of Aging,* **29,** 586-597.

Wang, Jiou, Slunt, Hilda, Gonzales, Victoria, Fromholt, David, Coonfield, Michael, Copeland, Neal G., Jenkins, Nancy A. & Borchelt, David R. (2003). "Copper-binding-site-null SOD1 causes ALS in transgenic mice: aggregates of non-native SOD1 delineate a common feature". *Human Molecular Genetics,* **12,** 2753-2764.

Wang, Jiou, Xu, Guilian, Li, Hong, Gonzales, Victoria, Fromholt, David, Karch, Celeste, Copeland, Neal G., Jenkins, Nancy A. & Borchelt, David R. (2005). "Somatodendritic accumulation of misfolded SOD1-L126Z in motor neurons mediates degeneration: αB-crystallin modulates aggregation". *Human Molecular Genetics,* **14,** 2335-2347.

Wang, Xiaoju, Fan, Huadong, Ying, Zheng, Li, Bin, Wang, Hongfeng & Wang, Guanghui (2010). "Degradation of TDP-43 and its pathogenic form by autophagy and the ubiquitin-proteasome system". *Neurosci Lett,* **469,** 112-116.

Warraich, Sadaf T., Yang, Shu, Nicholson, Garth A. & Blair, Ian P. (2010). "TDP-43: a DNA and RNA binding protein with roles in neurodegenerative diseases". *The International Journal Of Biochemistry & Cell Biology,* **42,** 1606-1609.

Watanabe, M., Dykes-Hoberg, M., Culotta, V. C., Price, D. L., Wong, P. C. & Rothstein, J. D. (2001). "Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues". *Neurobiology of Disease,* **8,** 933-941.

Watson, Melanie R., Lagow, Robert D., Xu, Kexiang, Zhang, Bing & Bonini, Nancy M. (2008). "A drosophila model for amyotrophic lateral sclerosis reveals motor neuron damage by human SOD1". *J Biol Chem,* **283,** 24972-24981.

Wegorzewska, Iga, Bell, Shaughn, Cairns, Nigel J., Miller, Timothy M. & Baloh, Robert H. 2009. TDP-43 Mutant Transgenic Mice Develop Features of ALS and Frontotemporal Lobar Degeneration. National Academy of Sciences.

Weirui, Guo, Yanbo, Chen, Xiaohong, Zhou, Kar, Amar, Ray, Payal, Xiaoping, Chen, Rao, Elizabeth J., Mengxue, Yang, Haihong, Ye, Li, Zhu, Jianghong, Liu, Meng, Xu, Yanlian, Yang, Chen, Wang, David, Zhang, Bigio, Eileen H., Mesulam, Marsel, Yan, Shen, Qi, Xu & Kazuo, Fushimi (2011). "An ALS-associated mutation affecting TDP-43 enhances protein aggregation, fibril formation and neurotoxicity". *Nature Structural & Molecular Biology,* **18,** 822-830.

Wiedemann, Falk R., Manfredi, Giovanni, Mawrin, Christian, Beal, M. Flint & Schon, Eric A. (2002). "Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients". *J Neurochem,* **80,** 616-625.

Wiggins, A. K., Shen, P. J. & Gundlach, A. L. (2003). "Delayed, but prolonged increases in astrocytic clusterin (ApoJ) mRNA expression following acute cortical spreading depression in the rat: evidence for a role of clusterin in ischemic tolerance". *Brain Res Mol Brain Res,* **114,** 20-30.

Wijesekera, Lokesh C. & Leigh, P. Nigel (2009). "Amyotrophic lateral sclerosis". *Orphanet Journal of Rare Diseases,* **4,** 3-3.

Williamson, Toni L. Cleveland Don W. (1999). "Slowing of axonal transport is a very early event in the toxicity of ALS?linked SOD1 mutants to motor neurons". *Nature Neuroscience,* **2,** 50.

Wilms, H., Sievers, J., Dengler, R., Bufler, J., Deuschl, G. & Lucius, R. (2003). "Intrathecal synthesis of monocyte chemoattractant protein-1 (MCP-1) in amyotrophic lateral sclerosis: further evidence for microglial activation in neurodegeneration". *Journal of Neuroimmunology,* **144,** 139-142.

Wils, Hans, Kleinberger, Gernot, Janssens, Jonathan, Pereson, Sandra, Joris, Geert, Cuijt, Ivy, Smits, Veerle, Ceuterick-De Groote, Chantal, Van Broeckhoven, Christine, Kumar-Singh, Samir & Appel, Stanley 2010. TDP-43 Transgenic Mice Develop Spastic Paralysis and Neuronal Inclusions Characteristic of ALS and Frontotemporal Lobar Degeneration. National Academy of Sciences.

Wilson, Jason M. B., Khabazian, Iraj, Pow, David V., Craig, Ulla K. & Shaw, Christopher A. (2003). "Decrease in glial glutamate transporter variants and excitatory amino acid receptor down-regulation in a murine model of ALS-PDC". *Neuromolecular Medicine,* **3,** 105-118.

Wilson, M. R. , Yerbury, J. & Poon, S. 2008a. Extracellular chaperones and amyloids. *In:* ASEA, A. & BROWN, I. (eds.) *Heat Shock Proteins and the Brain: Implications for Neurodegenerative Diseases and Neuroprotection.*: Springer Science.

Wilson, M. R. & Easterbrook-Smith, S. B. (2000). "Clusterin is a secreted mammalian chaperone". *Trends Biochem Sci,* **25,** 95-98.

Wilson, M. R., Yerbury, J.J. & Poon, S. (2008b). "Potential roles of abundant extracellular chaperones in the control of amyloid formation and toxicity.". *Molecular BioSystems,* **4,** 42-52.

Wilson, M.R. & Easterbrook-Smith, S.B. (1992). "Clusterin binds by a multivalent mechanism to the Fc and Fab regions of IgG.". *Biochimica Et Biophysica Acta,* **1159,** 319-326.

Wilson, Mark R., Yerbury, Justin J. & Poon, Stephen 2007. *Potential roles of abundant extracellular chaperones in the control of amyloid formation and toxicity,* Research Online.

Wolf, Matthew J. & Rockman, Howard A. (2011). "Drosophila, genetic screens, and cardiac function". *Circulation Research,* **109,** 794-806.

Wood, J. D., Beaujeux, T. P. & Shaw, P. J. (2003). "Protein aggregation in motor neurone disorders". *Neuropathology And Applied Neurobiology,* **29,** 529-545.

Wu, Lien-Szu, Cheng, Wei-Cheng, Hou, Shin-Chen, Yan, Yu-Ting, Jiang, Si-Tse & Shen, C. K. James (2010). "TDP-43, a neuro-pathosignature factor, is essential for early mouse embryogenesis". *Genesis (New York, N.Y.: 2000),* **48,** 56-62.

Wu, Yong, Zinchuk, Vadim, Grossenbacher-Zinchuk, Olga & Stefani, Enrico (2012). "Critical evaluation of quantitative colocalization analysis in confocal fluorescence microscopy". *Interdisciplinary Sciences, Computational Life Sciences,* **4,** 27-37.

Wyatt, A.R., Yerbury, J.J., Poon, S. & Wilson, M. R. (2009). "Therapeutic targets in extracellular protein deposition diseases". *Current Medicinal Chemistry,* **16,** 2855-2866.

Wyatt, Amy R., Yerbury, Justin J., Ecroyd, Heath & Wilson, Mark R. 2013. *Extracellular chaperones and proteostasis,* Research Online.

Xu, Ya-Fei, Gendron, Tania F., Zhang, Yong-Jie, Lin, Wen-Lang, D'alton, Simon, Sheng, Hong, Casey, Monica Castanedes, Tong, Jimei, Knight, Joshua, Yu, Xin, Rademakers, Rosa, Boylan, Kevin, Hutton, Mike, Mcgowan, Eileen, Dickson, Dennis W., Lewis, Jada & Petrucelli, Leonard (2010). "Wild-type human TDP-43 expression causes TDP-43 phosphorylation, mitochondrial aggregation, motor deficits, and early mortality in transgenic

mice". *The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience,* **30,** 10851-10859.

Yang, C.R., Leskov, K., Hosley-Eberlein, K., Criswell, T., Pink, J.J., Kinsella, T.J. & Boothman, D.A. (2000). "Nuclear clusterin/XIP8, an x-ray induced Ku70-binding protein that signals cell death.". *Proceedings of the National Academy of Science USA,* **97,** 5907-5912.

Yang, G.C., Nieto, R., Stachura, I. & Gallo, G.R. (1992). "Ultrastructural immunohistochemical localization of polyclonal IgG, C3, and amyloid P component on the congo red-negative amyloid-like fibrils of fibrillary glomerulopathy". *Am J Pathol,* **141,** 409-410.

Yang, W.Y. & Gruebele, M. (2003). "Folding at the speed limit.". *Nature,* **423,** 193-197.

Yerbury, J.J., Kumita, J.R., Meehan, S., Dobson, C. M & Wilson, M. R. (2009). "Alpha 2 macroglobulin and haptoglobin supress amyloid formation by interacting with prefibrillar protien species.". *J Biol Chem,* **284,** 4246-4254.

Yerbury, J.J., Poon, S., Meehan, S., Thompson, B., Kumita, J.R., Dobson, C.M. & Wilson, M.R. (2007). "The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with pre-fibrillar structures.". *The FASEB Journal,* **21,** 2312-2322.

Yerbury, J.J., Rybchyn, M.S., Easterbrook-Smith, S.B., Henriques, C. & Wilson, M.R. (2005a). "The acute phase protein haptoglobin is a mammalian extracellular chaperone with an action similar to clusterin.". *Biochemistry,* **44,** 10914-10925.

Yerbury, J.J., Stewart, E.M., Wyatt, A.R. & Wilson, M.R. (2005b). "Quality control of protein folding in extracellular space.". *European Molecular Biology Organization Reports,* **6,** 1131-1136.

Yerbury, Justin J., Gower, Dane, Vanags, Laura, Roberts, Kate, Lee, Jodi A. & Ecroyd, Heath 2013. The small heat shock proteins αB-crystallin and Hsp27 suppress SOD1 aggregation in vitro. Springer.

Yiwen, Cui, Katsuhisa, Masaki, Ryo, Yamasaki, Shihoko, Imamura, Suzuki, Satoshi O., Shintaro, Hayashi, Shinya, Sato, Yuko, Nagara, Kawamura, Mami F. & Jun-Ichi, Kira (2014). "Extensive dysregulations of oligodendrocytic and astrocytic connexins are associated with disease progression in an amyotrophic lateral sclerosis mouse model". *Journal of Neuroinflammation,* **11,** 1-30.

Yokoseki, A., Shiga, A., Tan, C. F., Tagawa, A., Kaneko, H., Koyama, A., Eguchi, H., Tsujino, A., Ikeuchi, T., Kakita, A., Okamoto, K., Nishizawa, M., Takahashi, H. & Onodera, O. (2008). "TDP-43 mutation in familial amyotrophic lateral sclerosis". *Ann Neurol,* **63,** 538-42.

Yuasa, Yoshihiro, Okabe, Masataka, Yoshikawa, Shingo, Tabuchi, Katsuhiko, Xiong, Wen-Cheng, Hiromi, Yasushi & Okano, Hideyuki (2003). "Drosophila homeodomain protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors". *Development (Cambridge, England),* **130,** 2419-2428.

Zhang, Bin, Tu, Pang-Hsien, Abtahian, Farhad, Trojanowski, John Q. & Virginia, M. Y. Lee 1997. Neurofilaments and Orthograde Transport Are Reduced in Ventral Root Axons of Transgenic Mice That Express Human SOD1 with a G93A Mutation. Rockefeller University Press.

Zhang, Fan, Kumano, Masafumi, Beraldi, Eliana, Fazli, Ladan, Du, Caigan, Moore, Susan, Sorensen, Poul, Zoubeidi, Amina & Gleave, Martin E. (2014). "Clusterin facilitates stress-induced lipidation of LC3 and autophagosome biogenesis to enhance cancer cell survival". *Nat Commun,* **5**.

Zhang, Yong-Jie, Xu, Ya-Fei, Cook, Casey, Gendron, Tania F., Roettges, Paul, Link, Christopher D., Lin, Wen-Lang, Tong, Jimei, Castanedes-Casey, Monica, Ash, Peter, Gass, Jennifer, Rangachari, Vijayaraghavan, Buratti, Emanuele, Baralle, Francisco, Golde, Todd E., Dickson, Dennis W., Petrucelli, Leonard & Snyder, Solomon H. 2009. Aberrant Cleavage of TDP-43 Enhances Aggregation and Cellular Toxicity. National Academy of Sciences.

Zinchuk, Vadim, Zinchuk, Olga & Okada, Teruhiko (2007). "Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena". *Acta Histochemica Et Cytochemica,* **40,** 101-111.

Zinkie, Samantha, Gentil, Benoit J., Minotti, Sandra & Durham, Heather D. 2013. Expression of the protein chaperone, clusterin, in spinal cord cells constitutively and following cellular stress, and upregulation by treatment with Hsp90 inhibitor. Springer.

Zoubeidi, Amina, Chi, Kim & Gleave, Martin (2010). "Targeting the cytoprotective chaperone, clusterin, for treatment of advanced cancer". *Clinical Cancer Research: An Official Journal Of The American Association For Cancer Research,* **16,** 1088-1093.

Zsila, F. (2010). "Chaperone-like activity of the acute-phase component human serum α1-acid glycoprotein: Inhibition of thermal- and chemical-induced aggregation of various proteins.". *Bioorganic & medicinal chemistry letters,* **20,** 1205-1209.

CHAPTER 8: APPENDIX

APPENDIX 1

Male dEAAT1-Gal4 driver line flies were crossed with the following virgin female "gene of interest" flies; TDP-43, clu, TDP;clu and 51D. Once the progeny reached the third instar larval stage they were dissected and immunohistochemistry performed according to the methods outlined in section 2.4.2, with the exception that the primary anti-HA antibody used for the detection of HA-tagged TDP-43 was omitted. This was to test whether any non-specific binding was occurring with the secondary anti-rat Alexa Fluor® 594 antibody. In all four crosses no signal corresponding to Alexa Fluor® 594 fluorescence was detected, indicating that the secondary antibody binds specifically to the primary anti-HA antibody (Figure 8.1).

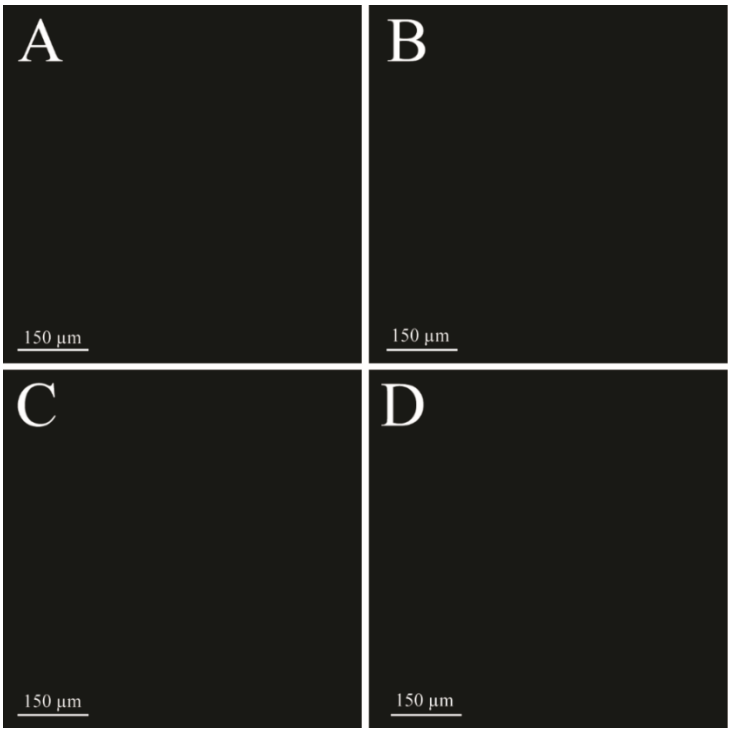

Figure 8.1: Immunofluorescence images of *Drosophila* third instar larva stained with the relevant secondary anti-rat Alexa Fluor® 594 antibodies only. Flies are expressing A) TDP-43, B) co-expressing TDP-43 and clusterin, or C) clusterin in glial cells under control of the dEAAT1-Gal4 driver. TDP-43 (if detectable) would be shown by anti-HA (red) staining. D) Non-transgenic 51D control. Images presented are representative of multiple images obtained from ~2-3 brains

APPENDIX 2

Third instar larvae from uncrossed reporter TDP-43 and TDP;clu lines were dissected and stained for TDP-43 as outlined in section 2.4.2. Immunofluorescence analysis did not detect any specific fluorescence that would indicate expression of TDP-43 in the uncrossed fly lines (Figure 8.2).

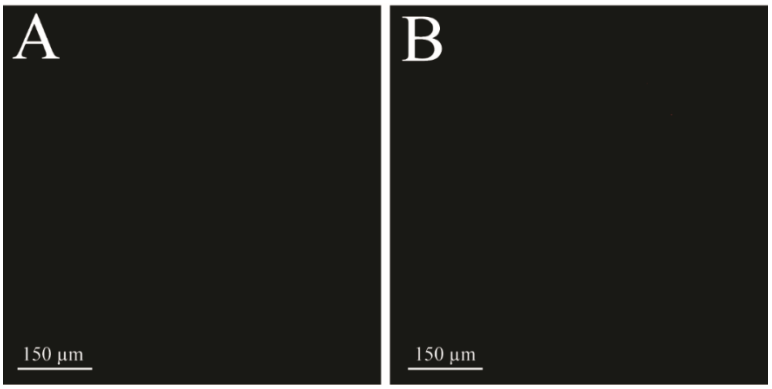

Figure 8.2: Immunofluorescence images of *Drosophila* third instar larva stained for HA-tagged TDP-43. Larvae from uncrossed A) TDP-43 and B) TDP;clu reporter lines were dissected and imaged. TDP-43 (if present) would be shown by anti-HA (red) staining. Images presented are representative of multiple images obtained from ~2-3 brains

APPENDIX 3

Thoracic spinal cord sections from SALS (09/066) patients were treated and stained for clusterin and TDP-43 as described in section 2.4.1, with the exception that the primary antibodies used for the detection of clusterin (goat anti-clusterin) and for TDP-43 (polyclonal rabbit anti-TARDBP) were omitted to test whether any non-specific binding was occurring with any of the secondary antibodies. When both the white and grey matter was examined, no specific fluorescence corresponding to clusterin or TDP-43 was detected, indicating that the secondary antibodies used bound specifically only to the primary antibodies used (Figure 8.3). Gains used were equivalent to those used to obtain Figure 5.5 in Chapter 5.

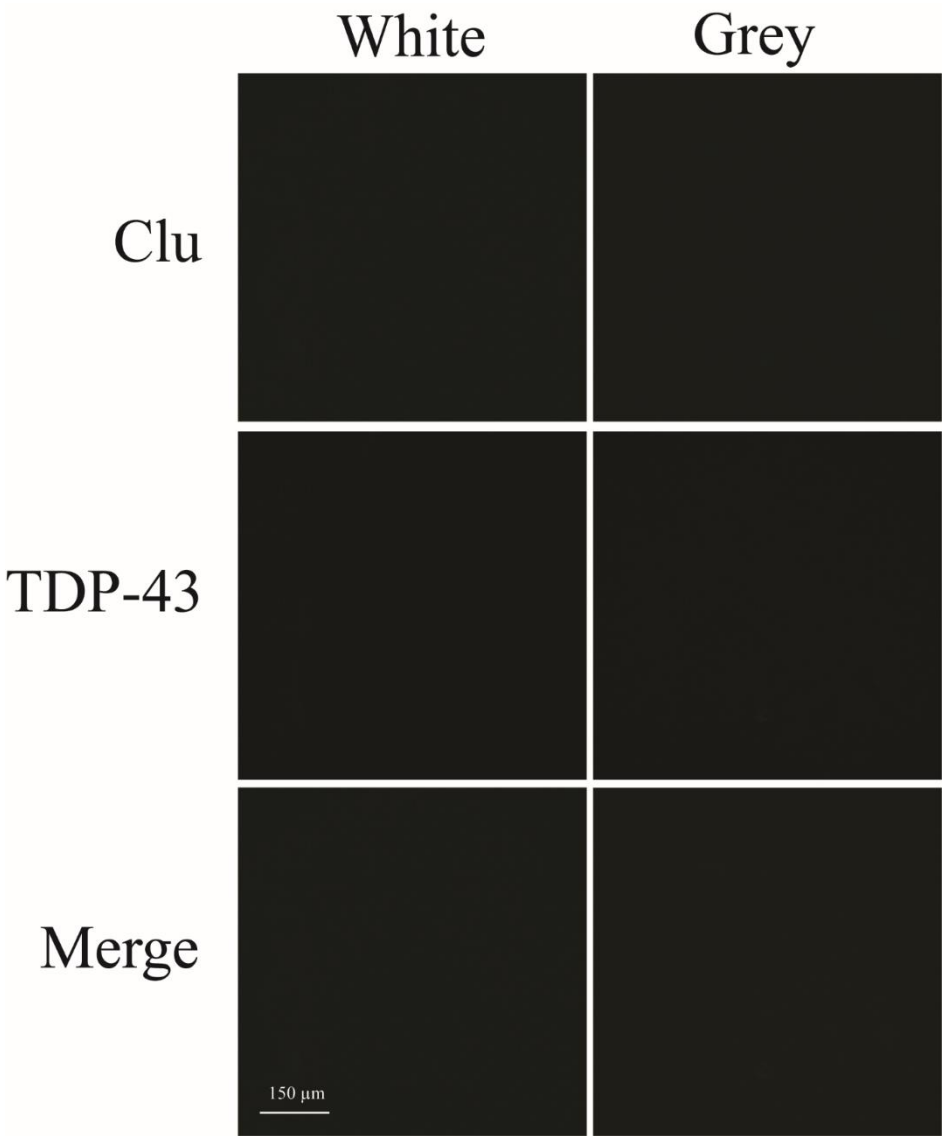

Figure 8.3: The white and grey matter of human thoracic spinal tissue from an ALS (09/066) patient stained as to detect clusterin and TDP-43 but with the omission of primary antibodies. Clusterin would be shown by green staining, and TDP-43 by red (if present).