THE INVESTIGATION OF OXIDATIVE DAMAGE TO NUCLEIC ACIDS DURING AGEING AND MOTOR NEURON DISEASE

by

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disorder, characterised by the degeneration of upper and lower motor neurons. Multiple mechanisms have been associated with ALS pathology, however the precise molecular events leading to selective motor neuron degeneration have yet to be understood. Prominent neuronal RNA oxidation has been reported during ageing and presymptomatic stage of ALS, with specific transcripts selectively modified during disease, which may contribute towards selective cellular degeneration.

Gene expression changes using microarray technology has been widely used to investigate pathways underlying ageing and neurodegenerative disease. The aim of our study was to investigate the gene expression profile of an oxidised fraction of RNA extracted from the anterior spinal cord of normal mice aged six, twelve, and eighteen months. In data presented here, we identify specific classes of genes to be enriched within the oxidised fraction at each age. Furthermore, genes previously linked to the pathogenesis of ALS and normal ageing, such as those involved in RNA processing and transcriptional regulation, are identified as being differentially oxidised in the anterior spinal cord.

The presence and distribution of oxidative damage to nucleic acids within an *in vivo* model of familial ALS and age-matched controls is demonstrated. The predominance of cytoplasmic 8-hydroxyguanosine reactivity within motor neurons supports previous data of RNA susceptibility to oxidative modification in neurodegenerative disease. Investigation of RNA oxidation in an *in vitro* model of fALS harbouring G93A and H48Q human *SOD1* mutations identified prominent levels of RNA oxidation in comparison to controls, which correlated with a reduction in human SOD1 protein expression within these cells. Subsequent work demonstrated the G93A mutation to be the most susceptible to oxidative stress related cellular decline, in terms of mitochondrial bioenergetics, mitochondrial morphology, and cell viability. The heterogeneity of various *SOD1* mutations on cellular function is demonstrated.

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2- oxodADP	2- hydroxydeoxyadenosine diphosphate
2-oxodATP	2- hydroxydeoxyadenosine triphosphate
8-OHdG	8- hydroxydeoxyguanosine
8-OHG	8- hydroxyguanosine
8-oxodADP	8- hydroxydeoxyadenosine diphosphate
8-oxodADP	8- hydroxydeoxyadenosine diphosphate
8-oxodATP	8- hydroxydeoxyadenosine triphosphate
8-oxodGDP	8- hydroxydeoxyguanosine diphosphate
8-oxodGMP	8- hydroxydeoxyguanosine monophosphate
8-oxodGTP	8- hydroxydeoxyguanosine triphosphate
8-oxoG	8- hydroxyguanine
8-oxoGTP	8- hydroxyguanosine triphosphate
AD	Alzheimers disease
Ad	Autosomal dominant
ALS	Amyotrophic lateral sclerosis
ALS-FTD	ALS with fronto-temporal dementia
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	Adenosine monophosphate-activated protein kinase
ANG	Angiogenin
ANOVA	Analysis of variance
APE/Ref-1	Apurinic/apyrimidinic endonuclease/redox factor-1
APP	Amyloid precursor protein
AR	Autosomal recessive
ARE	Antioxidant response element
aRNA	Complementary RNA
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATXN	Ataxin
BCA	Bicinchoninic acid
BER	Base excision repair
BOLD	Blood-oxygen-level-dependent
C.elegans	Caenorhabditis elegans
C9ORF72	Chromosome 9 open reading frame 72
Ca^{2+}	Calcium ion
CBP	CREB-binding protein
cDNA	Complementary DNA
CFAS	Cognitive
CNS	Central nervous system
CR	Calorific restriction
CREB	cAMP responsive element binding protein
Ct	Threshold cycle
DDR	DNA damage response
DMEM	Dulbeccos modified eagles medium
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	Double-stranded break
E.coli	Escherichia coli
EAAT	Excitatory amino acid transporter
ECAR	Extracellular acidification rate

ELP	Elongator protein
ER	Endoplasmic reticulum
fALS	Familial ALS
FC	Fold-change
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
fMRI	Functional magnetic resonance imaging
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
GAPDH	Glyceraldehyde 3-phosphate dehyrogenase
GluR	Glutamate receptor
GSK	Glycogen synthase kinase
GST	Glutathione S-transferase
H2AX	H2A histone family member X
hnRNP	Heterogeneous nuclear ribonucleoprotein
HOX-1	Haem oxygenase-1
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IIS	Insulin/insulin-like growth factor-1 signalling pathway
IMS	Intermembrane space
IP	Immunoprecipitation
IVT	*In vitro* translation
kDa	Kilo dalton
LMN	Lower motor neuron
MAPK	Mitogen activated protein kinase
MCI	Mild cognitive impairment
miRNA	microRNA
Mls	Millilitres
MM	Mismatch
MMR	Mismatch repair
MN	Motor neuron
Mn	Manganese
MND	Motor neuron disease
MRN	Mre11-Rad50-Nbs1
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTH1	Human oxidised purine nucleoside triphosphatase 1
MTH2	Human oxidised purine nucleoside triphosphatase 2
MUTYH	mutY homolog (E. coli)
Na^{2+}	Sodium ion
NAPDH	Reduced Nicotinamide adenine dinucleotide phosphate
ncRNA	Non-coding RNA
NEIL2	Nei endonuclease VIII-like 2
NER	Nucleotide excision repair
NF	Neurofilament
NFL	Neurofilament light
NHEJ	Non-homologous end-joining
NLS	Nuclear localization signal
nM	Nanomolar
NMDA	N-methyl-D-aspartate
NMJ	Neuromuscular junction
NO	Nitric oxide
NQO1	NADPH quinone oxidoreductase 1
NRF2	Nuclear erythroid-2-related factor2

NSC34	Neuroblastoma spinal cord hybrid cells
Nudix	Nucleoside diphosphate linked moiety X
NUDT	Nudix-type motif
NUDT5	Nucleoside diphosphate linked moiety X type 5
OCR	Oxygen consumption
OCT	Optimum cutting temperature
OGG1	Oxoguanine DNA glycosylase
PCI	Phenyl chloroform isoamyl alcohol
PCR	Polymerase chain reaction
PD	Parkinson's disease
PIKK	Phosphoinositide-3-kinase related protein kinase
piRNA	PIWI-interacting RNA
PLS	Primary lateral sclerosis
PM	Perfect match
PMA	Progressive muscular atrophy
PNPase	Polynucleotide phosphorylase
PolyQ	Polyglutamine
PSEN1	Presenilin 1
QPCR	Qualitative polymerase chain reaction
RBP	RNA binding protein
RCF	Relative centrifugal force
RNA	Ribonucleic acid
RNase	Ribonuclease
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revolutions per minute
RRM	RNA recognition motif
rRNA	Ribosomal RNA
sALS	Sporadic ALS
SAPE	Streptavidin phycoerythrin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SETX	Senataxin
siRNA	Small interfering RNA
sitRNA	Stress induced small RNA
SMA	Spinal muscular atrophy
SMN	Survival motor protein
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleoprotein
SOD1	Superoxide dismutase 1
SSB	Single-stranded break
TDP-43	TAR DNA-binding protein 43
TF	Transcription factor
TOR	Target of Rapamycin
tRNA	Transfer RNA
UMN	Upper motor neuron
UPS	Ubiquitin proteasome system
UTR	Untranslated region
v/v	Volume to volume
VAMP	Vesicle associated membrane protein
VAPB	VAMP-associated membrane protein B
VEGF	Vascular endothelial growth factor
w/v	Weight to volume

WNT	Wingless-related integration site
WT	Wild-type
YB-1	Y-box binding protein
µl	Microliter
µM	Micromolar
µm	Micrometer

Chapter 1
Introduction

1.1 Amyotrophic Lateral sclerosis

1.1.2 Clinical Presentation and Diagnostic Criteria

Motor neuron diseases are a series of rapidly progressive adult-onset disorders characterised by the selective degeneration of motor neurons (MNs) within the brainstem, spinal cord, or motor cortex, leading to paralysis of voluntary muscles. The major site of degeneration distinguishes the clinical subtypes of motor neuron disease (MND) (table 1.1), which are classified by clinical signs and symptoms (table 1.2).

Table 1.1: *Clinical Subtypes of Motor Neuron Disease*

Site of degeneration	*Disease classification*
Combined upper and lower motor neuron degeneration	Amyotrophic lateral sclerosis (ALS)
Predominantly lower motor neuron (LMN) degeneration	Progressive muscular atrophy (PMA)
Predominantly upper motor neuron (UMN) degeneration	Primary lateral sclerosis (PLS)
Motor neuron degeneration accompanied by neuronal degeneration in cognitive association areas	ALS with fronto-temporal dementia (ALS-FTD)

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder; with an incidence of 1-2/100,000 people, it is the most common form of MND. MNs are the cells

that form the basic functional unit of the motor system, which is the component of the central nervous system (CNS) responsible for voluntary movement of the musculature. The first description of ALS was by Charcot in 1869, and despite extensive research the aetiology and underlying pathogenesis of this MND remains unclear, as evident by the current lack of effective treatments. The impaired activation and degeneration of MNs, reduction in central motor drive, and atrophy of muscle fibres causes progressive weakness, plasticity, and wasting of the voluntary muscles, leading to progressive paralysis which is presented clinically (Wijesekera and Leigh 2009). Death of patients most frequently results from the failure of the respiratory muscles and diaphragm (Shaw 2005).

The onset of disease may present predominantly with lower motor neuron (LMN) degeneration, upper motor neuron (UMN) degeneration, or bulbar signs (Kirkwood and Austad 2000). Patients clinically present with limb-onset (70%), bulbar onset (25%), or with respiratory involvement (5%) (Ravits and La Spada 2009, Vucic 2007). The clinical presentation, rate of progression, and prognosis, often differ between patients, creating difficulties for diagnosis. These differences are likely due to the selective distribution of damage. The diagnosis of ALS is based on the presence of characteristic clinical features in conjunction with laboratory investigations to exclude overlapping conditions (table 1.2) (Oliveira and Pereira 2009).

Typical characteristics of ALS revealed from post-mortem examination are degeneration and loss of MNs in the anterior horn and in the motor nuclei of cranial nerves VII, X, XI, XII, accompanied by axonal loss in the lateral and anterior corticospinal tracts. There is, however, relative preservation of MNs in the nuclei supplying the extraocular muscles (III, IV, and VI). Corticobulbar and corticospinal tract degeneration is detected at the level of the internal capsule and cerebral peduncles in the midbrain (Brockington*, et al.* 2013, Mannen*, et al.* 1977). As the MNs degenerate they are replaced by gliosis, leading to hardening of the corticospinal tracts (Ravits and La Spada 2009). The progression of clinical features and symptoms in ALS relates to cumulative MN loss within a given region as well as anatomical spread of disease. This propagation of degeneration has given rise to two hypotheses regarding the onset of disease (Kiernan*, et al.* 2011). The "dying-forward" hypothesis proposes that cortical MNs are the primary sites of disease and damage is propagated through the monosynapses formed with LMNs, mediating anterior degeneration of anterior horn cells. Subclinical UMN involvement is invariably found in all forms of MND, and clinical observations that the oculomotor, abducens, and Onuf's nuclei do not synapse with cortical MNs and do not degenerate in ALS provide support for this hypothesis (Browne*, et al.* 2006, Eisen*, et al.* 1992). The "dying-back"

hypothesis proposes that the muscle cells or the neuromuscular junction (NMJ) are the primary site of disease, with damage propagated retrogradely from the distal synaptic and axonal compartments of the neuron, causing upper and lower MN loss. Evidence of synaptic denervation preceding MN degeneration and clinical manifestation of symptoms provides support for this hypothesis (Fischer*, et al.* 2004, Frey*, et al.* 2000).

Table 1.2: *Clinical presentation, clinical tests, and diagnosis criteria*

Signs and symptoms		*Clinical laboratory tests*	*Requirements for diagnosis*
UMN	*LMN*		
• Pseudobulbar features • Spastic Tone • Pathologic tendon reflexes • Pathologic responses	• Atrophy • Fasciculations • Weakness	• Blood count • Urinalysis • Neuromuscular-related tests • Cerebral spinal fluid • Muscle and bone marrow biopsy • Magnetic resonance investigation-brain & spinal cord • DNA analysis	• Evidence of LMN and UMN degeneration on clinical, electrophysiologic, or neuropathologic examination • Absence of any evidence suggesting other explanations for UMN and LMN signs • No neuroimaging evidence of other disease processes

There is no definitive diagnostic test or biomarker for ALS. Riluzole is the sole drug approved for use in ALS, and only moderately prolongs survival (Miller*, et al.* 2007). Understanding the molecular basis of ALS will hopefully lead to the development of diagnostics for improving the classification of different disease subtypes, and contribute effectively towards identifying specific therapeutic targets and treatments for patients (Gonzalez de Aguilar*, et al.* 2007).

1.1.3 Pathogenic mechanisms underlying MND

ALS is a multi-factorial disorder in which combinations of factors interact and contribute towards neuronal viability. The pathogenic mechanisms influencing susceptibility and resistance to neuronal degeneration are discussed in this section; determining how the distinct pathways involved in disease overlap and converge to cause similar phenotypes is pivotal (figure 1.1) (Ravits and La Spada 2009). These are important to consider when developing therapeutics and investigating other mechanisms underlying disease, as these may converge and influence susceptibility. The interaction between these proposed pathogenic mechanisms is complex, and they characterise the progressive neuronal degeneration and muscular paralysis. The majority of ALS cases are sporadic (sALS) but around 10% of cases are familial (fALS) (Andersen 2006). Studies of sALS and fALS patients, accompanied by use of *in vitro* and *in vivo* models, have extended the understanding of ALS pathophysiology. Multiple perturbations in cellular function have been identified in ALS MNs, which will be discussed in the following sections.

The complex nature of the disease allows for many potential targets of therapeutic intervention. Although MNs are selectively vulnerable to degeneration in ALS, other neuronal subgroups are affected in some patients, in particular following a prolonged disease course (Brockington*, et al.* 2013). Selective cellular vulnerability may be characterised by individual predispositions such as genetic background, systemic factors such as vascular lesions, glial reactions and the immune system, advancing age, and the accumulation of toxic species (Saxena and Caroni 2011). The basis of selective vulnerability of MNs remains unclear, which subsequently contributes towards the challenges faced in developing therapeutics.

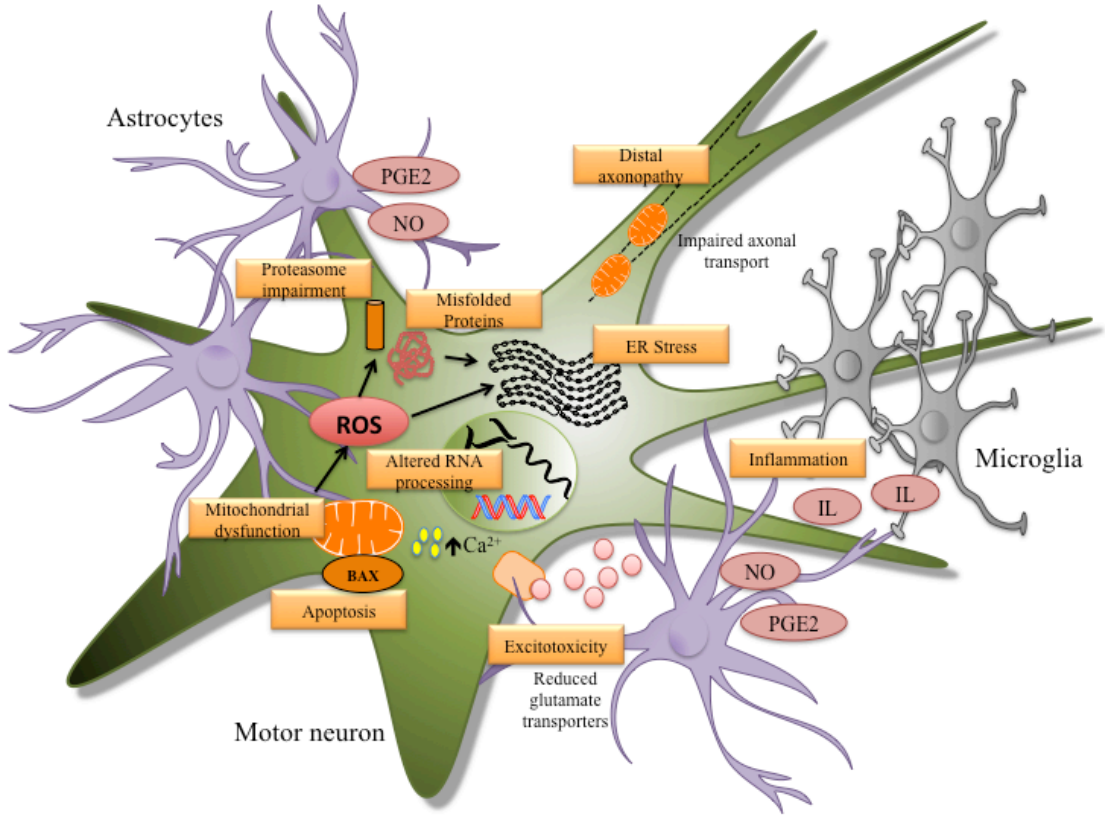


Figure 1.1: Molecular mechanisms of motor neuron injury in ALS

ALS is a complex disorder in which multiple mechanisms interact to cause cellular decline and degeneration. Several cellular pathways are activated in MNs and disease is propagated through interaction with glial cells. Molecular mechanisms contributing towards MN injury include mitochondrial dysfunction and oxidative stress, which along with dysregulated RNA processing and metabolism can reduce transcriptional fidelity leading to the production of aberrant proteins with a tendency to aggregate. Aberrant proteins can lead to endoplasmic reticulum stress and proteasome impairment, along with disruption of multiple metabolic pathways. Mitochondrial impairment and subsequent disruption to calcium homeostasis can cause activation of autophagy and apoptotic pathways, and an energy deficit leads to impaired axonal transport. The release of inflammatory mediators from glial cells and reduced expression of glutamate transporters results in excitotoxicity. Although a number of cases are familial, the pathology of both sporadic and familial disease appears consistent. Abbreviations: ALS, amyotrophic lateral sclerosis; BAX, BCL2-Associated X; Ca^{2+}, Calcium; ER, endoplasmic reticulum; IL, interleukin; NO, nitric oxide; PGE2, prostaglandin E2; ROS, reactive oxygen species (Adapted from Ferraiuolo*, et al.* 2011)

1.1.3.1 Genetic factors

The genetically diverse, rapidly progressive, heterogeneous nature of ALS makes it a difficult disease to study. Familial cases of ALS typically only account for 5-10%, but the clinical presentation of fALS and sALS can be very similar (Traynor*, et al.* 2000). The identification of genes implicated in fALS offers an approach to study common mechanisms underlying pathogenesis (table 1.3). Gene discoveries for juvenile-onset as well as typical ALS, which commences in later life, have been made (Valdmanis*, et al.* 2007). The limitation of working with genetic variants results from the small patient group carrying the mutations, bringing uncertainty to extending the findings in to the ALS population as a whole.

Autosomal dominant fALS was first linked to chromosome 21q22 and subsequently mutations in *Cu/Zn Superoxide Dismutase 1* (*SOD1*) (Rosen*, et al.* 1993). Mutations in *SOD1* account for approximately 2% of all ALS cases (Andersen*, et al.* 2003, Valentine*, et al.* 2005) and approximately 20% of fALS cases (Andersen 2006), though this varies depending on the population sampled. SOD1 is an ubiquitously expressed metalloenzyme that catalyses the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen. More than 150 individual *SOD1* mutations have been reported (ALS Online Genetic Database, ALSOD: http://alsod.iop.kcl.ac.uk/) (Wroe*, et al.* 2008), and the pathogenicity of mutant *SOD1* is thought to be toxic by a mechanism that is independent of its dismutase activity (Boillee*, et al.* 2006). This was determined in mouse studies where inactivation of *SOD1* did not lead to ALS, or alternatively, the transgenic expression of *SOD1* mutants in mice was pathogenic but did not alter enzyme activity. In addition, *SOD1* deficient mice do not develop ALS, and mutations in *SOD1* are not restricted to the active site of the enzyme (Deng*, et al.* 1993, Gurney*, et al.* 1994, Reaume*, et al.* 1996). However, despite these and other studies, the exact mechanisms by which mutant *SOD1* causes selective MN degeneration remains to be established. There is no clear correlation between SOD1 enzymatic activity, clinical progression and disease phenotype (Andersen*, et al.* 1997, Yamanaka and Cleveland 2005). The characterisation of mutations in *SOD1* has led to the development of *in vitro* and *in vivo* models, from which much understanding of the mechanisms involved in ALS pathology have been derived (Bendotti and Carri 2004).

Mice carrying a human mutant *SOD1* transgene develop a fatal neurological disease exhibiting muscle wasting and progressive paralysis, which clinically resembles human

ALS (Gurney*, et al.* 1994, Martin 2007). Histopathological features include aggregation of ubiquitinated proteins in MNs, microglial activation, and selective degeneration of MNs within the spinal cord (Cheroni*, et al.* 2005, J. Lee*, et al.* 2009). *In vivo* models are extremely valuable for investigating the manifestation of disease at different stages, and can be used to decipher mechanisms underlying sALS. However, in order to induce a pathological phenotype the level of mutant *SOD1* expression required is considerably higher than that in patients harbouring the mutation, which needs to be accounted for when using this model to study the pathology of ALS (Bendotti and Carri 2004).

Over 40 mutations in the glycine-rich carboxy terminal of the *TAR-DNA binding protein* (*TARBP*) gene encoding the TAR-DNA binding protein 43 (TDP-43) have been identified, which may account for up to 6% of fALS cases and 0-5% of sALS cases (Kabashi*, et al.* 2008, Mackenzie*, et al.* 2010, Van Deerlin*, et al.* 2008). TDP-43 functions as an RNA/DNA binding protein involved in transcriptional regulation, alternative splicing, and mRNA stabilisation. The discovery of mutations within genes coding for proteins involved in RNA processing provided evidence for defects in RNA processing/metabolism as a pathological mechanism underlying ALS (Sreedharan*, et al.* 2008). Wild-type TDP-43 is predominantly present in the nucleus, whereas pathological TDP-43 displays increased cytoplasmic localisation, inclusion formation, and stress granule association (Neumann*, et al.* 2006). A loss of nuclear function and a gain of cytoplasmic function have both been suggested as pathogenic activities of the mutant protein.

Fused in sarcoma (FUS) also encodes an RNA/DNA binding protein which is found to be mutated in a further 4% of fALS cases and 1% of sALS cases (Kwiatkowski*, et al.* 2009, Vance*, et al.* 2009). FUS is a component of the ribonucleoprotein complexes involved in transcriptional regulation, alternative splicing, and RNA transport. Like TDP-43, FUS is normally located within the nucleus; however, examination of post-mortem tissue from patients carrying the FUS mutations identified abnormal cytoplasmic inclusions within neurons and glia (Kwiatkowski*, et al.* 2009). These inclusions were not immunoreactive for TDP-43, indicating that the neurodegenerative process due to a mutation in *FUS* is independent of TDP-43 mislocalisation (Vance*, et al.* 2009). The precise roles of TDP-43 and FUS have not been elucidated, but they are both structurally related to the heterogeneous ribonucleoprotein family, and have been implicated at multiple levels of RNA processing, including transcriptional regulation, alternative splicing, microRNA (miRNA) processing, RNA subcellular localisation, translation, decay, and overall contributing towards genome stability.

A large hexanucleotide (GGGGCC) repeat expansion in a non-coding region of chromosome 9 open reading frame 72 (*C9ORF72)* was recently identified as the most common cause of familial FTD and ALS to date (DeJesus-Hernandez*, et al.* 2011, Renton*, et al.* 2011). The expansion is found in intron 1 of *C9ORF72* between two non-coding exons, and the minimum size of the pathogenic expansion is undefined. This expansion is present in around 40% of fALS and 7% of sALS cases, making it the most frequent genetic abnormality identified in ALS patients (Mahoney*, et al.* 2012, van Rheenen*, et al.* 2012). Whilst the unaffected control population is found to carry less than 30 repeats, FTD and ALS patients carry very large expansions containing hundreds of repeats. Familial cases of *C9ORF72* ALS are inherited in an autosomal dominant manner; with sporadic cases potentially arising due to the inherent genomic instability at the site of the expanded repeat sequence. Studies have found the repeat to form G-quadruplexes, which are highly stable nucleic acid secondary structures formed through association of short tracts of G-rich sequences (Fratta*, et al.* 2012). G-quadruplexes forming on the template or non-coding strand, could enhance transcription by keeping the template strand single-stranded (Bochman*, et al.* 2012). This suggests a mechanism that is consistent with the identification of nuclear foci containing the repeat in patient tissue (DeJesus-Hernandez*, et al.* 2011). Cases with histopathological correlation show TDP-43 deposition, and ubiquitin positive neuronal cytoplasmic inclusions in FTD-ALS cases linked to the *C9ORF72* expansion. A recent paper from our laboratory (Cooper-Knock*, et al.* 2012) identified a unique pathological signature in *C9ORF72* cases. In a cohort of 563 cases, all the cases with the hexanucleotide repeat expansion of *C9ORF72* displayed the features of a typical variant of ALS. The expansion was absent from most cases with a previously identified mutation, supporting independent pathogenesis. *C9ORF72* cases were distinguished from the rest of the cohort by the extra motor pathology in the frontal cortex and the hippocampal CA4 subfield neurons. Interestingly the inclusions present in CA4 neurons were limited to *C9ORF72* cases only, indicating this pathology is a relatively reliable indicator of mutation status (Cooper-Knock*, et al.* 2012). Despite the recent findings, the pathogenic mechanism whereby the expansion leads to neurodegeneration has yet to be defined.

Other genetic mutations have been identified in ALS cases, with some mutations present in both fALS and sALS cases (table 1.3). Those implicated in sALS cases could represent a novel mutation in the particular gene, or the sporadic forms may harbour mutations that have incomplete penetrance, with the onset of symptoms due to other underlying pathogenic mechanisms. It must also be noted that aberrant epigenetic

regulation/silencing of particular genes essential to MN function and survival could underlie sALS. Greater comprehension of genetic variants and how their pathologies converge will provide insight into prospective new targets for MND. Whole genome sequencing is becoming increasingly useful for identifying rare genetic variants underlying ALS, and identifying how genes potentially interact to produce a MN specific phenotype and how these may propagate other proposed pathogenic mechanisms is of importance when developing therapeutics.

Table 1.3: *Genetic loci associated with MND categorised by clinical phenotype*

ALS Disease Type	*Chromosomal Locus*	*Gene*	*Onset/ Inheritance*	*Action of Protein*	*References*
ALS1	21q22.1	Superoxide dismutase 1 (*SOD1*)	Adult/Ad	Detoxification enzyme	(Rosen, *et al.* 1993)
ALS2	2q33	Alsin (*ALS2*)	Juvenile/AR	GEF signalling, endosomal dynamics	(Hadano, *et al.* 2001)
ALS3	18q21	Unknown	Adult/Ad	-	(Hand, *et al.* 2002)
ALS4	9q34	Senataxin (*SETX*)	Juvenile/Ad	DNA/RNA helicase, RNA processing	(Chen, *et al.* 2004)
ALS5	15q15-21.1	Spatacsin (*SPG11*)	Juvenile/AR	-	(Hentati, *et al.* 1998, Orlacchio, *et al.* 2010)
ALS6	16q12	Fused in Sarcoma (*TLS/FUS*)	Adult/Ad	RNA binding and processing, DNA repair	(Kwiatkowski, *et al.* 2009, Vance, *et al.* 2009)
ALS7	20ptel-p13	Unknown	Adult/Ad	-	(Sapp, *et al.* 2003)
ALS8	20q13.3	VAMP-associated membrane protein B (*VAPB*)	Adult/Ad	Intracellular membrane trafficking, calcium metabolism	(Nishimura, *et al.* 2004)
ALS9	14q11.2	Angiogenin (*ANG*)	Adult/Ad	Neovascularisation, RNA processing	(Greenway, *et al.* 2006, Wu, *et al.* 2007)
ALS10	1p36.2	TAR-DNA binding protein (*TARDBP*)	Adult/Ad	DNA/RNA binding, splicing, transcriptional regulation	(Sreedharan, *et al.* 2008)
ALS11	6q21	Polyphosphoinositide phosphatase (*FIG4*)	Adult/Ad	Endosomal trafficking	(Chow, *et al.* 2009)
ALS12	10p15-p14	Optineurin (*OPTN*)	Adult/Ad&AR	Endosomal trafficking	(Maruyama, *et al.* 2010)
ALSX	Xp11-q12	Ubiquilin 2 (*UBQLN2*)	Adult/x-linked	-	(Deng, *et al.* 2011)
ALS-FTD	9p21-p22	Chromosome 9 open reading frame 72 (*C9ORF72*)	Adult/Ad	-	(DeJesus-Hernandez, *et al.* 2011, Renton, *et al.* 2011)
ALS-FTD	9q13-p12	Valosin-containing protein (*VCP*)	Adult/AD	-	(Johnson, *et al.* 2010)
ALS–FTD	9p13.3	σ _Non-opioid receptor 1 (*SIGMAR1*)	Adult/Ad Juvenile/AR	-	(Luty, *et al.* 2010)
ALS-dementa-PD	17q21	Microtubule-associated protein tau *(MAPT)*	Adult/Ad	Cytoskeletal dynamics	(Hutton, *et al.* 1998, Zarranz, *et al.* 2005)

1.1.3.2 Oxidative Stress

Oxidative stress is a biological phenomenon resulting from an imbalance between the production and detoxification of reactive oxygen species (ROS). ROS can arise as by-products of aerobic metabolism within cells, as a result of the leakage of electrons from the mitochondrial respiratory chain, by cellular oxidative enzymes such as cytochrome P450 and xanthine oxidase, from peroxisomes in the catabolism of long chain fatty acids, and they are released from phagocytes and lymphocytes (Lenaz*, et al.* 1998). ROS have important physiological roles in signalling, and variation of the normal redox state may lead to toxicity and damage (Ray*, et al.* 2012, Shukla*, et al.* 2011). Redox homeostasis is maintained through the antioxidant defence system, which targets and detoxifies or removes ROS, preventing free radical mediated damage (Inoue*, et al.* 2003, Pratico 2008). Cellular biomolecules are continuously modified by ROS, reactive nitrogen species (RNS), and non-radical species arising from environmental exposure and oxidative cellular metabolism. Some ROS mediated post-translational modifications are necessary for cells to function normally including physiological signalling; however, an excess of these molecules results in macromolecular damage. Numerous studies implicate increased intracellular oxidative toxicity in the process of biological ageing, and although oxidative stress is well documented as an early feature in neurodegeneration, the full extent of its interaction and contribution to the underlying pathology of disease remains to be elucidated (Chang*, et al.* 2008, Nunomura*, et al.* 1999, Xu*, et al.* 2011, Ying 1997, Zhang*, et al.* 1999).

The role of oxidative stress in ALS pathogenesis has received particular interest because of the high incidence of mutations in *SOD1,* which encodes a major antioxidant protein, in fALS cases. Widespread oxidative damage in familial and sporadic disease can be correlated to deterioration in neuronal function, and has been associated with many of the other pathophysiological factors that underlie neurodegenerative diseases, including mitochondrial dysfunction, excitotoxicity, and protein aggregation (figure 1.2) (Ferraiuolo*, et al.* 2011). Oxidative damage to proteins, lipids, and nucleic acids can occur during periods of cellular stress, and has been implicated in neurodegenerative pathologies. Elevated markers of free radical damage have been identified in sALS and fALS patients, including protein and lipid oxidation in the brain and spinal cord (Bowling*, et al.* 1993, Mitsumoto*, et al.* 2008, Shaw 2005, Simpson*, et al.* 2004). Increased levels of nucleic acid oxidation have also been detected in sALS cases and transgenic murine models of disease (Chang*, et al.* 2008, Fitzmaurice*, et al.* 1996). In the human mutant *SOD1* transgenic mouse model, oxidative damage to mRNA was

concomitant with reduced expression of encoded proteins. Interestingly oxidative damage of the mutant SOD1 protein is observed, which potentially causes further perturbations in its antioxidant properties and increases the likelihood of its aggregation, thereby sustaining a cycle of oxidative stress (Andrus*, et al.* 1998). The identification of misfolded and oxidised wild-type SOD1 in sALS patients suggests post-translational modifications of SOD1 may cause the protein to acquire toxic properties similar to fALS-linked mutant SOD1, and identifies a common SOD-dependent toxicity between fALS and a subset of sALS (Guareschi*, et al.* 2012).

Oxidative modification of biomolecules can depend upon a number of factors such as the location of ROS production, the relative ability of the molecule to be oxidised, and the availability of metal ions. These modifications may contribute towards disease pathogenesis by altering protein expression/function, disrupting cellular and metabolic processes, and causing a redundancy in the defence and repair pathways elicited by cells to protect against damage (Uttara*, et al.* 2009). A reduction in the functionality of the defence and repair mechanisms protecting cells from oxidative damage accompanies an increase in oxidative stress during ageing and neurodegeneration (Nakabeppu*, et al.* 2004), and will subsequently render cells vulnerable to insult whilst reducing the ability of cells to cope with physiological stress. The intrinsic selectivity of response to stress of neuronal subpopulations may be determined by their connectivity and excitability properties, with several stressors converging to form a vicious cycle driving dysfunction and degeneration.

Antioxidants have been investigated as potential therapeutics against neuronal loss, and some of the antioxidants tested in ALS clinical trials include Vitamin E, N-acetylcysteine, Coenzyme Q10, and Catalase. Vitamin E is the most potent scavenger of reactive oxygen known (Tucker and Townsend 2005). However, two clinical trials involving the oral administration of Vitamin E showed no effect on survival of ALS patients (Ascherio*, et al.* 2005, Desnuelle*, et al.* 2001). N-acetylcysteine when administrated orally replenished pools of glutathione (Burgunder*, et al.* 1989), which is an ROS scavenger. However clinical trials in ALS patients saw no improvement in disease progression or survival (Louwerse*, et al.* 1995). Although it remains unclear whether antioxidant therapies are indeed effective, trials have focused on the treatment of subsets of disease and have trialled individual compounds. Despite the ineffectiveness of these trials, the importance of oxidative stress in disease pathogenesis cannot be ignored, as it integrates with numerous other mechanisms proposed to underlie the pathogenesis of ALS and other

neurodegenerative pathologies (figure 1.2). Combinatorial therapies across a spectrum of patients with ALS may see greater improvements in prognosis.

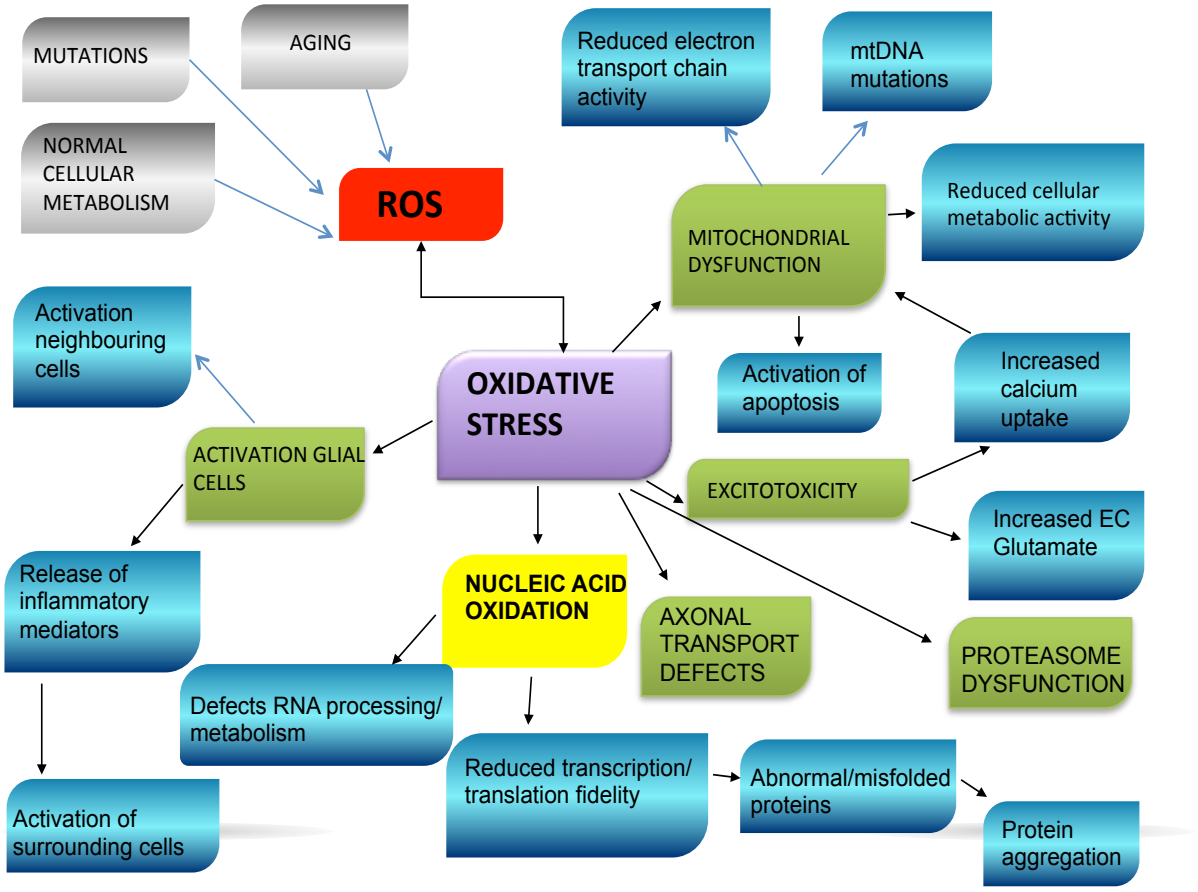


Figure 1.2: The interaction of oxidative stress with the other proposed mechanisms of ALS pathology

Oxidative stress is central to neurodegenerative pathologies and can interact and potentially exacerbate other mechanisms known to contribute towards neurodegeneration. Some of these mechanisms may interact and form vicious cycles of activation and damage. Abbreviations: EC, extracellular; mtDNA, mitochondrial DNA; ROS, reactive oxygen species. .

1.1.3.3 Mitochondrial Dysfunction

Mitochondria are responsible for oxidative phosphorylation and energy production, and have roles in calcium buffering and initiation of apoptosis. Neurons are dependent on a highly efficient electron transport chain to maintain their energy demands, and thus maintaining the functional integrity of mitochondria is essential for neuronal survival.

Evidence implicates oxidative stress and mitochondrial dysfunction in the ageing process and underlying the pathogenesis of neurodegenerative disease. Studies from both ALS patients and transgenic animal models have identified widespread morphological and functional changes to mitochondria including abnormal distribution and fragmentation, a decline in mitochondrial bioenergetic capacity, and increased generation of mitochondrial oxidants, as contributors towards disease pathogenesis (Navarro and Boveris 2007). Accompanying this, a loss of mitochondrial membrane potential, impaired electron chain transport, and a disruption of calcium homeostasis have also been identified during neurodegeneration (Borthwick*, et al.* 1999).

The accumulation of oxidative damage to mitochondrial proteins, lipids, and DNA has been demonstrated in tissues from both ALS patients and transgenic models of disease (Borthwick*, et al.* 1999, Kikuchi*, et al.* 2002, Mattiazzi*, et al.* 2002). This is associated with respiratory chain dysfunction and cellular energy deficits that can lead to paradoxical ROS production and changes at the transcriptional level (figure 1.3). Functional impairment of mitochondria including decreased activity of the electron transport chain have been observed in human patients, and *in vitro* and *in vivo* models of familial ALS-linked mutant *SOD1* (Cashman*, et al.* 1992, Fujita*, et al.* 1996, Mattiazzi*, et al.* 2002, Menzies*, et al.* 2002). Murine models of ALS demonstrate mitochondrial pathology as an early feature of MN injury, with vacuolation of degenerating mitochondria seen in the axons and dendrites of vulnerable neurons at presymptomatic stages of disease (Higgins*, et al.* 2003, Wong*, et al.* 1995). Abnormal mitochondrial distribution, swelling of cristae, and fragmentation of mitochondrial networks have been observed in sALS and fALS patients (Afifi*, et al.* 1966, Curtis 1971, Hirano*, et al.* 1984, Sasaki and Iwata 2007). Mitochondrial localisation of mutant SOD1 has been identified in isolated mitochondria and in MNs *in situ* (Higgins*, et al.* 2002, J. Liu*, et al.* 2004, Sasaki*, et al.* 2004), which consequently may impact the mitochondrial morphology and bioenergetic function. There is a strong argument for mitochondrial abnormalities participating in the disease causing mechanisms of neurodegenerative disorders, supported by an abundance of evidence for their dysfunction in neurodegenerative disease and during the ageing process.

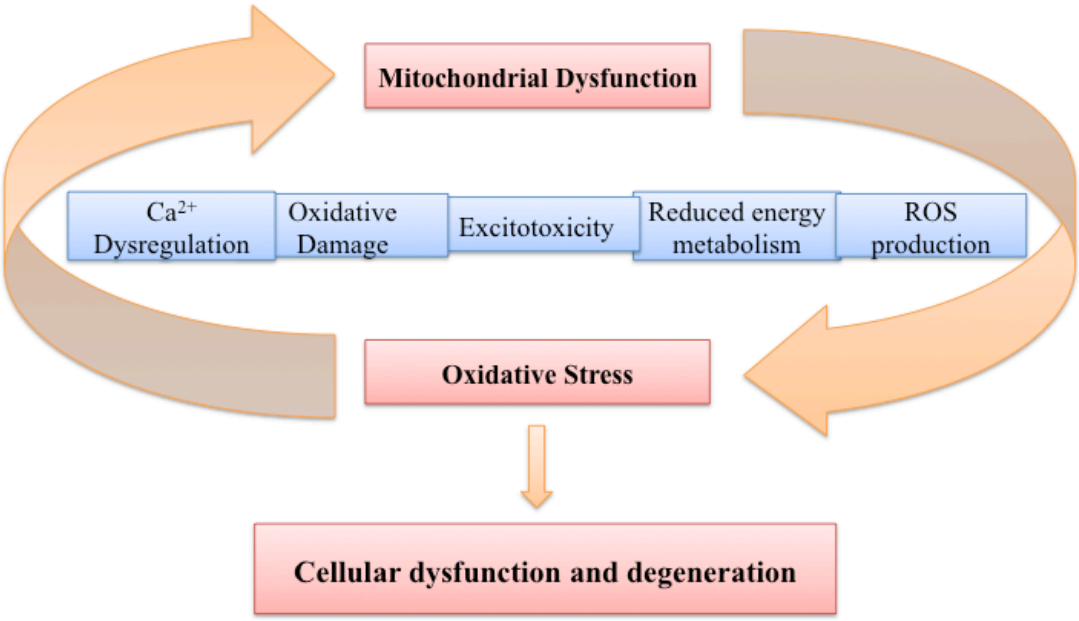


Figure 1.3: Mitochondrial dysfunction and oxidative stress interaction in neurodegeneration

Energy depletion due to mitochondrial defects may underlie the wider dissemination of energy metabolism defects that have been reported in patients and familial animal models of ALS. There is no clear evidence whether mitochondrial dysfunction is a primary or secondary defect in the pathological cascade of ALS.

1.1.3.4 Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. Upon membrane depolarisation, glutamate is released from the presynaptic terminals of neurons into the synaptic cleft where it can interact with the ionotropic and metabotropic receptors on the postsynaptic terminal. Glutamate reuptake transporter proteins, including excitatory amino acid transporters (EAAT), are located on surrounding neurons and perisynaptic astrocytes, and act to terminate the excitatory signal by removing glutamate from the extracellular space (Doble 1999). Excitotoxicity results from an increase in synaptic glutamate concentration in the synaptic cleft and the excessive stimulation of glutamate receptors, due to aberrant production of glutamate or impairment in its reuptake. This, in turn, leads to an excessive influx of calcium and an imbalance in calcium homeostasis. Levels of free intracellular calcium rise, leading to the activation of calcium dependent pathways and mechanisms to restore calcium homeostasis through compartmentalisation, transportation, and sequestration into organelles (Heath and Shaw 2002). Mitochondria are important reserves of calcium, and excessive calcium load

within cells will disrupt overall calcium homeostasis and may result in the diminished ability of mitochondria to sequester calcium (Bezprozvanny 2009). Mitochondrial dysfunction due to disturbances in calcium homeostasis leads to increased ROS production, which increases the likelihood of oxidative stress potentially initiating a vicious cycle leading to neuronal injury and death (Dykens 1994).

Substantial evidence supports excitotoxicity as a pathogenic mechanism of ALS, which either result as a primary defect within neurons, or occur as a secondary process of neurodegeneration. Abnormal editing of the GluR2 AMPA receptor subunit, which alters the calcium permeability of the channel, and low expression of calcium buffering proteins have been reported as contributory factors towards calcium mediated toxicity of MNs (Carriedo*, et al.* 2000, Kwak*, et al.* 2010). Mutant *SOD1* murine models also support a role for excitotoxicity, exhibiting altered electrophysiological properties and AMPA receptor subunit expression, accompanied by defective glutamate metabolism and increased glutamate signalling due to an accumulation of glutamate at the synapse (Meehan*, et al.* 2010, Milanese*, et al.* 2011, Van Damme*, et al.* 2007, Van Damme*, et al.* 2005). An increase in glutamate signalling has been observed in a subset of ALS patients, accompanied by reduced EAAT2 expression and activity in pathologically affected areas of the CNS (Rothstein*, et al.* 1990, Shaw*, et al.* 1995), suggesting a substantial contribution of astrocytes to the excitotoxic environment on MNs. Genetic mutations, aberrant RNA processing, or oxidative damage to EAAT2 protein may contribute towards a reduction in its expression (Bruijn*, et al.* 1997, Munch*, et al.* 2002, Trotti*, et al.* 1999).

Riluzole, the only approved drug for use is ALS, acts to ameliorate excitotoxicity, and provides a modest increase in patient survival (Cheah*, et al.* 2010, Miller*, et al.* 2012). A review of the literature found Riluzole to have a wide range of effects on factors influencing neuronal activity, including inhibition of the persistent sodium current, reduction of neuronal firing, potentiation of calcium-activated potassium currents, presynaptic reduction of neurotransmitter release, and enhanced production of neurotrophic factors through activation of the MAPK signalling pathway (Bellingham 2011).

1.1.3.5 Protein Aggregation

Protein aggregation is a pathological hallmark of numerous neurodegenerative diseases including ALS, and understanding the correlation between protein deposits, causes of

aggregation, neuronal dysfunction and subsequent loss are of interest (Ross and Poirier 2004). There is still debate over whether these aggregates are central to disease pathogenesis, harmless by-products, or potentially beneficial through the sequestration of toxic proteins. Protein aggregation may result as the consequence of a mutation that has changed the primary structure of the protein or resulted in the production of a short polypeptide. Aberrant protein folding or defects in post-translational modifications may result in proteins with a greater tendency to aggregate, and oxidative damage to nucleic acids may lead to the production of faulty proteins due to reduced fidelity of transcription/translation. In ALS, pathological protein aggregates that can be identified by immunoreactivity for ubiquitin and p62 are a fundamental feature of disease (Niwa*, et al.* 2002). TDP-43 has been identified as major constituent of these cytoplasmic aggregates (Neumann*, et al.* 2006). TDP-43 inclusions are not restricted to MNs and the redistribution of TDP-43 to the cytoplasm appears to be an early pathogenic event (Giordana*, et al.* 2010). SOD1 inclusions have been found within MNs of fALS patients and transgenic models of disease (Shibata*, et al.* 1996), and small eosinophilic Bunina bodies containing cystatin C are observed in MNs in the majority of cases (Okamoto*, et al.* 1993, Piao*, et al.* 2003). Some patients with FUS mutations exhibit cytoplasmic inclusions of the protein (Hewitt*, et al.* 2010). These aggregates have also been found to contain proteins such as neurofilament and peripherin (Arai*, et al.* 2006).

Evidence suggests that the protein aggregates formed in neurodegenerative diseases reflect the production of aberrant or misfolded proteins, which under disease circumstances or as a consequence of ageing may not be removed efficiently from cells. Intracellular aggregates may mediate MN degeneration through sequestration of essential cellular components or reduced functionality of defence mechanisms such as the molecular chaperones and the ubiquitin proteasome system (UPS) (Bruening*, et al.* 1999, Bruijn*, et al.* 1998).

1.1.3.6 Axonal Transport Defects

The communication between the cell body and its processes is essential to neuronal function and survival. Axonal transport serves to supply axons and synaptic structures with essential components, such as RNA, proteins, and organelles. Microtubule-dependent kinesin molecular motors are used for transport towards the neuromuscular junction (NMJ) (anterograde), and cytoplasmic dynein molecular motors transport components towards the cell body (retrograde). Impairments in axonal transport have

been proposed as a contributory mechanism towards MN dysfunction in ALS pathogenesis, and have been demonstrated in mouse models of ALS (Zhang*, et al.* 1997). Transgenic mice expressing human mutant *SOD1* manifest axonal defects early in disease (Williamson and Cleveland 1999), and the movement of neurofilaments, vesicles, and mitochondria are affected under these circumstances. Evidence has also shown that mutant SOD1 damage to mitochondria is associated with a reduction in their anterograde transport (Miller and Sheetz 2004), and promotion of their retrograde transport along axons (De Vos*, et al.* 2007). The consequence of the net accumulation of mitochondria within the cell bodies and a reduction in their distribution along the axon may result in a decline in energy production for the molecular motors, further restricting axonal transport. Damage or impairment of molecular motors, mitochondria, or microtubules, as consequences of oxidative damage or other defective cellular mechanisms, may all contribute towards the disruption of axonal transport (Mattson 2000). A recent study demonstrated the susceptibility of axons to exogenous ROS in comparison with nerve cell bodies and dendrites, and suggests increases in ROS generation and inflammation during neurodegenerative disease may preferentially affect axons (Fang*, et al.* 2012, Lucas*, et al.* 2006, Zipp and Aktas 2006).

1.1.3.7 Glial Pathology

Evidence for the involvement of non-neuronal cell populations in the cellular pathology of ALS suggests these are integral to neuronal dysfunction (Ince*, et al.* 2011). An increased understanding of glial cell function in the CNS accompanies the emerging role they have in disease. Glial cells provide structural, metabolic, and trophic support to neurons. They influence neuronal excitability by regulating neurotransmitter activity, integrate and process synaptic activity, and provide support to the blood brain barrier.

Glial cells may directly contribute to the underlying aetiology of ALS and thus the overall phenotype, or owing to their position in the surrounding environment of neuronal cells indirectly impact upon neuronal function and survival in disease states. A decline in functional glia would reduce trophic support for neurons, potentially increasing their vulnerability to extracellular damage and toxic insults, particularly from ROS released by inflammatory mediators (Lucin and Wyss-Coray 2009). Such inflammatory mediators include vasoactive amines, cytokines and nitric oxide, which are released by immune cells and are up-regulated in the brain and spinal cord of ALS patients (Almer*, et al.* 2002). This accompanies the increased activation of astrocytes and microglia, which may

initially result from the release of ROS from damaged MNs (Kawamata, *et al.* 1992). The further release of ROS from the activated glia continues to activate their neighbouring cells (Zhao, *et al.* 2004), and can disrupt the reuptake of glutamate by the surrounding astrocytes (Rao, *et al.* 2003) potentially producing a neurotoxic effect (Banati, *et al.* 1993), and promoting the spread of neuronal damage.

A non-cell autonomous mechanism of ALS is supported by studies of mutant *SOD1* transgenic mouse models, and it is widely accepted that toxicity of mutant *SOD1* is not confined to MNs. Initial attempts to generate disease by specific expression of mutant *SOD1* selectively in MNs or astrocytes failed (Gong, *et al.* 2000, Pramatarova, *et al.* 2001). However, a later study showed transgenic mice expressing G93A mutant *SOD1* specifically in neurons develop an ALS-like disease, characterised by the loss of MNs and muscle denervation (Jaarsma, *et al.* 2008). In this model, the disease developed at late stages and progressed slowly, without reaching the same degree of paralysis relative to those models where the mutant gene was ubiquitously expressed, supporting a non-cell autonomous element during ALS. Accompanying this, chimaeric mice with mixtures of normal and *SOD1* mutant expressing cells, showed that increased expression levels of mutant *SOD1* in MNs is not sufficient for early onset disease, implicating non-neuronal cells in driving disease initiation (Clement, *et al.* 2003, Yamanaka, *et al.* 2008). Pathological analysis has demonstrated astrogliosis as an accompanying factor to neuronal degeneration in the CNS, and cytoplasmic protein aggregates similar to those seen in neurons, are also present within glia in both ALS patients and models of disease (Ince, *et al.* 2011, Miller, *et al.* 2004, Nishihira, *et al.* 2008). In addition, these astrocytic inclusions are early indicators of mutant SOD1 toxicity, precede symptom onset and increase with disease progression (Bruijn, *et al.* 1997). Understanding how the support and regulatory functions of glia are altered during ALS is critical for determining how they might contribute towards the underlying pathogenesis of disease.

1.2 Ageing

Understanding the factors that lead to normal ageing is important, as ageing is associated with processes related to neurodegenerative disease. In addition, our study focuses on whether genes are differentially oxidised during ageing. The factors discussed here include oxidative stress, mitochondrial dysfunction, gene expression changes, RNA processing, and nucleic acid damage, along with how they relate to neurodegenerative disease and have links to our study on RNA oxidation.

1.2.1 The Ageing CNS

Ageing is an inevitable biological consequence which results from a combination of factors. The complexity of these contributory factors means there is no unifying theory to explain ageing, and this could be said to be true for age-associated diseases. Ageing is associated with a reorganisation of brain in structure accompanied by a general decline in cognitive and motor function, and the reduced ability to respond to and overcome physiological stress (Sun*, et al.* 2012). The success of research and the advances in medicine, along with improved socio-economic factors, have brought about an increase in the elderly population. While the achievements made should be recognised, the consequences of a population with greater survival rates must also be addressed, due to the huge economic implication it poses along with the increase in age-associated diseases. Although the processes underlying biological ageing remain controversial and poorly understood, it is generally accepted that at the cellular level ageing is associated with processes related to neurodegenerative disease including oxidative stress, mitochondrial dysfunction, and impaired DNA repair (Sahin and Depinho 2010). Investigation of these factors within the context of disease and their comparison to non-pathological ageing may provide valuable insights into the factors underlying neurodegeneration.

The gradual and progressive structural and functional deterioration of the CNS during ageing is accompanied by accumulated damage to a variety of cellular macromolecules and organelles that is not easily removed. As the molecular mechanisms associated with mammalian ageing become increasingly understood, it is clear there are changes that disrupt the homeostatic balance. The progressive decline in the functionality of defence and repair systems accompanying ageing leads to physiological vulnerability to endogenous and exogenous stresses imposed on tissues and cells, which in response can result in a loss of homeostasis and an overall increase in cellular vulnerability whilst inflicting a constraint on longevity (Kirkwood and Austad 2000). Although evidence points towards a deterioration of homeostatic control and an accumulation of damage during ageing, what actually causes these changes remains perplexing. The classical laboratory model species (such as flies, nematodes, and rodents) have been widely utilised by those seeking to understand the fundamental processes controlling ageing. New mutants and transgenics within these species have produced important insights into the nervous system and ageing itself. Whilst the use of model organisms is advantageous to understanding the processes of ageing, whether these changes reflect what happens under natural selection must be considered.

1.2.1.1 Free Radical Theory

Harman (1956) suggested that the production of free radicals by aerobic respiration leads to an accumulation of oxidative damage, resulting in ageing and culminating in cell death (Harman 1956). He hypothesised that endogenous oxygen radical formation occurs *in vivo*, as a by-product of enzymatic redox chemistry, and iron and other metals would catalyse oxidative reactions *in vivo* through Fenton-type chemistry (figure 1.4). This principle has received much attention in the last 50 years. The free radical/oxidative stress theory of ageing implicates oxidative stress, an imbalance between the production and detoxification of reactive oxygen species/reactive nitrogen species (ROS/RNS), as a prime candidate for causing ageing. It proposes that the cumulative damage to biological macromolecules by these species contributes to the functional decline of neurons and disrupts the support properties of glia (Andersen 2004). ROS and RNS can function as signalling species in many physiological processes through the selective activation/inhibition of other enzymes such as protein kinases and phosphatases (Trachootham*, et al.* 2008). Detrimental effects of ROS and RNS on cellular signalling may result from disruption of their normal physiological levels due to enhanced/reduced production, or as result of the direct attack of these species on biomolecules.

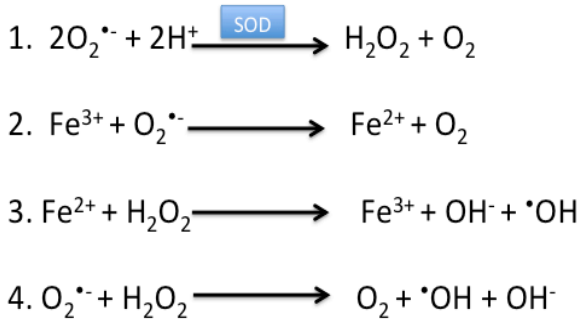


Figure 1.4: The generation of reactive oxygen species

The transfer of electrons to molecular oxygen results in the generation of partially reduced oxygen species. The generation of hydroxyl radical occurs from an interaction between superoxide and hydrogen peroxide (3). This superoxide anion radical can dismutate to water and oxygen, which is catalysed by superoxide dismutase (1). The Haber Weiss reaction generating the hydroxyl radical can be broken down in to two chemical reactions (2&3). The initial reaction catalyses the reduction of ferric ions to ferrous (2), then iron reacts with hydrogen peroxide to produce a highly reactive hydroxyl radical (Fenton Reaction, 3). The net reaction can be simplified (4).

Oxidative damage is identified by the accumulation of markers of oxidative injury such as lipoperoxides, carbonylated proteins, and oxidised nucleic acids. Modification of existing structures such as lipid peroxidation of membranes, and depletion of enzymatic activity and/or functional mitochondria are also indicators of oxidative damage due to increased stress (Finkel and Holbrook 2000). The CNS is particularly vulnerable to oxidative insults by ROS due to the high content of unsaturated fatty acids that are more liable to peroxidation, the abundance of redox-active metals (iron and copper), high oxygen consumption, and a low reserve of antioxidant capacity. Markers of increased oxidative stress have been extensively studied in animal models of ageing, along with oxidative stress-response gene expression, to determine whether ageing is the cumulative result of oxidative damage to cells. Several lines of evidence have been found to support the free radical theory including: (1) metabolic rate and antioxidant activity correlates with species life span; (2) expression of antioxidant enzymes in experimental animals can produce a significant increase in longevity; (3) cellular levels of free radical damage increase with age; and (4) dietary restriction leads to reduced ROS production and an increase in lifespan (Fontana*, et al.* 2010, Johnson*, et al.* 2013, Parkes*, et al.* 1998, Wickens 2001). Although, conflicting results from similar studies make it difficult to determine the actual impact of these on lifespan (Griswold*, et al.* 1993). Accumulation of deleterious effects caused by free radicals and the ability of an organism to cope with damage induced by ROS play an important role in determining organismal lifespan. The bulk of evidence suggests an overall age-associated reduction in the intrinsic ability of cells to degrade damaged products.

Antioxidants are classified as exogenous or endogenous compounds responsible for the removal of free radicals, scavenging ROS or their precursors, inhibiting formation of ROS, and binding metal ions needed for catalysis of ROS generation (Gilgun-Sherki*, et al.* 2001) (details on redox regulation can be found in section 3.1). Antioxidants are sorted in two major groups, enzymatic and non-enzymatic. Enzymatic antioxidants comprise superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase. Non-enzymatic antioxidants, which include ascorbic and lipoic acid, polyphenols and carotenoids, are majority derived from dietary sources (Poulsen*, et al.* 1998, Uttara*, et al.* 2009). Indirectly acting antioxidants include Chelating agents, which are indirect acting non-enzymatic antioxidants that bind to redox metals to prevent free radical generation (Gilgun-Sherki*, et al.* 2001). Given their functions in neutralising ROS and other kinds of free radicals, antioxidants have attracted attention because of their therapeutic potential. However, in general the overexpression of enzymatic antioxidants

and lifelong administration of non-enzymatic antioxidants in model organisms have failed to provide consistent and reproducible lifespan extension (Brewer 2010). One complication is that defence mechanisms/enzymes are induced in response to stress. Interactions between antioxidants are also complex, making it difficult to measure their activity and utilise them for therapies. Despite these challenges, multiple lines of evidence suggest progressive oxidative damage is a conserved central mechanism of age-related functional decline. How the cell responds to oxidative stress may change during ageing, making it particularly complicated to target therapeutically.

1.2.1.2 Mitochondrial Theory

Support for the free radical theory came from an increased understanding of mitochondrial bioenergetics, with evidence from such studies supporting the role of electron transport defects and increased ROS production during ageing and age-associated neurodegenerative diseases. In 1972, Harman extended his original studies to include the involvement of mitochondria in the physiological processes of ageing (Harman 1972). The premise of the mitochondrial free radical theory of ageing is that mitochondria are both producers and targets of ROS. Miquel et al. in the 1980s suggested that oxidative damage to mitochondrial DNA (mtDNA) in post-mitotic cells caused by an accumulation of ROS, would impact the mitochondrial respiratory complexes in terms of energy production and inflict damage upon mtDNA (Miquel*, et al.* 1983, Miquel*, et al.* 1980). Mutations and blocks to replication of the respiratory complex proteins as result of this damage, would consequently lead to mitochondrial dysfunction, further ROS production, and ultimately physiological decline. This vicious cycle of continuous mitochondrial dysfunction and chronic oxidative stress is considered to be one of the causative factors in the ageing process, as a result of insufficient supply of energy and/or increased susceptibility to apoptosis (Judge and Leeuwenburgh 2007). Energy transduction capacity of the mitochondria is essential for maintenance of neuronal function. Redox sensitive signalling between the mitochondria and the rest of the cell establishes a homeostatic balance of the redox environment whilst controlling cellular energy levels (Yin*, et al.* 2012). Impairments to the metabolic network during ageing would negatively influence cytosolic signalling, transcriptional regulation, and general homeostasis.

However, along with the support for this theory of ageing came conflict, also supported by advances in mitochondrial biology. The conserved nature of the respiratory chain

would suggest that the mechanisms for controlling ROS would have evolved to be highly efficient. Studies suggest that increased cellular oxidative metabolism could lead to beneficial effects that would expand lifespan and low levels of ROS are in fact important signalling molecules within many redox regulation pathways (section 1.3.1) (Calabrese*, et al.* 2010, Stranahan and Mattson 2012). The free-radical theory assumes oxygen radicals are generated in direct proportion to oxygen consumption, damaging cellular macromolecules and organelles causing them to lose functionality, however increased oxygen consumption does not inevitably increase mitochondrial radical production (Barja 2007). While it is still plausible that oxidative stress and mitochondrial dysfunction are contributory towards the ageing phenotype there are likely to be other interacting mechanisms that are instrumental in this process. Simply reducing oxidative stress is likely to have minimum impact on lifespan, unless specific gene expression changes are identified as particular targets or stress is targeted in combination.

Studies that link mitochondrial respiration/ATP production and longevity have given conflicting results that are difficult to reconcile in a unifying theory (Bratic and Trifunovic 2010). The mitochondrial theory of ageing extends from the free radical theory and proposes that the free radicals produced during oxidative phosphorylation inflict damage upon mitochondrial macromolecules such as mtDNA, proteins or lipids, and are thus responsible for ageing. Oxidative damage induced mtDNA mutations have been reported to accumulate with age (Ames*, et al.* 1993, Khaidakov*, et al.* 2003, Mecocci*, et al.* 1993). These can significantly impair the assembly and/or the function of the respiratory chain, which will in turn trigger further accumulation of ROS, resulting in a vicious cycle that leads to energy depletion in the cell and ultimately cell death. The abundance of mtDNA also declines with age in various tissues of humans and rodents (Barazzoni*, et al.* 2000). The abundance of mtDNA correlates with the rate of mitochondrial ATP production, suggesting age-related reduction in function due to loss of mtDNA. Whether and how mutations and deletions of mtDNA cause the ageing phenotypes are not clear; however, molecular events leading to altered expression of mtDNA encoded genes or impairment in biogenesis of mitochondria would cause a deficiency in energy metabolism in the affected cells/tissues. In the ageing brain reduced autophagic clearance of dysfunctional mitochondria and increased mtDNA damage may reduce ATP levels and elevate the levels of ROS (Batlevi and La Spada 2011). ROS can further damage cellular macromolecules resulting in reduced transcription, and damage to RNA and protein, leading to protein misfolding and aggregation. Aggregated proteins may accumulate in the brain as consequence of inefficient clearance through the autophagic and ubiquitin-protease pathways.

1.2.1.3 Gene Expression Changes

Microarray technology allows global gene expression analysis in humans and model organisms of ageing and disease, which not only leads to the identification of evolutionarily conserved changes in gene expression, but also those that may accompany neurodegeneration (Bishop*, et al.* 2010, Reuter-Lorenz and Park 2010). Microarray studies are used to detect the expression of specific gene transcripts or determine their activity within an organism, tissue, or specific cell population. It is used to compare gene expression between two or more states. Combining this with bioinformatics approaches allows an unbiased approach to uncover whole-organism wide or tissue specific gene expression profiles and identifies novel cellular pathways that are altered between the groups examined. The transcriptional effects of ageing have been studied in model organisms such as Caenorhabditis elegans (C.elegans) (Lund*, et al.* 2002, McCarroll*, et al.* 2004), Drosophila (Pletcher*, et al.* 2002), mice (Jiang*, et al.* 2001, Lee*, et al.* 2000), and humans (Fraser*, et al.* 2005, Lu*, et al.* 2004). The evidence shows that gene expression changes occur in only a fraction of the genes studied, which indicates specific pathways/classes of genes are targeted and altered during the ageing process. Stress response and DNA repair genes saw a common up-regulation with increasing age across the four organisms, and in the brains of mice and humans protein folding, metal ion homeostasis, and inflammatory or immune response genes were also up-regulated. Examples of some of the processes with genes that were down regulated with age across the organisms include those involved in protein turnover, neuronal plasticity, and energy metabolism. Transcriptional profiling of human prefrontal cortex revealed defined sets of genes with reduced expression over the age of forty. These included genes with fundamental roles in synaptic plasticity, vesicular transport and mitochondrial homeostasis. Gene expression patterns were also relatively homogeneous in both aged populations and young adults (Lu*, et al.* 2004), but varied between people in the middle years. This suggests that age-associated gene expression changes developed earlier in some individuals and the processes occurring at this time may be important in later ageing. This evidence provides support for altered gene expression within the CNS during ageing, and poses the question whether gene expression changes contribute towards the susceptibility of developing neurodegenerative disorders.

When investigating oxidative damage during ageing and age-related neurodegenerative disorders, it is important to consider whether some regions of the genome and particular transcripts are/become more vulnerable to oxidative modification (Yankner*, et al.* 2008). One study found markedly increased 8-oxoguanine levels in the promoters of genes

down-regulated with age (Lu*, et al.* 2004), suggesting promoters driving high levels of transcription may be more vulnerable to oxidative insult, resulting in reduced levels of transcription (Fraser*, et al.* 2005). The importance and consequences of nucleic acid damage, and the gene expression changes it leads to, may be more pronounced in disease states. Differences in transcriptional profiles may result from activation of compensatory or homeostatic genes during ageing to compensate for the ageing phenotype, or a loss of these mechanisms leading to cellular dysfunction and a predisposition to disease. The reduced ability or investment in cellular maintenance and repair are likely to underlie age-associated accumulation of damage and subsequent cellular changes. The up-regulation of transcriptional repressor activity genes and age-dependent methylation patterns suggests transcriptional activity decreases with age (Oberdoerffer and Sinclair 2007). A large number of oxidative stress responsive transcription factors and genes have been identified, and some of these have been implicated in the ageing process. A conserved feature appears to be an increase in the expression of genes involved in stress-response pathways, which may be used as a mechanism by the brain to protect against the pathology of neurodegenerative disorders. ROS may also induce the stress response by altering the expression of respiratory genes to uphold energy metabolism.

Whether the mechanisms of ageing are conserved remains to be established. Molecular genetics have identified mutations that affect longevity and gene expression changes associated with ageing. But whether the genes identified in the laboratory are under selection in natural populations remains controversial. Specific molecular pathways implicated in ageing appear conserved across species, however, the contribution of these to the ageing phenotype remains unresolved, along with identifying specific gene expression changes across studies, which are specific to a given organism or tissue. The pathways altered during ageing have also been implicated in neurological diseases, however, it remains unknown how normal ageing translates into neurodegenerative disease.

One of the remaining problems in the biology of ageing is an understanding of the genetic basis of variation in lifespan among species. This remains difficult as many theories of ageing exist. Ageing may not be dictated by genetics and might arise as a by-product of mutation accumulation or antagonistic pleiotropy; underlying mechanisms that have not been programmed or evolved by natural selection (Ackermann*, et al.* 2007, Partridge and Gems 2006, Rose and Graves 1989). Alternatively, different signalling pathways may act to regulate ageing, with roles in ensuring development and function and consequently fitness (Kenyon 2005, Paaby and Schmidt 2009, Reznick 2005); implicating the

mechanisms of ageing to be shaped by selection on pleiotropic functions that enhance early fitness (Flatt and Schmidt 2009).

1.2.1.4 RNA Processing

The identification of multiple species of non-coding RNAs (ncRNA) accompanied by a more complex understanding of RNA biogenesis has led to a shift in the study of the molecular mechanisms of ageing and neurodegenerative disease towards epigenetics and post-transcriptional regulation. The multiple layers of gene regulatory mechanisms may stabilise or disrupt networks during ageing. A study in C.elegans of the expression of the developmental timing microRNA (miRNA), lin-4, provided the first evidence for a role of miRNAs in ageing (Boehm and Slack 2005). Previously, studies had observed age-associated miRNA expression changes in discrete organisms and tissue but they have not provided a direct link (Ibanez-Ventoso*, et al.* 2006, Lund*, et al.* 2002). Recently, a deep-sequencing study in C.elegans revealed several classes of small ncRNAs that undergo age-related expression changes (Kato*, et al.* 2011). This study identified the majority of miRNA gene expression changes occur during early adulthood, rather than in mid to late stages of lifespan. Small ncRNAs showed significant differential expression with age, which appeared to be caused by aberrant transcriptional activity at miRNA promoters. Uncontrolled transcriptional activation would affect the activities of many target genes, disrupting homeostasis leading to cellular decline. Another deep-sequencing investigation identified numerous differentially expressed miRNAs during ageing in the mouse brain (Inukai*, et al.* 2012), providing further support for these gene expression changes and altered regulation during ageing. A global downward trend of miRNA expression was observed, which was in agreement with previous studies (de Lencastre*, et al.* 2010, Noren Hooten*, et al.* 2010).

Gene expression profiles can also be altered through ncRNA instability, impacting genome stability, and affecting post-transcriptional processing, leading to functional decline. This may be determined by a few key miRNAs and/or is tissue-specific during ageing (Bates*, et al.* 2010). Other types of ncRNA are also likely to be involved in the ageing process; however only a few tissues have been analysed, and this mostly has been limited to miRNA changes (Lukiw 2007). RNA surveillance genes including those associated with RNA editing and RNA interference pathways have been implicated in human longevity (Sebastiani*, et al.* 2009), suggesting unresolved complexity in the mechanisms underlying the ageing phenotype. Gene expression changes are accompanied

by changes in the regulation of RNA processing and metabolism. Deciphering how dysfunction of gene regulatory networks is associated with changes in gene expression and vice versa remains to be established.

1.2.1.5 Nucleic Acid Damage

Evidence in the literature identifies an accumulation of nucleic acid damage in the form of base modifications including oxidation, single-stranded breaks (SSBs), and double stranded breaks (DSBs), during the ageing process and in various neurodegenerative disorders (Coppede and Migliore 2010, Fishel*, et al.* 2007, Halliwell 2006, Weissman*, et al.* 2007b). Oxidative modifications to DNA and RNA can either occur through direct modification of the bases or base damage within the nucleotide pool. The most abundant and characterised oxidised bases are 8-hydroxydeoxyguanosine and 8-hydroxyguanosine for DNA and RNA respectively. Guanine has the lowest oxidation potential compared to adenine, thymine/uracil, and cytosine; consequently, it is more readily oxidised (section 1.6) (Fiala*, et al.* 1989, Wamer*, et al.* 1997).

Oxidative damage to DNA and RNA has been shown to increase in the human brain during ageing (Kregel and Zhang 2007, Nunomura*, et al.* 2012), and in the case of DNA, oxidative damage to both nuclear and mitochondrial DNA is significantly increased in all major tissues in aged organisms, including rodents and humans, suggesting that this is a widespread phenomenon and not tissue specific (Gianni*, et al.* 2004, Hamilton*, et al.* 2001, Takabayashi*, et al.* 2004). In long-lived post-mitotic neurons, the removal of oxidative lesions is crucial for maintaining genomic stability. Transcription coupled repair, a specialised sub-pathway of NER, removes DNA lesions from the transcribed strand of active genes within neurons (Bohr*, et al.* 1985, Mellon*, et al.* 1987). If DNA remains unrepaired within neurons it leads to an accumulation of lesions, which may interfere with DNA-dependent processes, affecting the fidelity of the information transferred during transcription and translation (McMurray 2005). The accumulation of damage to DNA as a mechanism of neurotoxicity has been demonstrated (Chen*, et al.* 2007), and DNA damage was found to be increased in the promoters of genes whose expression were reduced in ageing (Lu*, et al.* 2004). Impairments or redundancy of the transcriptional machinery and/or DNA damage responses during ageing and neurodegenerative disease may lead to an increased accumulation of damage resulting in genome instability. Evidence suggests that neurodegeneration is linked with aberrant neuronal cell re-entry into the cell cycle, which could lead to uncontrolled cell growth or,

more typically, induction of cell death and neurodegeneration (figure 1.5) (Liu and Greene 2001, Wartiovaara, *et al.* 2002). Cell cycle protein expression and activation has been identified in the dying neurons of patients with neurodegenerative disorders, supporting DNA damage initiated apoptosis in disease (Herrup and Busser 1995, Husseman, *et al.* 2000, Yang, *et al.* 2001).

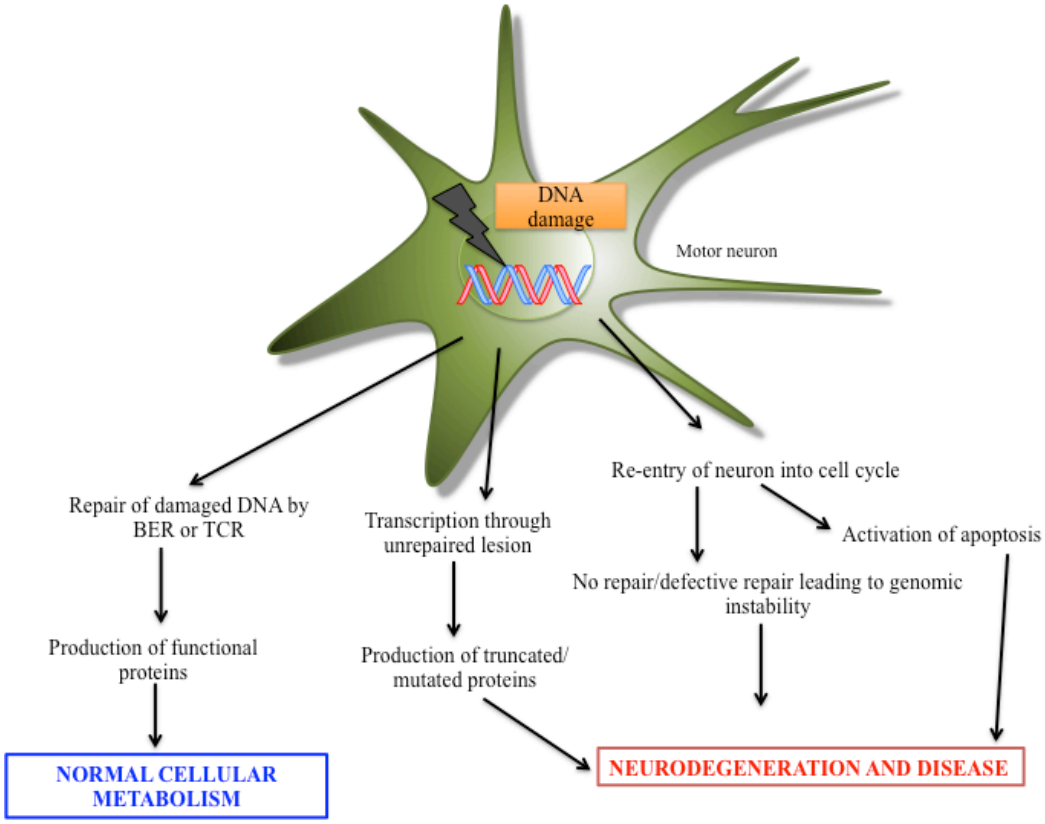


Figure 1.5: The consequences of DNA damage in post-mitotic neurons

Activation of the DNA damage response following insult would lead to initiation of DNA repair, resulting in the production of functional proteins and maintenance of cellular homeostasis. Transcription through the unrepaired lesion may occur if the transcription machinery fails to recognise a defect, which could lead to the production of aberrant proteins that have a tendency to aggregate. Attempted entry into the cell cycle has also been identified following DNA damage, which can either activate apoptotic pathways or lead to a redundancy in repair. A reduction in the level of functional proteins would lead to cellular decline, and potentially neurodegeneration.

Evidence for an increase in oxidative damage to RNA has also been documented in ageing brain (Liu, *et al.* 2002, Nunomura, *et al.* 2012). The effective removal of oxidised bases from RNA remains to be established, suggesting sub-lethal insults may be a major contributory factor to the ageing phenotype, as a reduction in RNA biogenesis and

fidelity would considerably impact metabolic processes. However, RNA quality control mechanisms can target aberrant RNAs for degradation. Oxidised RNA may simply be targeted for removal rather than repaired. RNA half-life varies for housekeeping and regulatory genes (Rabani, *et al.* 2011, Schwanhausser, *et al.* 2011, Sharova, *et al.* 2009, Yang, *et al.* 2003), and the rapid turnover of some transcripts may indicate that degradation of aberrant transcripts is more efficient than repair. This may be problematic if the machinery for surveillance and degradation is damaged. With the increasing understanding of RNA processing and metabolism, and as the repertoire of small RNAs grows, greater understanding of how damage to RNA may underlie the ageing phenotype should result.

1.2.2 Other contributory factors

1.2.2.1 Signalling Pathways

An alternative way of investigating genetic changes underlying and contributing towards the ageing phenotype is to study the signalling pathways and transcription factors (TF) that have been shown to influence longevity. Dietary calorific restriction is one of the best-known physiological mechanisms seen to extend lifespan in many species from yeast to primates (Colman, *et al.* 2009). Initially the processes proposed to be responsible for the anti-ageing effects of dietary restriction included a reduction in ROS production by the mitochondria. This would effectively reduce oxidative stress and subsequent damage to biomolecules. Another mechanism is hormesis, a process by which exposure to a sub-lethal level of stress increases the resistance of cells and tissues to a subsequently higher and otherwise lethal level of the same stress (Barja 2004, Mattson, *et al.* 2002). It is now recognised that although these may still have an influence, the longevity response is principally regulated by nutrient sensing pathways including rapamycin (TOR), adenosine monophosphate-activated protein kinase (AMPK), sirtuins and insulin/insulin-like growth factor (IGF-1) signalling (Greer and Brunet 2009, Greer, *et al.* 2007, Honjoh, *et al.* 2009, Rogina and Helfand 2004). Lifespan extension results from gene expression changes, with the TFs affected up-regulating or down-regulating diverse genes that cumulatively produce significant effects on lifespan. For example the inhibition of the TOR pathway or mutations that inhibit the IGF-1 signalling pathway, or its upstream regulators and downstream effectors, can extend lifespan through gene expression changes (Harrison, *et al.* 2009, Tullet, *et al.* 2008). In addition sirtuins are NAD-

dependent protein deacetylases whose overexpression also results in extended lifespan (Kenyon 2005). Studies demonstrate that these pathways coordinately regulate each other along with a variety of stress response pathways, which are proposed to participate in ageing (Sengupta*, et al.* 2010).

1.2.2.2 Insulin/insulin-like growth factor-1 signalling

The insulin/insulin-like growth factor-1 (IGF1) signalling pathway (IIS) is an evolutionary conserved pathway reported to be a key determinant of lifespan (Kaletsky and Murphy 2010, van Heemst, et al. 2005). The insulin receptor is expressed throughout the brain and signalling through IGF1 receptor functions as a nutrient sensor and controls the transcription of stress response genes. Information about the importance of the IIS pathway in ageing has come from genetic studies in nematodes, fruit flies, and rodents. It was the first pathway to be associated with lifespan, with mutations in several genes involved in the pathway shown to influence longevity (Kenyon 2005). Targeted deletion of specific genes has demonstrated that multiple components of the IIS pathway play a role in the ageing process (Bartke 2008). Mutations in *daf-2*, an insulin receptor ortholog in C.elegans, substantially extended lifespan, and mutation of *Chico*, an insulin receptor substrate which functions in an insulin-like growth factor pathway in Drosophila, extended lifespan significantly in both homozygotes and heterozygotes (Clancy*, et al.* 2001, Kenyon*, et al.* 1993, Kimura*, et al.* 1997, Tatar*, et al.* 2001). The long-lived mutants share some phenotypic characteristics including enhanced sensitivity to insulin, and reduced insulin signalling and IGF1 plasma levels coupled to reduced sensitivity to this growth factor (Piriz*, et al.* 2011). Although evidence supports the therapeutic advantage of IGF1 administration in model organisms of ageing and AD (Carro*, et al.* 2006, Carro*, et al.* 2002), contradictory reports suggest inhibition of IGF1/insulin signalling could be equally beneficial against AD pathology in animal models (Cohen*, et al.* 2009, Killick*, et al.* 2009), highlighting the complexity of these changes in ageing and disease. Differences in lifespan are seen between different species, which could have arisen due to changes in regulatory genes, adding further complexity. Further evidence also supports the notion that the beneficial effects of calorific restriction (CR) are mediated in part by the IIS pathway (Bishop and Guarente 2007, Greer and Brunet 2009), as CR does not extend the already long lifespans of mice with mutations in the gene for the growth hormone receptor (Arum*, et al.* 2009). However, a recent study in primates questions the robustness and nature of restricting calorie intake in studies relating to

lifespan, as no significant difference in lifespan was seen between rhesus monkeys given a normal diet and those with a 30% CR (Mattison*, et al.* 2012).

1.2.2.3 Inflammation

Inflammation is a localised response activating a complex network of molecular and cellular interactions to restore physiological homeostasis. The inflammation hypothesis of ageing describes a state of chronic, low-level inflammation that potentially is a convergent process linking ageing to neuropathological disease (Chung*, et al.* 2001). Disturbances in immune response and altered redox homeostasis during ageing support this hypothesis, in which both may lead to increased activation of inflammatory mediators. Inflammation is fundamentally a protective response, however the increased release of inflammatory mediators can cause direct damage to the surrounding cells. Microglia are the resident immune cells of the CNS, they constitutively express surface receptors and following cellular damage respond by inducing a protective immune response. This consists of a transient up regulation of inflammatory molecules such as cytokines and chemokines, along with neurotrophic factors. However, activated microglia are the most abundant source of free radicals in the brain and release radicals such as superoxide and nitric oxide. Microglia-derived radicals, as well as their reaction products hydrogen peroxide and peroxynitrite, can inflict damage on cells. With the ageing brain more susceptible to events associated with neuroinflammatory processes, this could provide an explanation for a source of toxins that account for damage and degeneration of neurons (Floyd 1999). The proinflammatory phenotype of astrocytes in the ageing brain despite having a neuroprotective response can also have several detrimental effects. Increased cytokine secretion can activate inflammatory neurodegeneration by triggering oxidative stress involving nitric oxide-provoked pathways (Brown and Bal-Price 2003). It remains to be established whether inflammation is a key pathogenic feature of neurodegenerative disease and contributes towards the ageing phenotype, or whether its detrimental effects result from increased activation of mediators trying to maintain cellular homeostasis resulting from perturbations of other processes.

1.2.2.4 Replicative Senescence

Cellular senescence is a growth arrest programme that limits the lifespan of proliferative mammalian cells, typically driven by a persistent DNA damage response (DDR)

(Campisi and d'Adda di Fagagna 2007). Replicative senescence is associated with changes in gene expression, nuclear structure, protein processing, and metabolism, but cells remain metabolically viable (Ben-Porath and Weinberg 2004, Itahana*, et al.* 2004). Senescent cells activate downstream signalling pathways, resulting in the induction of synthesis and release of ROS and pro-inflammatory cytokines and chemokines (Coppe*, et al.* 2008, Passos*, et al.* 2010). The cell hypothesis of ageing proposes that the progressive accumulation of senescent cells has a causal role in ageing and age-related pathology through their impact on their surrounding environment (Wang*, et al.* 2009). Studies report an increase in the expression of senescent markers in the brain during ageing, particularly in astrocytes, and suggest senescent cells induce a bystander effect that propagates DNA damage (Nelson*, et al.* 2012). Post-mitotic neurons are generally not considered in studies on senescence, as they do not proliferate. Despite this, neurons accumulate DNA damage during ageing and some of their phenotypic changes may be a result of this. A recent study in ageing mice reports DNA damage to be interconnected with other markers of the senescent phenotype in Purkinje cells (Jurk*, et al.* 2012), with neurons displaying pro-oxidant and pro-inflammatory characteristics. The data questions the conventional view of cell cycle arrest as a defining feature of the transition of a cell in to a state of senescence, and suggests senescence induced changes may be due to signalling pathways downstream of the DDR.

1.2.3 Effects of ageing on nervous system function

1.2.3.1 Cognitive Decline

Cognitive ageing can be described as a pattern of age-related impairment in cognitive functions. The aim of many cognitive ageing studies is to investigate 'normal' or non-pathological ageing, which rarely extends beyond the categorical exclusion of specific medical conditions known to impair cognition (for example dementia or stroke). This makes it difficult to separate the effects of normal ageing from the effects of age-associated diseases, which may have long, progressive preclinical histories (Hedden and Gabrieli 2004). Human cognitive studies have been considerably enhanced through the availability of neuroimaging technology. Functional imaging studies have given an insight in to neural activity and how this can change depending on the physiological circumstances (Bishop*, et al.* 2010, Reuter-Lorenz and Lustig 2005). There is the consensus that the blood-oxygen-level-dependent (BOLD) signal obtained from functional magnetic resonance imaging (fMRI) is a reasonable, although indirect, index

of neural activity, especially the synaptic activity reflected in local field potentials (Mukamel*, et al.* 2005). Neuroimaging can be combined with behavioural and genetic approaches to investigate the differences that underlie successful ageing. Age-related differences are seen in tasks involving working memory, attention, and task switching, with older adults generally displaying slower processing speeds (Madden 1990, McCabe*, et al.* 2010, Salthouse 1996). However, some aspects of cognition are maintained with age (Deary*, et al.* 2009), and this variability indicates how ageing has distinctive effects on the neural systems and the changes are not predetermined.

Challenges faced when exploring age-related differences in cognition include the difficulty in separating the effects of normal ageing from those of pathological processes that compromise cognition. Most adults experience some form of age-related neural pathology and brain functions may be perturbed by undetected neuropathological changes, and ageing is associated with a strong risk for AD, PD, diabetes, hypertension, and arteriosclerosis (Bowling and Beal 1995). Cross sectional comparisons between age-groups is often the basis for ageing studies, although these are time and money efficient and have contributed most of what we know to date about ageing of the brain, they are vulnerable to cohort effects. From the extensive literature on non-pathological brain ageing it is clear that ageing is influenced by a large number of factors that vary from individual to individual. A limitation of these studies is that inferences about age-related changes in cognition depend on how the changes are measured and the sample population.

Mild cognitive impairment (MCI) is a known transitional stage between normal ageing and dementia in which a patient has memory difficulties and poor performance on memory tasks but does not meet the diagnostic criteria for AD. Individuals with MCI display an increase in neurofibrillary tangles in the temporal lobes, which is correlated with their poorer memory performance. The precise neuropathological relationship between the biological changes of cognitive ageing and those of AD remains uncertain. Population based neuropathological studies such as the MRC Cognitive Ageing Study (CFAS) reveal a spectrum of neuropathological changes in ageing brains. CFAS studies have highlighted the coexistence of different pathologies across age-associated neurological disorders. The overlap in pathology between demented and non-demented individuals is such that thresholds of pathology for dementia are difficult to be established, and the overlap in pathology appears to increase with age (Lace*, et al.* 2009, Matthews*, et al.* 2009, Simpson*, et al.* 2010). Studies of age/disease related changes

carried out post-mortem makes identifying the sequence of events leading to pathological changes difficult as it only provides a fragment of information.

1.2.3.2 Structural brain changes

A reduction of brain volume accompanying ageing has been identified through post-mortem and *in vivo* animal studies. Originally this atrophy was thought to be the consequence of neuronal loss, however further investigations revealed only a slight reduction in total cell number during ageing (Esiri 2007, Raz and Rodrigue 2006). Atrophy appears not to result from cell death but rather is the result of cell shrinkage, dendritic regression, and reduced synaptic densities in older adults (Resnick*, et al.* 2003, Terry 2000). A significant reduction in synapses has been demonstrated in the prefrontal cortex during ageing, and synaptic loss is the best pathological correlate of dementia (Honer 2003, Peters*, et al.* 2008, Terry*, et al.* 1991). Regional changes in brain volume however are not uniform. Structural imaging techniques show age-related differences in the reduction of grey and white matter structures in the brain (Kaup*, et al.* 2011), with volume loss displayed in the prefrontal cortex and sub-regions of the hippocampus during human ageing (Rajah*, et al.* 2011). Functional connectivity can provide information as to how activity within a network of brain regions is correlated, or how activity in a particular brain area is correlated with the rest of the brain, and assessing the integrated activity among groups of brain regions may be used as a way of defining functional decline in brain networks with age. Indeed imaging studies have identified differences in activation patterns with advancing age, accompanied by a global loss of integrative function (Fling*, et al.* 2011).

It is also important to consider how brain activity is related to other aspects of brain ageing, such as changes in structure or neurotransmitters. Functional MRI studies aid this by providing evidence of age differences in task related brain activity (Eyler*, et al.* 2011, Spreng and Grady 2010). To what extent individual variability in behavioural, genetic, and neurobiological markers of cognitive ageing reflects normal and pathological ageing remains to be understood. Future studies should focus on variability in older populations rather than merely differences between age groups.

1.2.4 Relation to neurodegeneration

Intensive research on brain ageing has been partly driven by the effect it has on enhanced susceptibility to cardiovascular disease, cancer, and neurodegenerative disorders. Cellular and molecular changes induced by ageing are likely to interact with genes and environmental factors to influence which cells age successfully and which succumb to degeneration. It remains unclear how selective neuronal vulnerability arises, resulting in distinct patterns of neurodegeneration in different diseases (Hindle 2010). At the cellular level ageing is associated with processes additionally related to neurodegenerative disease including oxidative stress, mitochondrial dysfunction, and impaired DNA repair (Sahin and Depinho 2010). Investigation of these factors within the context of disease and their comparison to non-pathological ageing may provide valuable insights into the factors underlying neurodegeneration. One clear difference is that the number of neurons lost in normal ageing is substantially lower than those lost as a result of neurodegenerative disease, suggesting that although the mechanisms for neuronal deterioration may be similar, there are other influencing factors which differentiate between non-pathological ageing and neurodegenerative disease.

Oxidative stress is a prominent feature of neurodegenerative diseases, and is of considerable interest in brain ageing owing to the association of oxidative damage with many age-related diseases, and because it provides a plausible mechanism for cellular decline and degeneration. Several questions relating to the processes of normal ageing can be queried; does oxidative stress and subsequent damage increase during brain ageing, what are the underlying processes causing the oxidative stress changes observed with age, and are there potential therapeutic strategies to alter or inhibit these changes? Neurons are highly energetic which leads to increased oxygen consumption and a reliance upon efficient mitochondria for proper functioning. Mutations implicated in PD have directly linked mitochondrial dysfunction to disease, and pathological studies have demonstrated increased ROS in affected brain tissues of patients with neurodegenerative diseases (Andersen 2004, Filosto*, et al.* 2011). Studies have also shown activities of Cu/Zn SOD1, catalase, glutathione peroxidase, and glutathione reductase are reduced in the affected brain regions of AD patients (Pappolla*, et al.* 1992, Zemlan*, et al.* 1989). Interactions between oxidative stress and other molecular mechanisms involved in the process of neurodegeneration, such as protein misfolding, proteasomal malfunction, glial cell activation, and mitochondrial dysfunction have also been implicated in the ageing process.

Signalling pathways that play a part in regulating ageing and specifically lifespan have also been implicated in the development of age-related pathologies that themselves may be influenced by changes to the molecular mechanisms of ageing (Bishop*, et al.* 2010). The IIS pathway functions as a nutrient sensor and modulates cellular stress. Reduced IIS specificity has been shown to extend lifespan in model organisms of ageing (Broughton and Partridge 2009). In mammals, the levels of IGF1 are reduced with age, and administration of IGF1 has shown widely neuroprotective responses and ameliorates neurodegenerative disease in animal models (Sonntag*, et al.* 2000). However, recent observations suggest that insulin can be neuroprotective against oxidative stress, and insulin resistance impairs memory inhibition and may contribute towards amyloid neurodegeneration underlying AD (Cholerton*, et al.* 2011, Duarte*, et al.* 2008). This highlights the potential differences in signalling pathways in normal ageing of the brain compared to neurodegenerative disease, and how insulin signalling may have opposing effects on different aspects of disease aetiology (Niccoli and Partridge 2012).

Particular types of gene expression changes might increase/reduce the susceptibility of an ageing brain to neurodegeneration and neurological disorders. The molecular signatures of ageing may reflect transcriptional responses to ageing of healthy cells and their adaptation to degenerative processes (de Magalhaes*, et al.* 2009), and the influence of other interacting factors may predispose an individual to the development of an age-related neurodegenerative disorder.

Increasing evidence demonstrates the impact of aberrant of RNA processing and metabolism in neurodegeneration. Integrating ageing, maintenance of the brain, and susceptibility to neurological disorders is necessary to understand the connection between them. A study in Drosophila recently provided a molecular link between ageing and neurodegeneration, via the conserved miRNA miR-34. Liu et al. reported that a loss of miR-34 induced age-associated gene expression changes characteristic of accelerated brain ageing, and flies displayed defects in protein misfolding and reduced survival (Liu*, et al.* 2012). This not only links the ageing process to neurodegeneration, but also indicates that RNA processing is influential in the process. Theories of ageing include the antagonistic pleiotropy theory, in which certain genes are beneficial at one age but detrimental at another. miRNA pathways may provide a mechanism by which potentially harmful age-related activities of genes are suppressed through alteration of the transcriptome with age, and aberrant expression of these genes may promote age-associated decline and potentially lead to disease (de Lencastre*, et al.* 2010, Kirkwood

2005, Williams and Day 2003). The aberrant processing of transcripts, including transcription and translation, due to nucleic acid damage may increase cellular vulnerability to degeneration. Evidence supporting this includes an accumulation of DNA damage in post-mitotic neurons during ageing (Mandavilli and Rao 1996, Rutten*, et al.* 2007), and increased DNA damage and markers of cell cycle re-entry in models of disease and in AD and PD patient brains (Weissman*, et al.* 2007a). The attempted re-entry of neuronal cells into the cell cycle is most likely to activate apoptotic pathways potentially leading to neurodegeneration (figure 1.5) (Barzilai 2010). Oxidative modification to DNA and RNA has also been documented in neurodegenerative diseases, with the consequences of this damage shown to cause increased production of aberrant proteins and short polypeptides (section 1.6) (Ding*, et al.* 2005, Shan*, et al.* 2007, Tanaka*, et al.* 2007).

Selective neuronal vulnerability refers to the differential vulnerability of neuronal populations to stresses that cause cellular damage leading to different neurological diseases. Specific brain regions exhibit different vulnerabilities in various neurological disorders causing difficulties when comparing age-related changes to those seen in neurodegenerative disease. These differences may reflect the heterogeneity in neuronal responses to the cell damaging processes associated with each disease (Wang and Michaelis 2010). Evidence from some neurodegenerative diseases, particularly AD, suggests there is a lengthy preclinical period, providing further difficulties in distinguishing between normal ageing and early phases of disease progression. The development of therapeutic interventions for age-associated diseases requires an increased understanding regarding the processes contributing towards normal and pathological ageing of the brain, as the ageing process and age-associated diseases may be different manifestations of the same fundamental intracellular processes. Therapies targeting the ageing process may be advantageous in potentially attenuating the development of many diseases simultaneously rather than specifically targeting individual diseases in isolation.

1.2.5 Conclusions

The factors proposed to contribute towards the ageing phenotype have been outlined in this section. It is important to understand how particular cellular processes change during ageing and identify how this might influence the ageing phenotype. How these changes correlate to what is reported in neurodegenerative disease is also discussed, as

determining the difference between normal and pathological ageing may facilitate the development of therapeutics for neurological disorders. The complexity of ageing remains a significant challenge in determining the molecular basis underpinning it. Reductions in the fidelity and coordinated activities of signalling pathways and repair networks, which act to maintain cellular and organismal homeostasis, may modulate the rate of ageing and the appearance of age-related pathology. Controversy remains over a genetic basis of ageing (Antebi 2007, Martin*, et al.* 2007). Whilst some believe ageing could be genetically determined, others accept a more generalised concept that ageing is the result of an interaction between environment and genes (Bishop*, et al.* 2010, Kirkwood 2005), not a controlled process. The most studied negative environmental factor is stress, whilst cognitive demanding activities, diet, and physical exercise can lead to successful ageing of the brain.

Though ageing and longevity studies mainly focus on singling out the contribution of individual physiological traits to the ageing process, these remain difficult to interpret and consolidate. Furthermore, given the emergent complexity due to their co-dependence, these factors should not be studied in isolation and understanding the interplay between longevity traits is crucial.

The importance of studying ageing is an acknowledgement of the many underlying features the process shares with neurodegenerative diseases, and understanding these in the ageing processes may provide directions to pursue for therapeutic targets in these diseases. Studies investigating normal cellular metabolism and neurodegenerative disease are beginning to shape our knowledge of the ageing brain, however, the question of how the brain ages at the molecular level remains complex (Reuter-Lorenz and Park 2010). The studies in short lived animal models in ageing research have provided much knowledge on the process of ageing, but can also pose potential problems such as being definitive that the cause of death is actually due to an acceleration of ageing, as it can be difficult to disentangle underlying disease processes from normal ageing mechanisms.

1.3 Oxidative Stress

This section will focus on the importance and consequences of oxidative stress to the CNS during ageing and neurodegeneration. Under pathological conditions abnormally large concentrations of ROS may lead to permanent changes in signal transduction and gene expression, which is typical of disease states and important to consider when

developing therapeutics. An increase in oxidative stress may lead to increased nucleic acid oxidation which in turn may affect numerous cellular processes, so it is important to understand which cellular and metabolic processes may be affected with relation to oxidative stress.

Under normal physiological conditions reactive oxygen species (ROS) are involved in redox-sensitive signalling to maintain cellular homeostasis through signalling pathways and changes in gene expression. However, an imbalance between the production of free radicals such as superoxide, hydroxyl radical, hydrogen peroxide, peroxynitrite, and their detoxification by antioxidant processes can result in oxidative stress (figure 1.6). Oxidative damage is marked by lipid peroxidation, nitration, protein and nucleic acid oxidation, and reactive carbonyls, and can lead to cellular degeneration due to functional decline. Because the direct detection of ROS is difficult, oxidative stress is often measured by the alteration of antioxidant status or the accumulation of relatively stable products of lipid, protein, and nucleic acid interactions (Radak*, et al.* 2011).

Increased ROS generation has been associated with dysfunctional mitochondria in ageing and neurodegenerative disease, and exposure to chronic oxidative stress is considered a central contributory factor towards processes (Radak and Boldogh 2010). This section will focus on the importance and consequences of oxidative stress to the CNS during ageing and neurodegeneration. Under pathological conditions abnormally large concentrations of ROS may lead to permanent changes in signal transduction and gene expression, which is typical of disease states and important to consider when developing therapeutics.

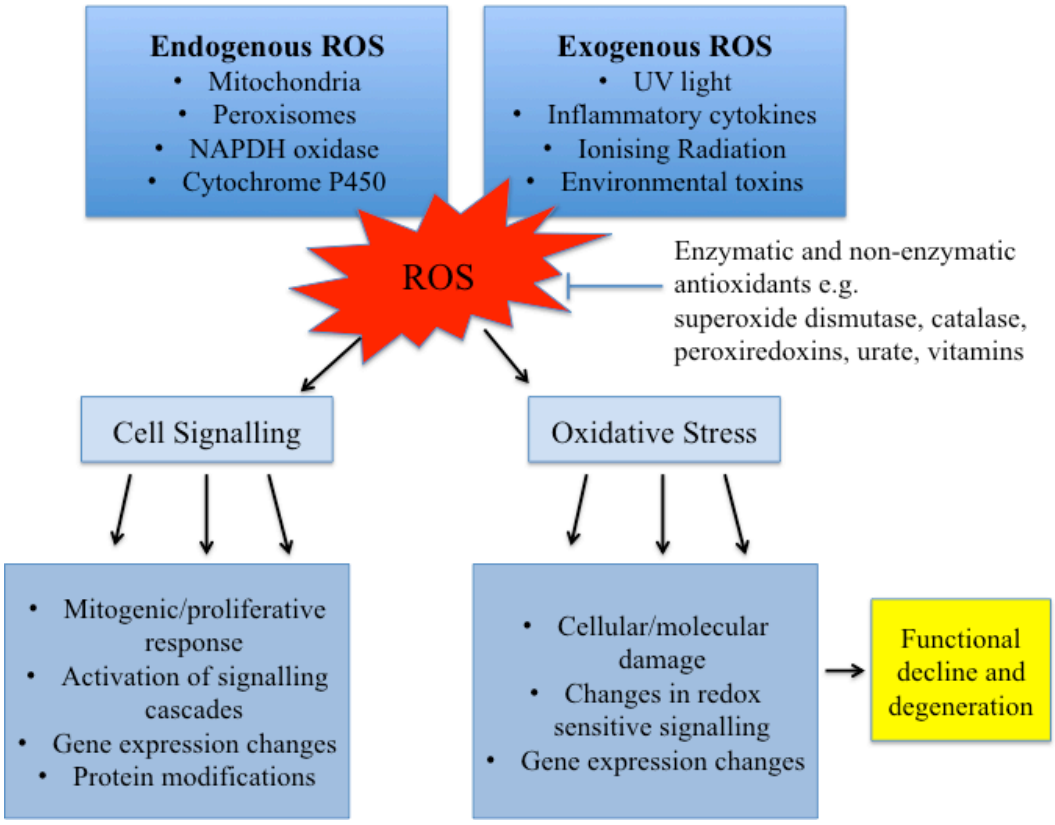


Figure 1.6: Redox sensitive signalling and oxidative stress responses to ROS

ROS are generated by endogenous and exogenous sources, and their detoxification by enzymatic and non-enzymatic antioxidants prevents the detrimental effects ROS can inflict on cells if their production exceeds their removal. ROS can modify cellular macromolecules leading to redox sensitive changes in cell signalling. An excess of ROS within cells can lead to oxidative stress and cellular and molecular mechanisms may be compromised, leading to cellular decline and degeneration.

1.3.1 ROS and cellular signalling

Endogenous ROS molecules can have differential effects depending on cellular context and specific modulators to their activity. It is important to consider ROS as signalling molecules as the modification of substrates may lead to different phenotypes. It may be plausible that RNA is reversibly oxidised as a regulatory mechanism so it is important to understand how these molecules act in signalling pathways.

The free radical theory of ageing originally implied that the targets of ROS were random, indiscriminate, and cumulative. However, evidence suggests ROS act as specific signalling molecules under both physiological and pathophysiological conditions (Cui, *et*

al. 2012, Finkel and Holbrook 2000). The generation of ROS within certain limits is essential to maintain homeostasis. Low and intermediary ROS levels have been reported to be physiologically important in intracellular signalling pathways encompassing redox regulation. Redox signalling is a well-recognised stress response that leads to a variety of downstream effects. The mitochondrial respiratory chain produces the majority of ROS and due to their importance in cellular signalling pathways the release of ROS into the cytosol is a tightly regulated process (Bae*, et al.* 2011). ROS can directly interact with critical signalling molecules to activate signalling in a variety of diverse cellular processes, including proliferation and survival (MAP kinases, PI3 kinase, PTEN, and protein tyrosine phosphatases), antioxidant gene regulation (thioredoxin, peroxiredoxin, Ref-1, and Nrf-2), mitochondrial oxidative stress, apoptosis, iron homeostasis (IRE–IRP), and DNA damage response (ATM) (as reviewed in Ray*, et al.* 2012). The activation and inactivation of transcription factors by ROS also acts to alter gene expression patterns in response to the cellular environment, which may be important in ageing and neurodegenerative disease. In addition, ROS can modulate the activity of membrane channels and metabolic enzymes, and regulate calcium dependent and phosphorylation dependent signalling pathways (Suzuki*, et al.* 1997, Trachootham*, et al.* 2008). The degree to which given pathways/transcription factors are targeted by ROS is dependent on the nature and duration of the stress, as well as the cell type. Determining which intracellular signalling pathway specific free radicals are involved in, and the precise mechanisms by which they alter the activity or stability of components of these pathways will improve our knowledge of ROS instigated cellular signalling under homeostatic conditions (Janssen-Heininger*, et al.* 2008, Poyton*, et al.* 2009).

The modification of proteins resulting in their activation/inactivation may depend upon redox sensitive amino acids, and evidence suggests the activation of oxidative stress response pathways consists mainly of the redox regulation of redox-sensitive cysteine residues on proteins by ROS (Barford 2004, Trachootham*, et al.* 2008). The reversible oxidation of proteins enables a dynamic regulatory process that varies in accordance with the redox conditions of the cell. The successive and reversible transfer of electrons and protons alternatively triggers functionally active or inactive states in many proteins and enzymes (Stolc*, et al.* 2011). Target proteins are transiently oxidised to enable transmission of the signal and then reduced to their basal oxidation state, with the ratio of oxidised to reduced forms dependent upon the redox potential of the cell. The oxidation and reduction of proteins is a major mechanism by which reactive oxidants influence cellular signal transduction pathways. The change in enzymatic activity or binding characteristics due to oxidation provides a mechanism for transduction of the signal.

Alternatively there may be a subset of sensor proteins, which serve as intermediary molecules, and once oxidised facilitate the oxidation of other protein targets through selective protein-protein interactions and thiol exchange.

1.3.2 Oxidative stress and gene expression changes

Changes in gene expression are another feature of cellular response to stress, as rapid adaptation to stress is crucial for maximising cell survival. Cellular adaptation mechanisms include induction of efficient changes in gene expression by intracellular signalling networks. Post-transcriptional effects provide immediate responses and the regulation of gene expression is essential for slower long-term adaptation and recovery phase. The control of gene expression is tightly regulated, and has fast response kinetics, enabling the cell to rapidly change its transcriptional capacity in the presence of stress (de Nadal*, et al.* 2011). Transcription factors stimulated by ROS may mediate gene expression induction by oxidative stress and subsequently regulate protein homeostasis. Global transcriptional responses to stress have been studied by gene expression profiling in worms, flies, and mammals (Lee*, et al.* 2000, Lund*, et al.* 2002, McCarroll*, et al.* 2004, Zou*, et al.* 2000). Microarray based methods also enable investigation of transcript decay and translation rates to identify targets under specific conditions. Sophisticated regulatory mechanisms adjust the induction and decay rates of mRNA to control mRNA stability, depending on the nature of the stress and the phase of response (Miller*, et al.* 2011, Shalem*, et al.* 2008). Stress response mRNAs are selectively stabilised or selectively degraded independently from global mRNAs, highlighting the importance of gene expression changes in response to cellular stress (de Nadal*, et al.* 2011). Investigating the molecular basis of gene expression regulation in response to stress has provided insight into the transcriptional processes regulated during stress, the importance of gene expression changes, and how stress-signalling molecules influence chromatin structure (Petesch and Lis 2008). Regulation specifically depends on the particular stress, cell type, and organism (Lopez-Maury*, et al.* 2008, Ni*, et al.* 2009).

Defence genes encode detoxifying enzymes, such as NAD(P)H:quinone oxidoreductase 1 (Nqo1), glutathione S-transferases (GST), and haem oxygenase 1 (Hox-1), antioxidant and related proteins such as thioredoxins, and γ-glutamate cysteine ligase (γ-GCS), ubiquitination enzymes and proteasomes. These have a wide-range of functions involving antioxidant response. The antioxidant response element (ARE) is found in the promoter of genes encoding antioxidant proteins and detoxifying enzymes, and acts to mediate the

transcriptional induction of genes during oxidative stress (Reddy 2008). Activation of gene transcription through ARE is primarily mediated by nuclear factor erythroid 2-related factor 2 (Nrf2) (Nguyen*, et al.* 2009). Nrf2 is a major transcription factor activated by oxidative stress. It acts to regulate the expression of several important antioxidant enzymes such as superoxide dismutases, peroxiredoxins, glutathione peroxidases and haem oxygenases (de Vries*, et al.* 2008, Itoh*, et al.* 2003, Lee*, et al.* 2003). Nrf2 binds to the ARE site in the promoter of these genes leading to their activation. Studies on the role of Nrf2 in ARE-mediated regulation of Nqo1 gene expression provided evidence for the role of Nrf2 in protection against oxidative and/or electrophilic stress (Venugopal and Jaiswal 1996). Nrf2 null-mice exhibit a marked decrease in the expression and induction of Nqo1, indicating that Nrf2 plays an essential role in the *in vivo* regulation of Nqo1 (Itoh*, et al.* 1997). Post-translational modification of Nrf2 results in ARE-induction by cysteine modification or serine phosphorylation. Modifications in Keap1, the protein that retains Nrf2 in the cytoplasm, have also been proposed to be important for the activation of Nrf2. The activation and repression of Nrf2 expression has been demonstrated to protect cells from free radical damage, prevent apoptosis and promote cellular survival (Copple*, et al.* 2008, Jaiswal 2004, Kirby*, et al.* 2005).

The p53 tumour suppressor produces different downstream gene expression responses according to the level and type of stress encountered by a cell. It exerts transcription dependent pro-apoptotic effects through induction of pro-oxidant genes during increased cellular stress and high ROS concentrations, leading to the inhibition of the cell cycle or initiation of apoptosis (Polyak*, et al.* 1997, Vousden and Lane 2007). p53 also presents a pro-survival role in response to low ROS levels by activating several antioxidants, including glutathione peroxidase, SOD2, ALDH4, and activating key signalling pathways and transcription factors (Hussain*, et al.* 2004, Tan*, et al.* 1999, Yoon*, et al.* 2004).

1.3.3 Oxidative stress and ageing

The susceptibility to developing cardiovascular and neurodegenerative diseases increases with age. The basis for this remains undetermined but one explanation is that such diseases may share common mechanisms with ageing. An age-dependent increase in the rate of ROS generation or reduction in cellular repair or degradation mechanisms has been reported, which will increase the oxidative load on the cell, resulting in a corresponding increase in oxidised macromolecules. There is abundant evidence for an age-related increase in products of oxidative damage within the mammalian brain, which

is in good agreement with findings in other tissues (reviewed in Droge 2002). How the cell responds to oxidative stress may change during ageing. A large number of oxidative stress responsive transcription factors and genes have been identified, and some of these have been shown to influence the ageing process. A conserved feature of ageing appears to be an increase in expression of genes involved in stress-response pathways (Haigis and Yankner 2010), and the brain may use conserved mechanisms of stress resistance during ageing to protect against the pathology of neurodegenerative disorders.

A reduction in cellular repair or degradation mechanisms, possibly due to the loss of physiological responses associated with stress tolerance, has also been reported, which further increases the oxidative load on the cell (Squier 2001). Experimental evidence suggests reduced mitochondrial function during ageing is due to age-dependent gene expression changes and the subsequent impairment of respiratory chain function and progressive oxidative damage is a conserved central mechanism of age-related functional decline. Autophagy enables the removal of misfolded proteins and dysfunctional organelles from cells, and although ROS act as signalling molecules in the early events of autophagy induction, if the pro-survival attempt fails, ROS induces cell death. Whether autophagy is a non-specific degradation process or rather some proteins/organelles being targeted for clearance is still being deliberated (Mammucari and Rizzuto 2010). A decline in GSH peroxidase activity has been found in the hippocampus and hypothalamus of aged rats (Rodrigues Siqueira*, et al.* 2005). A decrease in the activities of Manganese (Mn) and Cu/Zn superoxide dismutase isoenzymes and catalase has been found in the brain of ageing mice (Navarro and Boveris 2007). However, this has not been confirmed by other studies and remains controversial (Serrano and Klann 2004). The proinflammatory phenotype of astrocytes in the ageing brain can have both detrimental and neuroprotective responses. For instance increased cytokine secretion can activate inflammatory neurodegeneration by triggering oxidative stress involving nitric oxide-provoked pathways (Brown and Bal-Price 2003).

The coordinated action of redox signalling accompanied by other signalling pathways, gene expression changes, and RNA processing events maintain cellular and organismal homeostasis. Multiple lines of evidence suggest the regulation and execution of critical cellular and metabolic processes gradually decline, affecting the rate of ageing and the appearance of age-related pathologies. Although a great deal is known about ROS, the relationship between ROS induced damage on cellular metabolism, the damaging effects of redox imbalance, and the redox levels critical to regulation, remain unanswered.

1.3.4 Oxidative stress and neurodegeneration

The accumulation of intracellular oxidative toxicity has been linked to the degeneration of selective loss of neuronal populations in neurodegenerative disease. As previously discussed, increased intracellular levels of ROS, beyond the clearance capacity of the cell causes oxidative stress, potentially leading to cellular dysfunction and death. The underlying mechanism of oxidative stress has been reported in ALS, PD, and AD (Alam*, et al.* 1997, Ferrante*, et al.* 1997a). Oxidatively modified products of nucleic acids, proteins, and lipids are increased in the brains of patients with neurodegenerative diseases, and this can lead to functional disruption of these biomolecules. This has led to extensive research particularly in ALS, as mutations in antioxidant enzyme Cu/Zn SOD1 and the subsequent gain of function of this enzyme, is accountable for around 20% of fALS cases. In most neurological diseases overproduction of ROS has been linked to other mechanisms, such as cellular damage to proteins, lipids, and nucleic acids, mitochondrial dysfunction, neurotoxic aggregates, excitotoxicity, and endoplasmic reticulum stress (figure 1.2) (Duffy*, et al.* 2011, Kanekura*, et al.* 2009, Wood*, et al.* 2003). These features create an additive effect making the neurons and glia highly susceptible to damage by free radical species leading to a progressive reduction in neuronal structure and function (Shukla*, et al.* 2011).

Whether oxidative stress is a secondary effect of a pre-existing disease condition or whether it is a central mechanism of disease remains unknown. The continuous increase in ROS generation during neurological disorders due to dysfunction of related mechanisms and an insufficient antioxidant response might cause a shift in the redox homeostasis of cell. Equilibrium may still be reached; however, signal transduction and gene expression may be persistently modified, potentially giving rise to pathology. Underlying mechanisms of disease pathogenesis may be due to the loss of normal biological function of proteins due to mutation, or structural and functional changes due to ROS modification may lead to aggregation, mislocalisation, or aberrant activation of signal transduction cascades.

1.4 Mitochondrial Dysfunction

Mitochondria are the primary site of ATP production, maintain calcium homeostasis, influence calcium signalling, and participate in apoptotic cascades, making them critical to cellular function. Mitochondrial dysfunction is documented in normal ageing and

neurodegenerative disease. This section will discuss the relationship between mitochondrial dysfunction and neurodegenerative disease. Understanding the link between mitochondria and the oxidative stress hypothesis is important as our study investigates mitochondrial bioenergetics in a cellular model of ALS, with relation to susceptibility to oxidative stress. This is also an attractive area of research for targeting therapeutic approaches.

1.4.1 Mitochondrial dysfunction and neurodegeneration

Evidence of mitochondrial dysfunction in ALS comes from studies of familial and sporadic patients and transgenic models of disease (Cozzolino*, et al.* 2009, Sasaki and Iwata 2007, Wiedemann*, et al.* 2002). Changes in mitochondrial bioenergetics, clustering of abnormal mitochondria, calcium buffering, and induction of mitochondrial apoptosis are all features of ALS (Navarro and Boveris 2007). Although evidence implicates mitochondrial dysfunction in the pathogenesis of various neurodegenerative diseases, support is lacking for exactly what perturbations may lead to their physiological malfunction, and whether diminished function plays a primary role underlying the pathogenesis of neurodegenerative disorders such as ALS. In neurodegenerative diseases the selective loss of distinct neuronal populations is often observed, which in combination with mitochondrial dysfunction may place a greater responsibility on the surviving tissue to maintain an adequate energy supply to meet cellular demand.

Histological analysis of samples from ALS patients and murine models of the disease have revealed the presence of swollen and vacuolated mitochondria as a morphological feature of pathology (Higgins*, et al.* 2003, Sasaki and Iwata 2007, Sasaki*, et al.* 2004, Siklos*, et al.* 1996). Reduced mitochondrial function is correlated with altered mitochondrial morphology; a reduction in the activity of respiratory chain complexes has been observed in post-mortem tissue from ALS (Borthwick*, et al.* 1999, Wiedemann*, et al.* 2002). The expression of human mutant *SOD1* in cultured primary MNs has also been shown to increase mitochondrial depolarisation, impair calcium homeostasis, and reduce ATP production (Damiano*, et al.* 2006, Jung*, et al.* 2002, Menzies*, et al.* 2002). A reduction in the energy generation capacity of cells will lead to impaired electron chain transport and increased ROS production. The accompanying gene expression changes subsequently cause a shift in redox potential, leading to compromise of normal cellular processes associated with a reduction in the ability to adapt to physiological stress (Shigenaga*, et al.* 1994).

SOD1 can localise to the mitochondria, despite it normally having predominantly a cytosolic localisation. Targeting SOD1 to the mitochondrial intermembrane space (IMS) prevented motor neuropathy in mice lacking SOD1 (Fischer, *et al.* 2011). The function that mitochondrial localised SOD1 provides remains unclear, however mutations in SOD1 do not prevent the ability of the protein to accumulate in mitochondria (Bergemalm, *et al.* 2006, Ferri, *et al.* 2006, C. Y. Liu, *et al.* 2004, Pasinelli, *et al.* 2004, Vijayvergiya, *et al.* 2005). Mutant SOD1 associates with the mitochondria in an oligomeric and aggregated form (Deng, *et al.* 2006, Ferri, *et al.* 2006, Furukawa, *et al.* 2006), which may have a direct role in mitochondrial damage and cellular dysfunction in ALS. The presence of oligomerised mutant SOD1 in the IMS has been linked to an impairment of respiratory chain complexes, via alteration of the redox state of the mitochondria (Ferri, *et al.* 2006). Studies have also indicated the recruitment of mutant SOD1 to the inner mitochondrial space leads to the aberrant production of ROS (Ahtoniemi, *et al.* 2008, Goldsteins, *et al.* 2008).

In vivo mouse studies have demonstrated disturbances to calcium buffering and storage in the presence of human G93A mutant *SOD1* is linked to mitochondrial dysfunction (Jaiswal and Keller 2009). Studies of mitochondria isolated from the brain and spinal cord of mutant *SOD1* transgenic mice demonstrated an early reduction in the calcium buffering capacity, leading to a reduction in their membrane potential and possible dysfunction (Damiano, *et al.* 2006). In addition, nerve terminals of ALS patients have displayed chronic calcium overload (Siklos, *et al.* 1996). An elevation of intracellular calcium has been associated with depolarisation of the mitochondrial membrane potential and subsequent ROS generation, excitotoxicity, and ATP depletion (Dykens 1994, Gunter, *et al.* 1994, Schinder, *et al.* 1996).

Mitochondria are the major sites of ROS formation, with disturbances to their function often exacerbating this process. The uncoupling of mitochondrial respiration and oxidative phosphorylation during neurodegenerative disease, due to disturbances in mitochondrial function, can lead to a reduction in the activity of respiratory enzymes, an increase in proton leak, and subsequent increase in ROS production (Wei, *et al.* 1998). Mitochondria are particularly vulnerable to ROS damage, which can lead to disruption of their membrane due to lipid peroxidation, oxidation of iron-sulphur clusters in their proteins, and oxidative modification to mtDNA (Beckman and Ames 1998, Cozzolino and Carri 2012, Harman 2006, Vina, *et al.* 2003). Despite the evidence, this 'vicious cycle' hypothesis has been challenged, with controversy surrounding the extent of ROS mitochondrial damage required to functionally impact the electron transport chain due to

its highly conserved nature. The mtDNA genome is dependent upon nuclear-encoded proteins for its maintenance and transcription. Alterations of mitochondrial gene expression associated with disease or due to the accumulation of unrepaired mtDNA damage would cause respiratory chain dysfunction. Transcription of mtDNA determines the rate of assembly of new respiratory chain complexes (Lane 2011). Respiratory insufficiency, indicated by free radical leak, will activate the necessary mitochondrial transcription factors for the generation of new complexes. A threshold of free radical leak has been suggested, where a drop below the threshold would stimulate mitochondrial biogenesis. However a breach of the threshold may activate apoptotic pathways (Lane 2011). This may be affected by mutation and provide an explanation for the differences in mitochondrial bioenergetics seen between cells harbouring different mutant *SOD1* transgenes. Mitochondrial pathology is an early preclinical feature of MN injury in *SOD1* transgenic mouse models of ALS (Wong*, et al.* 1995) suggesting an importance in pathogenesis, however, whether mitochondrial dysfunction is a result of oxidative stress, or whether oxidative stress arises due to mitochondrial dysfunction remains to be established (figure 1.3).

1.5 RNA Processing and Disease

This section discusses RNA processing and metabolism dysfunction and its relation to neurodegenerative disease, as this has become increasingly linked to pathogenesis. RNA oxidation may have extensive effects on RNA processing and metabolism, so understanding these under normal cellular conditions is pertinent to understanding how they may be perturbed in ageing and neurodegenerative disease.

1.5.1 RNA processing and metabolism

The large number of proteins and regulatory RNAs in post-transcriptional RNA processing and the complex network of interactions among them provide cells with the ability to adjust their transcriptome and hence rapidly alter their proteome in response to stimuli. However, this provides significant opportunities for pathological alterations to RNA metabolism and extends cellular vulnerability to dysregulation that may lead to numerous diseases (Cooper*, et al.* 2009). The study of RNA processing and metabolism has revealed complex pathways in the generation, maturation and maintenance of functional RNA, and research has focused on how these may contribute towards neuronal dysfunction and death. The human transcriptome is comprised of protein-coding

messenger RNA (mRNA) and multiple classes of different structural and regulatory non-coding RNAs (ncRNA). The identification and characterisation of a non-coding portion of genome generates further complexity and mechanisms by which disturbances at the RNA level may contribute towards disease (Wapinski and Chang 2011). Maintaining the proper processing of these different RNA molecules is fundamental for maintaining sufficient levels of protein synthesis and limiting translational errors (figure 1.7).

Mutations in genes that encode factors important for ribonucleoprotein biogenesis and RNA processing, or perturbations of RNA processing, including splicing regulation, transcript stabilisation, translational repression and localisation of mRNA can result in MN degeneration. Many proteins involved in RNA processing are connected to a variety of neurodegenerative disorders. Perturbations of RNA processing events can have wide-ranging downstream effects on the expression of multiple genes. Complete understanding of how these pathways interact and elucidation of specialised mechanisms for mRNA targeting and processing in MNs are likely to produce new targets for therapy in ALS and related disorders. RNA processing was initially linked to MN degeneration by the identification of mutations in the survival motor protein (*SMN1*) in spinal muscular atrophy (SMA) (Lefebvre*, et al.* 1995). Since then RNA processing perturbations in transcription, mRNA stabilisation and transport, and translational regulation have been described in ALS, accompanied by a greater understanding of the importance and complexity of RNA processing events in many cellular processes and other complex diseases.

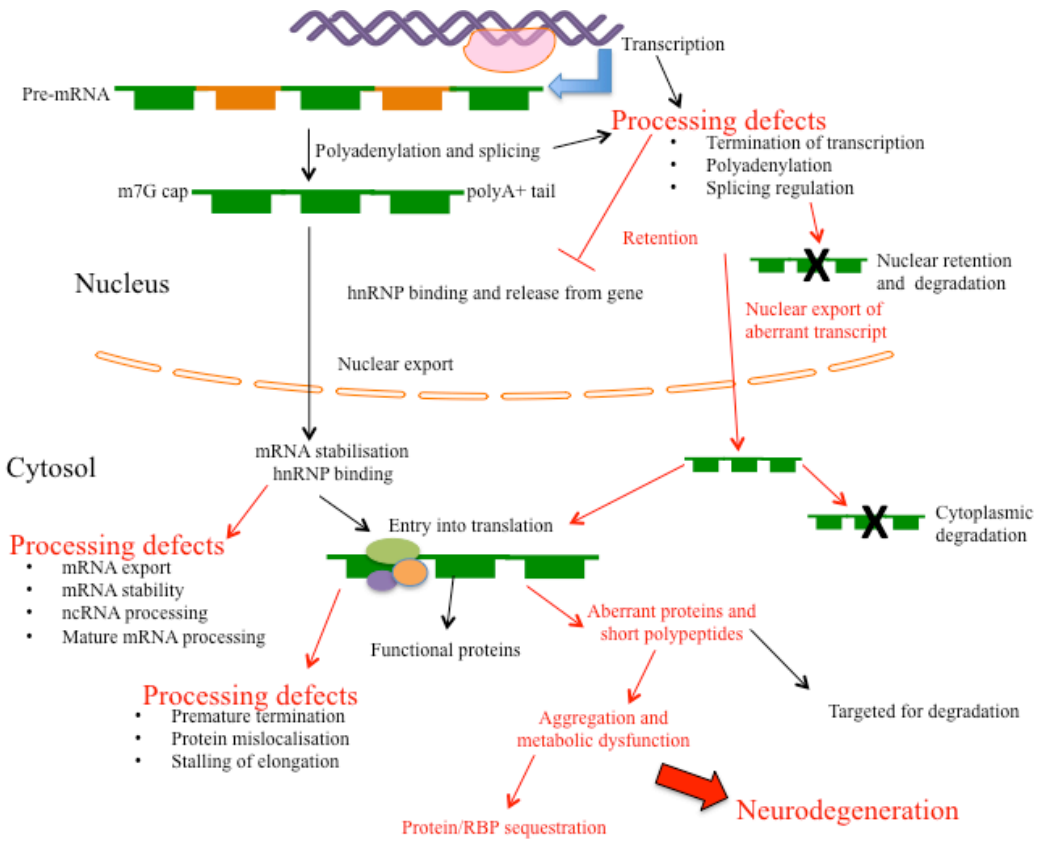


Figure 1.7: RNA processing and metabolism

Displayed here are some of the points at which aberrant RNA processing can lead to cellular dysfunction. There are multiple checkpoints and quality control mechanisms within the cell to initiate a response to aberrant processing through targeted sequestration or degradation of the transcript. The biogenesis and functioning of RNAs involves a series of transitions through different complexes and cellular compartments, all of which pose a potential threat to the cell if disrupted. Aberrant RNAs can arise through multiple events including gene mutations, transcriptional/translational error, nuclear pre-RNA processing, and ribonucleoprotein (RNP) assembly amongst others.

1.5.1.1 Transcription

For coding RNAs, RNA polymerase II carries out transcription to produce heterogeneous nuclear pre-mRNA (hnRNA) transcripts containing introns and exons. Pre-mRNA is spliced into its mature form through removal of the introns, and fusing of the exons by the spliceosome machinery, which is composed of small nuclear RNAs (snRNA), splicing factors, and RNA binding proteins (RBP) (Chen and Manley 2009, Smith and Valcarcel 2000). Splicing is part of the post-transcriptional processing of transcripts.

Alternative splicing allows cellular control over the protein isoforms expressed and is a key process in determining the properties of individual neurons within the CNS (Li*, et al.* 2007). These proteins, generated from a single pre-mRNA precursor, differ in their peptide sequence and thus can have different biochemical characteristics. Failure of splicing efficiency may result in the production of truncated or dysfunctional proteins, which may arise due to the presence of a premature stop codon or inaccurate peptide sequence.

1.5.1.2 Post-transcriptional regulation

Following transcription, RNA undergoes complex processing including splicing and editing, and associates with specific proteins that determine its subcellular localisation and stability. Post-transcriptional regulation of different RNA molecules is fundamental for generating diversity and maintaining sufficient levels of protein synthesis through limiting translational errors. ncRNA increases the dimension of control over spatial and temporal gene expression patterns. ncRNA molecules include microRNAs (miRNA) which function to control mRNA translation and stability, small nucleolar RNAs (snoRNA) important for post transcriptional modifications of ribosomal RNA, PIWI-interacting RNAs (piRNA) which suppress transposable element expression and mobility, and the heterogeneous family of long non-coding RNAs (lncRNA) involved in transcriptional regulation of gene expression, including mediating epigenetic modifications (Huttenhofer*, et al.* 2005, Taft*, et al.* 2010). ncRNAs are important regulators of RNA processing and oxidative modification or dysfunction of these transcripts may lead to disease phenotype.

Alternative splicing generates different protein isoforms through variation in splice site selection. Proteins generated by alternative splicing differ in their chemical and biological characteristics. RNA editing is an additional post-transcriptional modification, which involves base substitution/ modification of the RNA transcript. RNA editing is prominent in neuronal tissues, altering gene and adding further diversity to the proteasome (Paul 2008). The extent and specificity of RNA editing is co-regulated with pre-mRNA splicing, with RBPs influencing the capacity of the transcript to be edited or spliced (Farajollahi and Maas 2010, Reenan*, et al.* 2000, Rosenthal and Seeburg 2012, Ryman*, et al.* 2007). Adenosine to inosine base modification is the most prevalent change in base sequence, and is catalysed by double stranded RNA specific adenosine deaminases (ADARs) (Bass 1997). The inosine subsequently behaves like a guanosine in RNA

folding and is recognised as this base by the translation machinery resulting in the site-specific substitution of an amino acid and altered secondary/tertiary structures. The development of RNA sequencing technology has enabled investigation in to genome wide RNA editing (Lee*, et al.* 2013, Ramaswami*, et al.* 2013).

Ribonucleoprotein complexes (RNPs) formed within cells are composed of one or more types of RNA and typically numerous RNA-binding proteins (RBPs) (Dreyfuss*, et al.* 1993, Glisovic*, et al.* 2008). The RNPs are the functional forms of the corresponding RNAs, and their normal activity depends on both the specific composition and the precise arrangement of their protein constituents. As there are numerous RNAs and a very large number of RBPs, the biogenesis of RNPs must be orchestrated with great fidelity. Various RBPs are involved in the maintenance of splicing and processing, export of mRNA to the cytoplasm and subsequent retention for translation, and ultimately mRNA decay. These post-transcriptional RBP-mediated regulatory mechanisms allow a precise spatio-temporal control of mRNA translation, associated with transport and subcellular compartmentalisation of mRNAs in dendrites and axons (Besse and Ephrussi 2008).

1.5.1.3 mRNA stability and turnover

The rate of transcription is pertinent in the regulation of gene expression; however equally important is mRNA half-life (Bolognani and Perrone-Bizzozero 2008). mRNA stability is important for the temporal order of gene induction (Hao and Baltimore 2009), but also forms part of the quality control mechanisms of cells to ensure defective RNA molecules are rapidly degraded. The rate of mRNA turnover is determined in part by RBPs that directly interact with mRNAs to form mRNA-protein complexes. After transcription, RBPs recognise and bind to cis-regulatory RNA elements within the precursor mRNA sequence to form mRNP complexes, which can regulate gene expression by stabilising or destabilising a particular mRNA (reviewed in Kapeli and Yeo 2012). The aberrant expression of RBPs involved in the regulation of mRNA stability may be associated with disease (Hollams*, et al.* 2002).

mRNA decay is a part of post-translational mechanisms that play a critical role in modulating gene expression by adjusting the abundance of transcripts available for translation. This provides a cell specific transcriptome, increasing or decreasing degradation rates in response to development/differentiation cues, hormonal stimulus, and stress (Guhaniyogi and Brewer 2001). Evidence suggests the specific half-life of each mRNA is closely related to its physiological function and sequence features (Rabani*, et*

al. 2011, Schwanhausser*, et al.* 2011, Sharova*, et al.* 2009). mRNAs with long 3' untranslated regions (UTR) on average are less stable and the density of AU-rich elements negatively correlates with mRNA stability (Schwanhausser*, et al.* 2011). RNA structure has also been identified in influencing the transcription, splicing, cellular localisation, translation and turnover of the RNA. mRNA half-life is in part related to the functional role of the protein they encode, and this can change in response to a variety of stimuli including environmental factors, mitogens, growth factors and intracellular messengers (Hollams*, et al.* 2002). mRNAs of most house-keeping genes and those involved in constitutive cellular processes have been reported to be relatively stable, whereas mRNAs encoding proteins that are required for short-periods of time for example transcription factors, signalling genes and cell-cycle specific proteins often have short half-lives (Schwanhausser*, et al.* 2011, Tani*, et al.* 2012).

1.5.2 RNA processing and neurodegeneration

1.5.2.1 Transcriptional regulation

The expression of the Per 28 isoform of peripherin is an example of the expression of a toxic splice variant in ALS. Peripherin is a type III intermediate filament that is associated with spinal motor neuron inclusions in ALS (Corbo and Hays 1992). The Per 28 isoform retains exons three and four; with exon three encoding for a premature stop codon that subsequently results in the production of a truncated protein (Xiao*, et al.* 2008). Its expression within cells leads to the formation of peripherin inclusions (Sanelli*, et al.* 2007), formed through selective aggregation of the aberrant proteins.

ANG encodes an angiogenic ribonuclease whose expression is increased in response to hypoxic/ischaemic events, and is a known target gene of hypoxia inducible factor 1 (HIF1) (Kishimoto*, et al.* 2005, Sebastia*, et al.* 2009). ANG acts as a transfer RNA (tRNA) specific ribonuclease, regulates ribosomal RNA (rRNA) transcription, is required for tRNA derived production of stress-induced small RNAs (sitRNAs), and binds directly to DNA thereby regulating gene expression (Fu*, et al.* 2009, Xu*, et al.* 2003, Yamasaki*, et al.* 2009). Its normal function is to prevent cell death by inhibiting the translocation of apoptosis inducing factor to the nucleus. *ANG* mutations have been identified in both sALS and fALS patients, and are likely to have a deleterious effect through loss of function in ALS cases. The mutations result in reduced rRNA biogenesis, impacting subsequent protein synthesis and affecting cell proliferation, angiogenic activities and

nuclear localisation (Ainscow and Brand 1999, Gellera, *et al.* 2008, Greenway, *et al.* 2006).

Missense mutations in *SETX* are associated with MN degeneration and ALS4 (Chen, *et al.* 2004). The C-terminus contains a classical seven-motif domain characteristic for RNA/DNA helicases. RNA helicases are frequently found in large ribonucleoprotein complexes and in general maintain genome integrity and regulate RNA biogenesis (Hickson 2003, Tanner and Linder 2001, Tuteja and Tuteja 2004). SETX is involved in the modification of chromatin structure and interacts with proteins associated with transcription and pre-mRNA spicing, and although the precise mechanism underlying ALS pathogenesis remains to be elucidated, dysfunction of transcriptional termination and pre-mRNA splicing is suggested (Chen, *et al.* 2004, Suraweera, *et al.* 2009). This is supported in part by the homology of SETX to the regulator of nonsense transcripts-1 (RENT1 or Upf1), Immunoglobulin Mu-binding protein 2 (IGHMBP2) and splicing endonuclease1 (Sen1p), which are all involved in RNA processing (Guenther, *et al.* 2009, Mendell, *et al.* 2002).

Elongator protein is a complex that associates with RNA polymerase II and has histone acetyltransferase activity conferred by the elongator protein complex subunit 3 (ELP3) (Svejstrup 2007). Genetic variants of *ELP3* have been associated with ALS, and its knockdown in zebrafish embryos strongly implicated *ELP3* in axonal biology and as a gene that confers a risk of neuronal degeneration (Simpson, *et al.* 2009). ELP3 is primarily cytoplasmic in neurons and is part of the RNA Polymerase II complex, with roles in RNA elongation (Winkler, *et al.* 2001), modification of tRNA wobble nucleosides (Huang, *et al.* 2005), and when part of the Elongator complex has histone acetyltransferase (HAT) activity directed towards core histones H3 and H4 (Winkler, *et al.* 2002). It remains unknown which loss of function associated with reduced ELP3 expression is responsible for the increased risk of developing ALS (Lemmens, *et al.* 2010).

1.5.2.2 Post-transcriptional regulation

A significant advance in the understanding of the pathophysiology of ALS and also placing RNA metabolism as a key pathogenic mechanism was the discovery of mutations in the RNA processing genes Tar DNA binding protein 43 (*TARDBP)* and Fused in Sarcoma/Translocated in Liposarcoma (*FUS/TLS).* TDP-43 is homologous to the

heterogeneous nuclear ribonucleoproteins (hnRNPs) and was initially discovered to be a major component of the ubiquinated inclusions within motor neurons in ALS cases (Neumann, *et al.* 2006). FUS/TLS is structurally related to Ewing's sarcoma and TATA binding protein-associated factor 15, and has been found in cytoplasmic inclusions within the brain and spinal cord of ALS and FTLD patients (Kwiatkowski, *et al.* 2009, Tan, *et al.* 2012, Vance, *et al.* 2009).

TDP-43 and FUS/TLS are both RNA/DNA binding proteins involved in alternative splicing, transcriptional regulation, mRNA stabilization and microRNA processing. Investigations have shown that mutations in the *TARDBP* gene, which encodes TDP-43, and *FUS/TLS* mutations, targeted RNA processing and are causative of some cases of this disease, highlighting the importance of RNA metabolism for normal cellular functioning. There is evidence from both cellular and animal models to support both loss of nuclear function and gain of cytoplasmic function as pathogenic mechanisms conferred by these mutant proteins, however, the exact cause of the selective vulnerability of MNs carrying these mutations remains to be elucidated (Da Cruz and Cleveland 2011, Feiguin, *et al.* 2009, Kabashi, *et al.* 2010). In a subset of ALS and FTLD patients TDP-43 positive cytoplasmic inclusions are present in the CNS, even though the gene is not mutated (Lagier-Tourenne, *et al.* 2010, Renton, *et al.* 2011). How wild-type TDP-43 is pathogenic in these cases is also of interest.

TDP-43 has a primary structure that is characteristic of other hnRNPs, containing two RNA recognition motifs (RRM1 and RRM2) that are evolutionary conserved and involved in both RNA and DNA binding, nuclear export and cellular localisation sequences, and a glycine rich region enabling protein-protein interactions (Buratti and Baralle 2001, Wang, *et al.* 2004). Interestingly, nearly all the mutations identified in *TARDBP* in ALS patients are localised in exon 6, coding for the C-terminal domain (Arai, *et al.* 2010, Johnson, *et al.* 2009, Kabashi, *et al.* 2010, Lagier-Tourenne, *et al.* 2010). TDP-43 is intrinsically aggregation prone, and *in vivo* studies revealed increased aggregation when mutations were present in the C-terminal domain. In both ALS and FTD cases there is an abundance of cytosolic TDP-43, some of which forms aggregates or skeins, some of which are ubiquitinated (Arai, *et al.* 2006, Benajiba, *et al.* 2009, Neumann, *et al.* 2006). The redistribution of TDP-43 to the cytoplasm potentially causes a loss of function leading to neurodegenerative disease (Barmada, *et al.* 2010, Nonaka, *et al.* 2009). In response to stress and neuronal injury TDP-43 localises to stress granules, which are transient, dynamic cytoplasmic sites of mRNA triage, and act to sort mRNAs for storage, degradation or translation when required in neuronal repair (Anderson and

Kedersha 2006, Moisse*, et al.* 2009). Stress granules contain a myriad of mRNAs and proteins including RBPs, transcription factors, RNA helicases, nucleases, kinases, and signalling molecules (Anderson and Kedersha 2008, Anderson and Kedersha 2009). TDP-43 has also been found in processing bodies (P-bodies), which are spatially, compositionally, and functionally linked to stress granules (Kedersha*, et al.* 2005, Parker and Sheth 2007). P-bodies contain components of the mRNA decay pathways and proteins involved with translational control, suggesting they function as mediators of mRNA biogenesis including translation and decay. P-bodies can intermittently and transiently dock at stress granules, potentially facilitating the transfer of selected mRNPs. The numerous processes linked to TDP-43 have generated differing hypotheses about the pathogenic mechanism of the mutant protein (Strong 2010, Volkening*, et al.* 2009, Wang*, et al.* 2004), with its principal roles linked to mRNA processing and stress-granule formation. Cytoplasmic mislocalisation and aggregation of TDP-43 may itself be toxic to cells, and/or it could sequester other proteins important for normal cellular function. Alternatively, the loss of nuclear TDP-43 may be detrimental to the processing of pre-mRNA transcripts.

FUS/TLS protein structure is characterized by a N-terminal domain composed of sequences enriched in glutamine, glycine, serine and tyrosine residues (QGSY-region), a glycine-rich region, an RRM domain, two multiple Arginine-Glycine-Glycine (RGG) repeats flanking a zinc finger motif, and a C-terminal NLS region (Iko*, et al.* 2004). FUS/TLS has been demonstrated to bind to RNA targets in specific sequences recognised by the zinc finger domain, while the RGG and RRM domains mediate the specificity of this interaction (Iko*, et al.* 2004). The NLS-containing C-terminal region of the protein, in which the majority of ALS mutations occur in a similar fashion to TDP-43 mutations, has been implicated in the cytoplasm retention of FUS/TLS, since it has been found as extranuclear cytoplasmic aggregates in affected individuals (Gal*, et al.* 2011, Ito*, et al.* 2011, Vance*, et al.* 2009). FUS/TLS is a component of the hnRNP complex involved with pre-mRNA splicing and mRNA export (Iko*, et al.* 2004), which may be disrupted in the presence of a mutation. Although FUS/TLS is an RNA processing gene, the precise mechanism leading to MN degeneration remains unclear (Blair*, et al.* 2010).

A motor neuron disease linked directly to splicing failure is spinal muscular atrophy (SMA), caused by mutations in the survival motor neuron *(SMN)* gene. In humans, the *SMN* gene exists as two homologous copies, *SMN1*, which encodes the full-length protein, and *SMN2*, which encodes a truncated isoform (Lorson*, et al.* 1999, Monani*, et al.* 1999). The SMN complex consists of SMN and other proteins such as the gemins, and

it functions in the assembly of snRNP complexes. A risk factor reported for ALS is abnormal copy numbers of SMN1, with studies demonstrating a significant increase in the frequency of abnormal SMN copy number in ALS patients (Corcia*, et al.* 2006, Corcia*, et al.* 2002). Genotypes resulting in low levels of SMN protein are also thought to be instrumental in ALS pathogenesis, with studies in human G93A mutant SOD1 transgenic mice reporting that a reduction in levels of spinal cord SMN protein which contributes towards MN degeneration (Corcia*, et al.* 2006, Turner*, et al.* 2009, Veldink*, et al.* 2005). Alternative splicing in SMN interacting protein 1 (SIP1, now known as gemin2), which is essential for snRNP biogenesis, were reported in tissue from ALS and SMA patients (Aerbajinai*, et al.* 2002). The disruption of alternative splicing regulation can affect gene expression and subsequently impact the production of protein isoforms, potentially leading to disease. Homozygous deletions of *SMN2* are also suspected to act as a susceptibility factor for ALS, and as a prognostic factor affecting survival time in sALS patients (Echaniz-Laguna*, et al.* 2002, Kim*, et al.* 2010, Moulard*, et al.* 1998, Veldink*, et al.* 2001).

Ataxin 2 *(ATXN2)* has been implicated in RNA processing, possibly through direct RNA binding, translation, or transport and stability of mRNAs. Intermediate length polyglutamine (PolyQ) expansions (27-33 glutamines) in *ATXN2* are associated with ALS. The mechanisms by which *ATXN2* expansions contribute towards ALS pathogenesis remain unknown; however the PolyQ expansion has enhanced interaction with TDP-43, promoting its sequestration to the cytoplasm increasing stress-induced TDP-43 C-terminal cleavage and phosphorylation (Hart and Gitler 2012, Nonhoff*, et al.* 2007). Intermediate-length ataxin 2 PolyQ expansions have also been linked to the stress-dependent activation of multiple caspases, including caspase 3, suggesting cells with the expansion have a lower threshold for such activation in response to stress (Hart and Gitler 2012).

RNA editing is an alteration in the primary nucleotide sequence, and is observed in mRNA, ncRNA, tRNA, and rRNA. The most common modification in the mammalian CNS is adenosine to inosine (A to I). A to I editing has been reported to be associated with excitotoxicity in ALS. The glutamate receptor subunit Glur2 undergoes editing at the Q/R site, during which the glutamine (Q) codon is substituted by an arginine (R) codon. RNA editing of this subunit alters the calcium permeability of the receptor (Seeburg*, et al.* 1998). A decrease or loss of editing for this receptor could lead to increased calcium permeability of these AMPA receptors. The excitotoxicity associated with ALS would result in excessive activation of these receptors, resulting in excessive

calcium influx and disruption to calcium homeostasis (Akbarian*, et al.* 1995). A reduction in editing of the Q/R site in GluR2 has been identified in ALS patients (Kwak and Kawahara 2005, Kwak*, et al.* 2008). Aberrant RNA processing of *EAAT2* in ALS has also been reported, with a correlation between increased editing of *EAAT2* and activation of alternative polyadenylation site demonstrated in ALS patients (Flomen and Makoff 2011). RNA processing defects may lead to loss of protein function and activity, leading to functional decline.

A noncoding GGGGCC hexanucleotide repeat expansion in intron 1 of the *C9ORF72* gene has recently been identified in significant amounts of both sALS and fALS cases (DeJesus-Hernandez*, et al.* 2011, Renton*, et al.* 2011). Hypotheses surround *C9ORF72* pathogenesis however the precise mechanism underlying disease remains to be discovered. One possible mechanism is described in the sequestration model, in which the repeat expansion results in a toxic RNA gain of function. The expanded RNA may form pathogenic foci that sequester RBPs disrupting other RNA processing and metabolic events, or the reduction could results in a loss of normal protein function. The removal of RNA and RBPs from the active pool can impact RNA metabolism potentially leading to pathogenesis. Another model that of haploinsufficiency suggests a mechanism by which the expansion leads to loss of *C9ORF72* function, affecting its ability to be processed/translated, with one copy insufficient to retain its function. A 50% reduction in *C9ORF72* transcript has been observed in patients with the expansion (DeJesus-Hernandez*, et al.* 2011).

ncRNAs have diverse roles in RNA processing and metabolism and are increasingly being investigated in relation to disease (Delay and Hebert 2011, Enciu*, et al.* 2012). MicroRNAs (miRNA) regulate mRNA stability and translation (Eacker*, et al.* 2009), and studies have investigated the impact of global miRNA synthesis on neuronal function in neurodegeneration, the consequences of alterations in miRNA expression in models of disease, and disruption of miRNA regulatory networks due to mutated disease proteins (Gehrke*, et al.* 2010, Packer*, et al.* 2008, Schonrock*, et al.* 2010). For mature miRNAs to be generated transcripts must undergo two rounds of cleavage by two separate RNases, Drosha and Dicer, which reside in the nucleus and cytoplasm respectively. Studies have shown TDP-43 to be important in pre-miRNA synthesis through its interaction with Drosha. Mislocalisation to cytoplasmic aggregates or the presence of a mutation in TDP-43 is likely to affect processing by Drosha and Dicer, potentially affecting miRNA expression (Haramati*, et al.* 2010). An miRNA profiling study on skeletal muscle from human G93A mutant SOD1 transgenic mice discovered the skeletal muscle specific

miRNA miR-206 was up-regulated in the lower limbs. However mice deficient for miR-206 displayed accelerated disease progression, suggesting the induction of miR-206 acts as a compensatory mechanism by sensing motor neuron injury and promoting regeneration of neuromuscular synapses to delay MN degeneration (Williams, *et al.* 2009).

1.5.2.3 mRNA stability

Neurofilament (NF) aggregates have been identified within MNs of both fALS and sALS patients (Troost, *et al.* 1992). In transgenic mice carrying human G93A mutant *SOD1,* p190 Rho guanine nuclear exchange factor (p190RhoGEF) was identified in aggregates containing the low molecular weight NF (NFL) (Lin, *et al.* 2005). p190RhoGEF forms part of an RNA-protein complex that stabilises NFL mRNA through interaction with the 3'UTR. Aggregation of p190RhoGEF appears to trigger neurotoxicity, and the human homologue RGNEF was immunoprecipitated with NFL mRNA in ALS, but not control, lysates (Volkening, *et al.* 2009). RGNEF is a RNA binding protein that acts as a destabilising factor for human NFL mRNA and can regulate NFL protein levels in cells (Droppelmann, *et al.* 2013). The aggregation of NFL and p190RhoGEF reduces the availability of p190RhoGEF to stabilise NFL mRNA, which could contribute towards pathogenesis but this remains to be determined. The presence of mutant SOD1 has also been shown to impair a network of RBPs and causes the destabilisation of mRNA species such as human hNFL and VEGF mRNAs (Ge, *et al.* 2005, Lu, *et al.* 2007).

1.5.3 Conclusion

RNA metabolism refers to evolutionally conserved processes involved in RNA biogenesis from transcription initiation through to mRNA decay. As discussed here as the regulatory roles of RNA begin to be uncovered, RNA processing is becoming increasingly associated with human disease, including neurodegenerative disease. The spatial and temporal separation of transcription and translation allows eukaryotes to specifically regulate gene expression in accordance with cellular requirements. The proper processing of RNA molecules is fundamental for maintaining transcriptome and proteome integrity and overall homeostasis. Alternative splicing expands the versatility of genomic sequences and with RNA editing is used as a mechanism to generate many different messages from the same gene (Farajollahi and Maas 2010). The rate of transcription and mRNA stability and turnover are the key determinants of the gene

expression profile, by adjusting the abundance of transcripts available for translation and targeting aberrant mRNAs for degradation to maintain quality control of RNA biogenesis. Improper processing of mRNA can lead to the degradation or nuclear retention of transcripts, or lead to the production of nonsense protein products. RNA surveillance has an integral role in regulating gene expression, and adds to the complexity of RNA processing. Non-coding RNAs and RBPs are both essential to RNA processing and gene expression patterns by increasing the dimension of control over the transcriptome.

Greater understanding and characterisation of RNA populations, their complexity, and the processes in which they are involved, will hopefully provide valuable insights in to how their dysregulation can lead to human disease and identify potential therapeutic targets. Here we have discussed the increasing evidence of RNA metabolism and processing defects in the pathogenesis of ALS. Dysregulated RNA processing, loss of function, and RNA toxicity are previously established in neurological disorders and are ever more being related to pathogenic mechanisms of degeneration. The intricate networks of interaction and functional control increase the opportunity for exposure to mutations and misregulation that cause disease.

1.6 Nucleic Acid Oxidation

The basis of our study was formed from the substantial evidence demonstrating an increase in nucleic acid oxidation during ageing and other neurodegenerative diseases. This section describes some of the key findings linking oxidative damage to nucleic acids and neurodegeneration. The consequences of nucleic acid modification and the repair processes identified are also discussed.

1.6.1 Oxidative modification to nucleic acids

The high content of unsaturated fatty acids, elevated oxygen consumption, and the lack of antioxidant defences in comparison to other organs, are believed to contribute towards CNS vulnerability to oxidative damage (Nunomura*, et al.* 2006). The accumulation of oxidative damage to cellular macromolecules including nucleic acids during ageing and in age-associated diseases identifies the importance of studying oxidative stress related damage and its association with neuronal degeneration (Ding*, et al.* 2004).

RNA may be more vulnerable to oxidative damage, in comparison to nuclear DNA, due

to its lack of protective histones, associated proteins, and single-stranded nature; or as a consequence of the abundance and subcellular distribution of RNA, locating in the vicinity of mitochondria (Bregeon and Sarasin 2005). It could also be the consequence of differential turnover/repair rates for RNA and DNA damage. As our understanding of RNA processing and metabolism develops it is becoming increasing evident that RNA is essential for controlling gene expression and maintaining cellular homeostasis. The susceptibility to oxidative damage might also vary among the different classes of non-coding RNAs and mRNA species. The degree of protein association or structural conformation of RNA may protect some species against damage. Different RNAs have varying degrees of expression, turnover rates and temporal patterns of association with proteins and while evidence suggests differing levels of oxidative damage between transcripts the reasons behind this potentially selective vulnerability is not fully understood.

The oxidation of mRNA can occur through direct damage to the bases and by the incorporation of the oxidised base from the cytosolic pool through the normal action of RNA polymerase II (Ishibashi*, et al.* 2005, Yanagawa*, et al.* 1992). Guanine, due to its high oxidation potential, is the most abundant and characterised oxidised base (Burrows and Muller 1998). Guanines are susceptible to base attack by singlet oxygen and electron transfer reactions. Modification of the base at carbon 8 by hydroxyl radicals, results in the production of a reducing neutral radical, which through reaction with oxygen or electron transfer leads to the formation of 8-hydroxyguanine (8-oxoG) (Burrows and Muller 1998, Candeias and Steenken 2000). Other oxidised purine and pyrimidine bases have been identified (reviewed in Bjelland and Seeberg 2003), and at least two other modifications have been identified in yeast RNA (Yanagawa 1992). Our study focuses on guanine oxidation products, as this a common oxidation product which is often used as a biomarker for oxidative stress (Kasai 1997, Mosley*, et al.* 2006) .

8-hydroxydeoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG) are guanine oxidation products (figure 1.8), and can be formed by direct oxidation of the base in DNA and RNA respectively or, as has been stated, through incorporation of the oxidised base from the nucleoside pool (Fiala*, et al.* 1989, Wamer*, et al.* 1997). The oxidised nucleoside when present in DNA is particularly important since it can pair with either adenine or cytosine during DNA synthesis, leading to potential base mispairing and hence erroneous protein production (Shibutani*, et al.* 1991). Our work focuses on 8-OHG and 8-OHdG so the literature reviewed will correspond to understanding these two oxidation products. We chose these modifications due to extensive reporting of them in the literature with

regards to age-associated diseases. They have also been identified as biomarkers for oxidative stress.

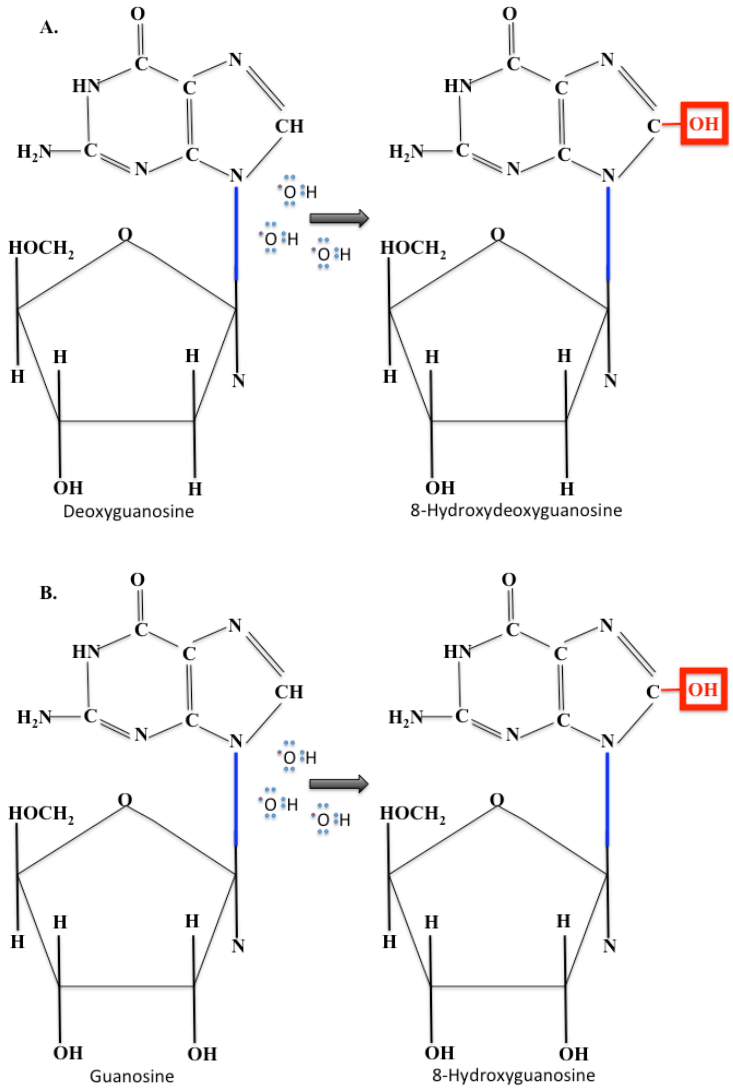


Figure 1.8: Hydroxyl radical attack of deoxyguanosine (A) and guanosine (B) nucleosides leading to the formation of 8-Hydroxydeoxyguanosine and 8-Hydroxyguanosine respectively

There is substantial evidence to associate RNA oxidation with the pathogenesis of neurodegenerative disorders. Further work involving the elucidation of the processing, surveillance and removal mechanisms related to this damage will provide greater understanding of their consequences and potential therapeutic strategies.

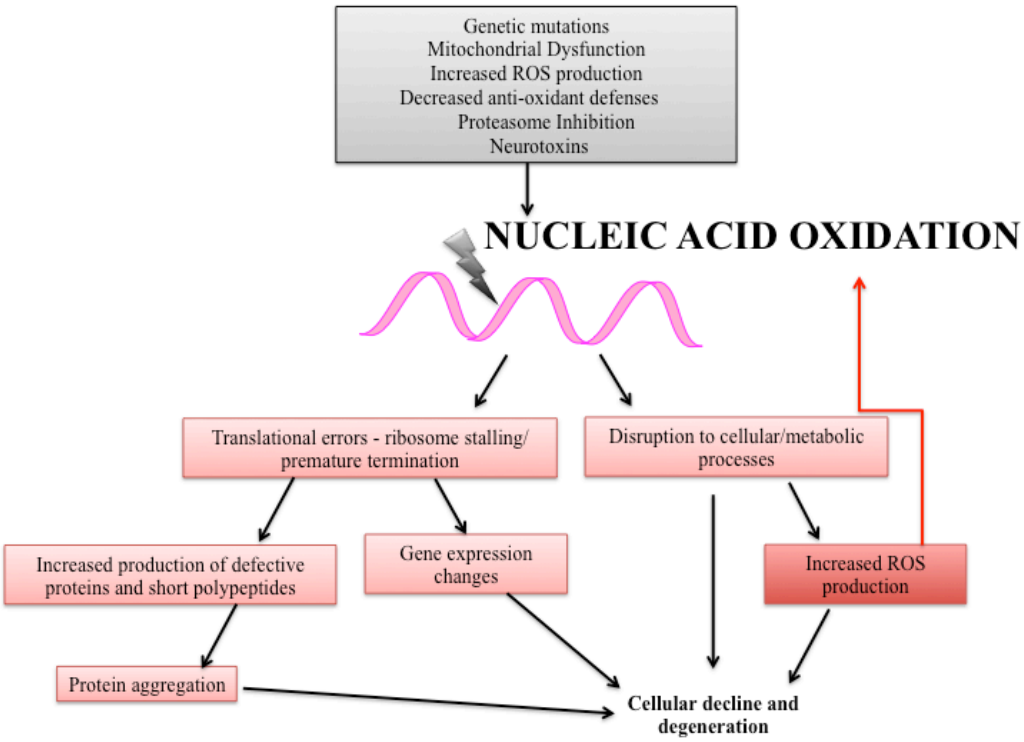


Figure 1.9: The consequences of nucleic acid oxidation

Oxidative damage to nucleic acids may alter the genetic information carried by nuclear and mitochondrial DNA and RNA. The potential detrimental effects of nucleic acid modification to nucleic acids are shown here. Oxidative damage may affect the fidelity of transcriptional and/or translational processes, lading to erroneous protein production and disturbances to cellular and metabolic processes.

1.6.2 Nucleic acid oxidation prevention and repair

1.6.2.1 Oxidative DNA damage

Cells have evolved intricate mechanisms for DNA and RNA damage prevention, detection, and removal. The DNA repair mechanisms constitute a complex system and defects in maintaining genome integrity are associated with aging and disease development (Hoeijmakers 2009). DNA damage in neurons can be repaired through the direct reversal of the modified bases, the removal of a modified base or small lesions by a base excision repair (BER), the removal of bulky, helix distortion lesions by nucleotide excision repair (NER), the correction of single base mismatches or deletions by mismatch repair (MMR), or repair of DSBs by the non-homologous end joining (NHEJ) pathway (Karran 2000, Kolodner and Marsischky 1999, Krokan*, et al.* 2000, Nakabeppu*, et al.*

2004). Oxidative damage to DNA includes single strand breaks (SSBs), double strand breaks (DSBs), base modifications, abasic sites, and DNA-protein cross-links. The BER pathway is believed to be the major pathway for repairing oxidative modifications to DNA (Fishel*, et al.* 2007). The first step in BER is the recognition of the damaged base by a substrate specific DNA glycosylase. Most DNA glycosylases have broad substrate specificities but can have preferences for either purines or pyrimidines. The major bifunctional glycosylase for purines is OGG1, which initiates excision of 8-OHdG from resting DNA, in which the oxidised guanine is paired with cytosine (Klungland*, et al.* 1999, Minowa*, et al.* 2000). Inefficient removal of the oxidised base may lead to mutagenesis and gene expression changes if the transcript is transcribed. Investigations into the coding properties of 8-OHdG demonstrated that the modified transcript does not block DNA replication catalysed by DNA polymerase and the enzyme can incorporate either adenine or cytosine opposite 8-oxoG (Grollman and Moriya 1993). MUTYH, the human homolog of the MutY protein in E.Coli, was discovered to act as an additional step of surveillance in mammalian cells to prevent mutagenesis by excising adenine incorporated opposite 8-OHdG from the progeny strand during replication (Michaels and Miller 1992, Slupska*, et al.* 1999). Endonuclease III-like protein UNG, and Nei-like DNA glycosylases NEIL-1 and 2 remove most of the oxidised pyrimidine bases (Krokan and Bjoras 2013).

The NHEJ pathway is a predominant form of DSB repair in mammalian cells, and of essential importance in post-mitotic neurons (Subba Rao 2007). Initiation of the pathway is governed by phosphoinositide-3-kinase related protein kinases (PIKKs) and accessory factors that directly sense the DNA damage and initiate a cascade of protein phosphorylation events that ultimately determine cell fate (Ciccia and Elledge 2010, Sancar*, et al.* 2004). The PIKKs participate in signal transduction pathways involved in DNA repair genome maintenance. They function as redox sensors, and respond to various stresses by phosphorylating targets in appropriate pathways. DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated (ATM), which are members of the superfamily of PIKKs, transduce the signal for DNA damage. The members of the superfamily are grouped on the basis of a high degree of sequence similarity in the C-terminal region and possess serine/threonine kinase activity (Abraham 2004). The Mre11-Rad50-Nbs1 (MRN) complex is a DNA damage sensor in mammals that is rapidly recruited to DSB sites in DNA (Stracker*, et al.* 2004), and is involved in the early steps of propagating the DNA damage signal. DSBs are recognised and bound by the heterodimeric regulatory subunits of DNA-PK (Ku80/Ku70) thereby recruiting the catalytic DNA-PK (Sawchuk*, et al.* 2004, Yoo and Dynan 1999), which can activate p53

through phosphorylation of the amino terminal site and trigger the onset of DNA repair or apoptosis (Soubeyrand*, et al.* 2004). H2A histone family member X (H2AX) is a substrate of some of the PIKKs, and is phosphorylated on serine 139 in response to DSB formation to become γ-H2AX (Rogakou*, et al.* 1998, Stiff*, et al.* 2004), with which many DDR proteins interact to initiate repair and maintain genome stability (Fernandez-Capetillo*, et al.* 2004, Stucki and Jackson 2006).

1.6.2.2 Oxidative RNA damage

By comparison, studies of mechanisms for the protection or removal of RNA damage have been limited to investigation into RNA surveillance and degradation. It remains to be determined whether repair processes for the removal of RNA lesions exist. Controversy surrounds the action of repair processes for RNA as it may be more efficient for cells to degrade damaged RNA rather than repair it (Krokan*, et al.* 2004). Multiple enzymes are able to recognise the same target RNAs, making redundancy a general feature and enhancing the overall efficiency of degradation pathways (Houseley and Tollervey 2009). RNA turnover may also represent an additional surveillance mechanism for the removal of aberrant RNA transcripts, thereby increasing the quality control of mRNA biogenesis (section 5.1.4). The emerging role of ncRNAs in gene expression regulation, chromatin structure, and translation highlights the importance for rapid turnover of damaged transcripts in cellular homeostasis. RNA surveillance pathways appear to act on all classes of RNA, efficiently identifying and degrading defective RNA transcripts. Insights into how nuclear RNA surveillance play an important role in regulating eukaryotic gene expression, adds to the complexity of RNA metabolism and presents potentially unexplored ways of cells to regulate the quality of RNA.

Although uncertainty surrounds the direct repair of RNA, specific enzymes known as ribonucleases (RNases) efficiently remove damaged RNA transcripts from the cell to ensure efficiency of RNA metabolism and processing. The three major classes of intracellular RNases are the endonucleases that cut RNA internally, the 5' exonucleases, and the 3' exonucleases. Whether selective degradation by specific RNases act to repair or remove the oxidised bases in RNA remains unresolved (Deutscher 2006, Li*, et al.* 2006). The E.coli polynucleotide phosphorylase (PNPase), a 3'-5' exonuclease, is important for mRNA turnover and degradation of aberrant non-coding RNAs (Deutscher 2006). *In vivo* experiments have suggested human PNPase (hPNPase) is a candidate for the turnover of oxidatively damaged RNA, demonstrating binding specificity for the

modified transcripts (Hayakawa, *et al.* 2001, Hayakawa and Sekiguchi 2006). The knockdown of PNPase in HeLa cells and their subsequent exposure to hydrogen peroxide resulted in increased levels of 8-oxoG containing RNA and reduced cell viability (Wu and Li 2008). Y-box binding protein (YB-1) is another candidate shown to be active in the handling of oxidatively damaged RNA. In E.coli the gene confers high resistance of the bacterial cell to oxidative stress, and has been found to regulate processes including transcription and translation (reviewed in Kohno, *et al.* 2003), and to aid in the winding and unwinding of RNA duplexes (Skabkin, *et al.* 2001). *In vitro* experiments have demonstrated YB-1 to bind specifically to 8-oxoG-containing oligonucleotides (Hayakawa, *et al.* 2002), and knockdown of YB-1 in mouse embryonic fibroblasts results in increased sensitivity to oxidative stress (Lu, *et al.* 2005). YB-1 has also been found to localise to P-bodies and stress granules where active mRNA degradation occurs (Yang and Bloch 2007), further indicating a potential role in RNA surveillance. However, the numerous roles of YB-1 make interpretation of the phenotypes difficult, as it may be involved in stress resistance at multiple levels. YB-1 has the capacity to stimulate the base excision repair activity of the nei endonuclease VIII-like 2 (NEIL2) protein, a DNA glycosylase which removes oxidised bases from DNA (Das, *et al.* 2007), demonstrating its involvement in the handling of oxidatively damaged nucleic acids. While oxidatively damaged RNAs are considered to be degraded rather than repaired these findings suggest a model wherein YB-1 binding to 8-oxoG-containing RNA may assist removal of these RNAs from the cells through the action of other proteins. However, this remains uncertain and it could be another surveillance mechanism generally targeting defective transcripts for removal.

The discovery of a mechanism of RNA alkylation repair (Aas, *et al.* 2003) suggested that other unidentified processes for the repair of RNA might exist, and cells may invest more into the protection of RNA than initially thought (Bregeon and Sarasin 2005, Krokan, *et al.* 2004). This would be understandable considering the identification of multiple metabolic and processing features of RNA required for normal cellular function. Alkylation damage in RNA is repaired by the human homologue hABH3 of AlkB, a DNA repair enzyme for E.coli, by oxidative demethylation of the damaged DNA and RNA bases (Aas, *et al.* 2003, Duncan, *et al.* 2002). The major apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) participates in base excision repair of DNA and activation of stress inducible redox transcription factors (Jayaraman, *et al.* 1997). APE/Ref-1 has also been suggested to possess activity toward RNA (Barnes, *et al.* 2009) and plays a role in the rRNA quality control process (Vascotto, *et al.* 2009b). Human APE/Ref-1 in APE/Ref-1 knockdown cells the ability to remove 8-hydroxyguanine-

containing rRNA upon oxidative damage is observed, along with impaired translation and reduced cell growth rate (Vascotto*, et al.* 2009b).

1.6.2.3 Oxidative damage to the nucleotide pool

Since the persistence of 8-oxoG in RNA would cause a reduction in the fidelity of gene expression, organisms must have a mechanism for scavenging oxidised molecules. Oxidation of nucleotides can occur in the cellular nucleotide pool, which can then be incorporated into newly synthesised DNA or RNA. Avoidance of such incorporation would provide a mechanism of coping with and possibly preventing nucleic acid damage (Bellacosa and Moss 2003, Bregeon and Sarasin 2005, Li*, et al.* 2006). Here we focus on what is known about 8-oxoG, however the nucleotide pool is likely to contain numerous oxidised nucleoside, nucleotides, and many of the intermediate products formed from these.

Proteins that specifically bind to oxidatively damaged RNA and selectively eliminate mRNA containing 8-oxoG have been reported to preferentially discriminate the oxidised transcripts (Hayakawa*, et al.* 2010, Hayakawa*, et al.* 2001, Hayakawa and Sekiguchi 2006, Hayakawa*, et al.* 2002). Preventing the incorporation of oxidised bases into nucleic acids may also be an essential mechanism for minimising damage in both DNA and RNA (Li*, et al.* 2006). The MutT protein in E.Coli hydrolyses 8- hydroxydeoxyguanosine triphosphate (8-oxodGTP) and 8- hydroxyguanosine triphosphate (8-oxoGTP) to their monophosphate forms thus removing the oxidised bases from the nucleotide pool and preventing their incorporation into DNA and RNA respectively (Maki and Sekiguchi 1992). Several mammalian MutT homolog hydrolases, including human oxidised purine nucleoside triphosphatases 1 and 2 (MTH1, MTH2) and nucleoside diphosphate linked moiety X type 5 (NUDT5) have been identified (Ishibashi*, et al.* 2003). These proteins all share a nudix motif, and are collectively called nudix proteins because most of them possess magnesium requiring enzyme activities to catalyse the hydrolysis of nucleoside diphosphates that are linked to another moiety. MTH1 efficiently hydrolyses 2-hydroxydeoxyadenosine triphosphate (2-oxodATP), 8-hydroxydeoxyadenosine triphosphate (8-oxodATP), 8-oxodGTP, and their corresponding ribonucleotides thus preventing their incorporation into DNA or RNA (Furuichi*, et al.* 1994, Nakabeppu 2001, Nakabeppu*, et al.* 2004, Sakumi*, et al.* 1993). Two other proteins containing the nudix motif, MTH2 (NUDT15) and NUDT5, were identified with the potential to hydrolyse either 8- hydroxydeoxyguanosine triphosphate (8-oxodGTP) or 8-

hydroxydeoxyguanosine diphosphate (8-oxodGDP) to 8-hydroxydeoxyguanosine monophosphate (8-oxodGMP), respectively (Cai, *et al.* 2003, Ishibashi, *et al.* 2005, Ishibashi, *et al.* 2003). A study investigating the hydrolysing properties of the NUDT18 protein, found the protein to hydrolyse 8-oxodGDP and 8-oxoGDP to their monophosphate forms. NUDT18 was subsequently found to hydrolyse 8-hydroxydeoxyadenosine diphosphate (8-oxodADP) and 2-hydroxydeoxyadenosine diphosphate (2-oxodADP), and has a sequence closely related to those of MTH1 and MTH2, suggesting it is to be named MTH3 (Takagi, *et al.* 2012). This presents an alternative to repair mechanisms, where supplementary support is in place to maintain high-quality RNA ensuring transcriptional fidelity. Evidence in support of these proteins comes from *in vivo* studies demonstrating an up-regulation of MTH1 expression in response to oxidative stress. Another study showed significantly higher levels of RNA oxidation in the hippocampus of MTH1-null rats (Kajitani, *et al.* 2006). Increased expression of MTH1 in vulnerable neuronal populations of the brain has been reported in AD (Furuta, *et al.* 2001) and PD (Shimura-Miura, *et al.* 1999) patients, which may represent an up-regulation in response to oxidative stress.

1.6.3 Nucleic acid oxidation in neurodegenerative disease

Previous work investigating RNA oxidation in neurological disorders forms the basis for its importance and investigation in ALS. Immunocytochemical and biochemical studies have shown that RNA oxidation is increased in neurological disorders, and the regional distribution of the damage is consistent with selective neuronal vulnerability (Nunomura, *et al.* 2002, Nunomura, *et al.* 1999, Zhang, *et al.* 1999). The presence of oxidatively damaged DNA and RNA in the post-mortem brains of AD (Nunomura, *et al.* 1999) and PD (Zhang, *et al.* 1999) cases has been demonstrated through *in situ* approaches using the 8-hydroxydeoxyguanosine/8-hydroxyguanosine (8-OHdG/8-OHG) specific antibodies. In both studies immunoreactivity was predominantly localised in the cytoplasm, and there was a significant increase in intensity in patients, compared to age-matched controls. Immunostaining of DNase treated post-mortem brain sections from these AD and PD patients revealed increased 8-OHG content of RNA in vulnerable neuronal populations. RNA oxidation appeared prominent in the frontal, temporal, and entorhinal cortex, hippocampus, and subiculum in AD patients, and in the substantia nigra in PD patients. Double labelling using an 8-OHG antibody and either neuron or astrocyte specific markers further demonstrated that oxidation occurred in the distinct groups of vulnerable

neurons that degenerate and have previously been shown to degenerate in these neurological disorders.

Immunocytochemistry of neurons from the cerebral cortex of double knock-in mice expressing familial AD linked mutations in amyloid precursor protein (APP) and Presenilin 1 (PS1) displayed increased RNA oxidation (Lovell and Markesbery 2008, Lovell*, et al.* 2011). Studies on post-mortem brain tissue from mild cognitive impairment (MCI) subjects have also demonstrated increased oxidation/nitration to proteins, lipid peroxidation, and DNA oxidation (Butterfield*, et al.* 2006, Keller*, et al.* 2005). A study of familial PD revealed elevated 8-OHG immunoreactivity in cells which had not developed α- synuclein aggregation, a hallmark of neuronal degradation (Nunomura*, et al.* 2004), supporting suggestions that RNA oxidation is an early event in the pathogenesis of neurodegenerative diseases, and not merely a consequence of dying cells. However, recent studies of RNA oxidation in vulnerable neurons of preclinical AD have shown inconsistent results. One study reported a significant increase in the level of 8-OHG immunoreactivity in preclinical AD patients in comparison to controls (Lovell*, et al.* 2011), which was not observed in another study (Nunomura*, et al.* 2012). The differences seen may have been due to the areas of the brain studied. The former study, that identified differences, was investigating damage in the hippocampus whereas the latter study was carried out in the cerebral neocortex. This result coincides with other work, which has also shown increased levels of oxidation products in hippocampus but not neocortex, and highlights the potential selective neuronal vulnerability to oxidative damage during different disease situations (Aluise*, et al.* 2011, Bradley*, et al.* 2010).

In a mixed astrocyte and neuron culture model, increased RNA and DNA oxidation was observed following proteasome inhibition, which also has been described as a feature of several neurodegenerative disorders (Ding*, et al.* 2004). Exposing primary rat cortical cultures to hydrogen peroxide and other oxidative insults caused prominent oxidative damage to RNA four hours post-treatment, as shown by immunoreactivity for 8-OHG, with diminished reactivity at ten hours post-treatment. RNA oxidation occurred at an early stage and was identified in a distinct group of neurons that later died. Treatment of cultures with RNase diminished fluorescence confirming it was RNA that been primarily oxidised (Shan*, et al.* 2007). The cytoplasmic predominance of oxidised RNA suggests that during disease states enhanced ROS production by the mitochondria inflicts damage on surrounding species.

Biochemical approaches have additionally been used to isolate and identify oxidised RNA species. Northwestern blotting using a monoclonal 8-OHG antibody demonstrated significant amounts of poly(A)+ mRNAs are oxidatively damaged in the brains of AD patients. Subsequent Southern blotting of the oxidised and non-oxidised RNA species revealed selective oxidative damage in AD frontal cortex but not in AD cerebellum or control samples. Quantitative polymerase chain reaction (RT-qPCR) and filter analysis revealed some mRNA species to be more susceptible to damage (Shan*, et al.* 2003). The transcripts targeted had either been characterised in AD or their protein functions had been implicated in AD pathogenesis. Among these were p21ras protein (Gartner*, et al.* 1999), carbonyl reductase (Balcz*, et al.* 2001), SOD1 (Omar*, et al.* 1999), Apo D (Terrisse*, et al.* 1998) and transferrin (Loeffler*, et al.* 1995). Further quantification of oxidised mRNAs by immunoprecipitation revealed 52.3 ± 6.15% of mRNAs contained 8-oxoG in the frontal cortices of AD patients compared to 1.78 ± 0.56% in age-matched control (Shan and Lin 2006). However, the magnitude of RNA oxidation in patients diagnosed with advanced stage AD was lower, which may be expected due to RNA loss as the neurons degenerate.

Oxidation of mRNA has also been observed in neurons in the motor cortex and spinal cord of ALS patients (Chang*, et al.* 2008), demonstrating this to be a feature common to neurodegeneration. Using a mouse model of ALS, oxidation was observed to be most prominent at the early pre-symptomatic stage and then to have subsided at later stages of disease. This supports previous studies of RNA oxidation in other neurodegenerative disorders, and demonstrates oxidative damage in vulnerable motor neurons and glia of the ALS-affected areas precedes MN death. Further investigation of the oxidised mRNA species by microarray analysis revealed that some species are more susceptible to oxidation. Furthermore, the identified oxidised mRNAs had already been described in relation to ALS. The role of mRNA oxidation in disease progression appears complex, as vitamin E treatment blocked mRNA oxidation and delayed disease onset but did not alter mean lifespan.

The data presented provides a firm basis for implicating oxidative damage to nucleic acids as a pathogenic mechanism underlying neurodegenerative disorders, including ALS. Oxidation as an early event in ALS pathogenesis suggests it may reduce the affected neurons resistance to other forms of damage. ALS is a multi-factorial disease and it is unlikely that there is any one initiating factor. It may be a multitude of disorders with a common phenotype; there might be many overlapping initiating factors rather than a primary cause for disease; alternatively an as of yet unidentified single specific factor

may cause ALS. These proposed mechanisms are described in the previous section, and although the relative importance of oxidative stress, the variety of damage it may cause, and the relationship of these consequences to neurodegeneration remains unknown, the complexity of ALS suggests it acts in combination.

1.6.4 Consequences of oxidative damage to RNA

Little work has focused on the relationship between oxidant-induced impairments in cellular function and the selective neuronal death that follows. Generally oxidative modifications may contribute towards disease pathogenesis by reducing protein expression/function, disrupting cellular and metabolic processes, and causing a redundancy in the defence and repair pathways elicited by cells to protect against damage (Uttara*, et al.* 2009). Possibly the most abundant result of oxidation is direct strand breaks, which have been suggested to constitute 40-90% of the reactions (Poulsen*, et al.* 2012). One mechanism for this, involves the initial formation of a nucleobase radical, which subsequently attracts a hydrogen atom from the ribose ring, ultimately resulting in strand scission (Jacobs*, et al.* 2011).

After the description of RNA oxidation as being a feature of neurodegeneration, work began to characterise the consequences RNA oxidation and how these may contribute towards the pathogenesis of neurodegenerative diseases. Luciferase mRNA was synthesised *in vitro* then treated with hydrogen peroxide in order to cause oxidative damage. Rabbit reticulocyte lysate was used to translate the oxidised RNA into protein, and their functional activities and protein levels subsequently analysed. In the oxidised mRNA samples, a reduction in both luciferase activity and protein level of the damaged mRNA was seen (Shan*, et al.* 2003). Furthermore, immunostaining of cells transfected with either oxidised or non-oxidised luciferase mRNA displayed the presence of protein aggregation in the cells containing the oxidised mRNA, potentially due to the formation of truncated or abnormal proteins that have a tendency to aggregate. The production of short polypeptides from the oxidised mRNA was observed in the presence of proteasome inhibitors, which may be due to both premature termination of translation and protease degradation of aberrant full-length protein (Tanaka*, et al.* 2007). Polyribosomes isolated from vulnerable brain regions of AD patients displayed a reduction in protein synthesis in an *in vitro* assay suggesting that oxidation of ribosomal RNA (rRNA) leads to defects in rRNA processing and ribosomal function (Ding*, et al.* 2005), associated with a reduced rate and capacity for protein synthesis. Other studies show that oxidation of specific

classes of mRNAs in the affected regions of AD brains is associated with low protein expression for those genes (Shan*, et al.* 2007), which may be cause by ribosomal stalling on the oxidised transcripts, and oxidised mRNA can induce translational errors leading to short polypeptides and/or degradation of the truncated protein (Shan*, et al.* 2007, Tanaka*, et al.* 2007). While oxidation of RNA transcripts may not fully inhibit protein synthesis it is clear that the fidelity of translation is affected.

1.7 Summary of Aims

Oxidative stress has been extensively studied with relation to the mechanisms contributing towards ageing and ALS pathology. Nucleic acid oxidation has been reported to increase during ageing and be prominent in areas of selectively neuronal vulnerability in ALS and other neurological disorders. The hypothesis that increased oxidative stress and nucleic acid oxidation contributes towards cellular decline and degeneration in ageing and ALS was to be investigated. Specific classes of RNA are targeted for oxidative modification in a murine model of familial-ALS; however it is currently unknown whether similar classes are also targeted for modification during ageing. The aim was to investigate differential gene expression changes due to oxidative modification of RNA during ageing, and identify genes selectively enriched within the oxidised fraction. We wanted to investigate whether the differentially enriched genes are enriched in pathways previously implicated in neurodegeneration, to link our gene expression profiling data in an aging study to age-associated diseases. The second aim was to investigate whether the presence of different *SOD1* mutations influences the amount of oxidative damage to RNA and determine the susceptibility of the mutations to oxidative stress related mitochondrial dysfunction and cellular decline.

Chapter 2
Materials and Methods

2.1 Materials

All commonly used chemicals were purchased from Melford laboratories (UK), unless otherwise specified. Analytical grade solvents ethanol, methanol and isopropanol were purchased from Fisher Scientific (UK). RNase free water was purchased from Ambion (UK). Filtered pipette tips were purchased from Fisher Scientific (UK). Primers for PCR and quantitative PCR (qPCR) were purchased from Eurofins MWG Operon (Germany).

For RT-qPCR, the Quantitect Reverse Transcriptase kit from Qiagen (UK) was used for cDNA synthesis. SYBR green master mix was purchased from Agilent (UK). PCR plates and sealing caps were purchased from BIOplastics (The Netherlands). SYBR Green was purchased from Agilent Technologies (UK). For PCR product electrophoresis, agarose was purchased from Melford Laboratories (UK), DNA Hyperladders IV and V, and 5x DNA loading buffer were from Bioline (UK).

For cell culturing of the mouse motor neuron-like hybrid cell line (NSC34), Dulbecco's Modified Eagle Medium (DMEM) media was purchased from Gibco (UK), fetal calf serum was from Biosera (UK), and Geneticin Selective Antibiotic (G418 Sulfate) was from Invitrogen (UK). Transfection of NSC34 cells was carried out using the mammalian expression vector pIRESneo from Clontech, Saint-Germain (France), using Lipofectamine 2000 from Invitrogen (UK) for delivery of the vector into cells. For cell viability assays, hydrogen peroxide and trypan blue were purchased from Sigma (UK).

For RNA extraction from NSC34 cells and mouse tissue, the RNeasy Mini Kit from Qiagen (UK) was used. GlycoBlue reagent for RNA precipitation was purchased from Ambion (UK). RNA concentration was measured on the Nanodrop Spectrophotometer (ND1000) supplied by Labtech International (UK).

For RNA immunoprecipitation, protein L beads were purchased from Thermo Scientific (UK). Pheynl chloroform isoayml alcohol (PCI) was purchased from Sigma (UK), and the 8-hydroxyguanosine 15A3 mouse monoclonal antibody was from Abcam (UK).

For the microarray study, 3' IVT Express amplification kits were purchased from Affymetrix (UK). All the materials and reagents required for assessing RNA quality on the Agilent 2100 Bioanalyzer were purchased from Agilent (US). Mouse Genome 430 2.0 GeneChip Microarrays and reagents used for RNA labelling, fragmentation, GeneChip hybridisation, and GeneChip stringency washes were purchased from Affymetrix (UK).

For Western blotting, protein concentration was measured by BCA assay, with reagents and protein standards purchased from Pierce (UK) and Thermo Scientific (UK) respectively. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was prepared using SDS from Melford Laboratories (UK), 30% acrylamide from National Diagnostics (UK), ammonium persulphate (APS) from Sigma (UK), and Tetramethylethylenediamine (TEMED) from Melford Laboratories (UK). Polyvinyl difluoride (PVDF) Immobilin transfer membrane was purchased from Millipore (US). Anti-mouse and anti-rabbit secondary antibodies were from DakoCytomation (Denmark). An enhanced chemiluminescence (ECL) kit for chemiluminescence based-immunodetection of horseradish peroxidase (HRP) was purchased from Biological Industries (UK). Development of membranes was performed on the G:BOX, purchased from Syngene, a division of the Synoptics Group (UK).

For metabolic activity assays, XF-24 Flux Packs and XF-assay media were purchased from Seahorse Bioscience (US). Gelatin was purchased from Sigma (UK). Oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone were purchased from Sigma (UK), and Calcein was purchased from Invitrogen (UK). Glucose and glutamine added to the XF-assay media were both purchased from Sigma (UK).

For mitochondrial morphology assessment rhodamine 123 was purchased from Invitrogen (UK). Cells were plated in to Lab-Tek chamber slides from Fisher Scientific (UK). Imaging was performed on a resonant scanning confocal microscope supplied by Leica Microsystems (Germany).

For immunohistochemistry, Haematoxylin and Eosin stains were purchased from Leica (UK) and cresyl violet stain purchased from Raymond A. Lamb, now Fisher Scientific (UK). Vectastain Elite ABC kits for mouse IgG and rabbit IgG along with the bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) alkaline phosphatase substrate kit were purchased from Vector Laboratories (UK). Imaging was carried out on the BX61 upright microscope supplied by Olympus (UK).

2.1.1 Solutions

50mM Phosphate buffered saline (PBS)

3.2mM Na_2HPO_4, 0.5mM KH_2PO_4, 1.3mM KCl, 135mM Nacl, pH 7.4

PBS-Tween (PBST)

0.05% (v/v) Tween-20 (Sigma, UK) in 50mM PBS

PBS-NP40

0.04% NP-40 in 50mM PBS

10% Sodium Dodecyl Sulphate (SDS)

10% (w/v) SDS in deionised water

Sodium Acetate

3M NaOAc, adjust to pH 5.2 with glacial acetic acid

Tris-acetate-EDTA (TAE) (1x)

40mM Tris base (Sigma, UK), 20mM acetic acid (Fisher Scientific, UK), 1mM EDTA (Sigma, UK), pH 8.0

Extra strong lysis buffer

100mM TrisHCL, 7mM NaCl, 0.5% (w/v) SDS, 1% (v/v) Triton X-100, 2mM Na_3VO_4, 1.25mM NaF, 1mM $Na_4P_2O_7$, 10mM EDTA

Running buffer (10x)

144g Glycine, 30.2g Tris, 10g SDS, made up to 1L with deionised water, 1x working solution

Transfer buffer (10x)

144g Glycine, 30.2g Tris, made up to 1L with deionised water, 1x working solution

Resolving buffer (4x)

1.5M Tris-HCL, 0.4% (w/v) SDS

Stacking buffer (4x)

0.5M Tris-HCL, 0.4% (w/v) SDS

10% Ammonium Persulphate (APS)

0.9g APS in 9ml deionised water

Laemlli sample buffer (2x)

1.5M Tris-HCL, 4% (w/v) SDS, 100mM dithiothreitol (DTT), 20% (w/v) Glycerol, 0.02% (w/v) Bromophenol blue (Fisher Scientific)

50mM Tris-buffered saline (TBS)

50mM Tris, pH 7.6, 150mM NaCl

TSB-Tween (TBST)

0.1% (v/v) Triton X-100 in 50mM TBS

4% Paraformaldehyde (PFA)

4% (w/v) PFA in 50mM PBS

Blocking buffer

5% (w/v) powdered milk in PBS-Tween (0.05%)

Trisodium Citrate buffer (TSC)

3g $Na_3C_6H_5O_7$, made up to 1L with deionised water, pH6

Cresyl Violet stain

0.1% (w/v) Cresyl Violet, 0.05% (v/v) acetic acid in distilled water

Blocking solution

15% (v/v) normal goat serum or 15% (v/v) normal horse serum (Vector Laboratories UK, antibody dependent) in 50mM TBST

Avidin-biotin peroxidase complex

Vectastain Elite ABC Kit (Vector Laboratories, UK) in 50mM TBS

Protein molecular weight marker

Precision plus protein dual colour standards (Bio-Rad, UK)

Supplemented XF-assay media

XF-assay media (Seahorse Biosciences), 25mM glucose, 2mM glutamine (pH 7.4 at 37°C)

2.1.2 Antibodies

Table 2.1: *IHC primary antibodies*

Antibody	*Species*	*Dilution and conditions*	*Antigen retrieval*	*Source*
8-OHG	Mouse monoclonal	1/200 1 hour RT	Pressure cooker TSC pH6	Abcam
H2AX	Rabbit polyclonal	1/2000 1 hour RT	Pressure cooker TSC pH6	R&D Systems
GFAP	Rabbit polyclonal	1/1000 Overnight 4°C	Pressure cooker TSC pH6	DakoCytomation
OGG1	Rabbit polyclonal	1/50 1 hour RT	Pressure cooker TSC pH6	Abcam
DNA-PK	Mouse monoclonal	1/100 1 hour RT	Pressure cooker TSC pH6	Abcam

Table 2.2: *SDS-PAGE primary antibodies*

Antibody	*Species*	*Dilution*	*Source*
SOD1	Sheep monoclonal	1/1000	Calbiochem
Actin	Mouse monoclonal	1/1000	Abcam
Tubulin	Mouse monoclonal	1/1000	Sigma

Table 2.3: *SDS-PAGE secondary antibodies*

Antibody	*Species*	*Dilution*	*Source*
Goat anti-mouse HRP	Mouse polyclonal	1/5000	Abcam
Goat anti-rabbit HRP	Rabbit polyclonal	1/5000	Dako

2.1.3 Mice Details

All mouse experiments were carried out in accord with the Animals (Scientific Procedures) Act 1986 under a UK Home Office project license. Animals were housed and cared for according to the Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (http://www.homeoffice.gov.uk/publications/science-research-statistics/animals/transposition_of_eudirective/codeofpracticegeneralsection).

2.1.3.1 G93A mutant *SOD1* transgenic mice

G93A mutant *SOD1* transgenic mice, B6SJL-Tg (SOD1-G93A) Gur/J (002726), were obtained from the Jackson Laboratory (USA) and backcrossed onto the C57BL/6 background from Harlan (UK) for 20 generations to create a line with a inbred C57BL/6 genetic background. This reduces genetic variation to improve the data generated using this model. The *SOD1* G93A transgene is maintained as a hemizygous trait by breeding hemizygous males with wild-type females.

The disease course and motor function of these mice were investigated in comparison to normal mice (Mead*, et al.* 2011). These *SOD1* G93A mice typically survive for 140 days. Mice are routinely checked daily from 125 days of age for signs of distress. 30-60 day old transgenic mice are classed as presymptomatic stage of disease; however a reduction in motor function is observed from approximately 45 days of age (Mead 2011). 90-120 day old transgenic mice are classed as symptomatic, and can be identified readily by their phenotype by 90 days of age. The natural lifespan of a non-transgenic mouse in the lab is typically about 24 months.

2.1.3.2 Aged mice

For the microarray study, the spinal cord from the common inbred laboratory C57BL/6 (Harlan, UK) mice aged six, twelve, eighteen months was used. These mice typically live for two years in a laboratory (Curtis 1971, Goodrick 1975), and therefore six-month animals were classified as the young animals, twelve month animals were classified as middle aged, and eighteen month old mice were classified as old.

2.2 Methods

2.2.1 Microarray

2.2.1.1 Preparation of materials for gene expression analysis

2.2.1.1.1 Tissue preparation

Mice were euthanased with an overdose of anaesthetic (intraperitoneal injection of approximately 2.5ml/kg pentobarbitone). Mice were perfuse-fixed with 30% sucrose in PBS whilst under terminal anesthesia. The whole spinal cord was dissected and split into upper and lower sections, before mounting and frozen on dry ice. Subsequent storing of tissue was at -80°C.

2.2.1.1.2 Dissection of anterior horn

Dr. Ke Ning kindly dissected the anterior part of the thoracic spinal cord from the aged mice. This was performed in PBS and samples were transferred to ice-cold lysis buffer (RNeasy kit, Qiagen), and RNA extraction carried out immediately.

2.2.1.1.2 RNA extraction from anterior horn

RNA extraction was carried out using the RNeasy kit (Qiagen), according to the manufacturer's protocol. Briefly, tissue was disrupted in lysis buffer using a hand-held homogeniser. An equivalent volume of ethanol was added to the lysate and this was transferred to a spin column. The RNA is bound to the column by centrifugation. The column is subsequently washed three times and the RNA eluted in RNase free water.

2.2.1.1.3 Determination of RNA concentration

RNA concentration was determined using the Nanodrop Spectrophotometer (Labtech International). For this 1µl of sample was pipetted on to the spectrophotometer pedestal. Fibre optic technology and surface tension hold the sample in place between two optical surfaces, forming a liquid column. The sample is assessed at both a 1mm and 0.2mm path, as determined by the gap between the optical surfaces (Gallagher and Desjardins 2007). The software automatically calculates the RNA concentration.

2.2.1.1.4 Immunprecipitation of oxidised RNA

Fractions of oxidised and non-oxidised RNA were separated by immunoprecipitation using the 8-hydroxyguanosine (8-OHG) antibody (Abcam). Total RNA was extracted from the mouse cord using the RNeasy mini kit as described in section 2.2.1.1.2. The tissue was homogenised in lysis buffer using a hand-held homogeniser and RNA was extracted as outlined in section 2.2.1.1.2. Following extraction, RNA concentration was determined on the Nanodrop Spectrophotometer (section 2.2.1.1.3). 1µg total RNA was incubated with 1.5µg 8-OHG antibody for six hours at 4°C, with regular mixing. 40µl of protein L beads (Thermo Scientific, UK) were added to each sample and mixed thoroughly. Samples were incubated for 16 hours at 4°C. Samples were centrifuged at 4°C for five minutes at 15,000 rcf. The supernatant i.e. the non-oxidised fraction was aspirated in to a fresh eppendorf and stored at -20°C. Two washes in 200µl of PBS-NP40 were carried out. Samples were centrifuged at 4°C for five minutes at 15,000 rcf. 300µl PBS-NP40, 30µl 10% SDS, and 1ml phenyl chloroform isoamyl alcohol were added to each oxidised fraction, and incubated at 37°C for 30-40 minutes, vortexing every five minutes, until the aqueous fraction was clear. Samples were centrifuged at 4°C for five minutes at 15,000 rcf, and the aqueous fraction aspirated in to a fresh eppendorf. The organic phase was discarded. 1ml 95% ethanol, 40µl 3M sodium acetate, and 2µl of 5mg/ml glycoblue were added sequentially to each sample. The samples were frozen at 80°C for one hour and centrifuged for 25 minutes at 4°C. The pellet was washed with 75% ethanol, air-dried and then resuspended in 10µl of nuclease free water.

2.2.1.1.5 Quality assessment of RNA

Following immunoprecipitation, the quality of the RNA was analysed on the Agilent 2100 Bioanalyser (Agilent, Palo Ato, CA) using an RNA 6000 Nano kit. This allows analysis of 5-500ng/µl RNA by calculating the 28s/18s ribosomal ratio and providing a qualitative assessment of RNA integrity.

2.2.1.1.6 Linear Amplification of RNA

Oxidised and non-oxidised fractions of RNA were linearly amplified and biotin labelled using the 3' IVT Express Kit (Affymetrix) according to the manufacturer's protocol. Briefly, an oligo-dT primer containing a T7 RNA polymerase promoter anneals to the poly-A tail of mRNA, and a first strand cDNA is synthesised. Following second strand synthesis, a double stranded cDNA molecule with the T7 polymerase promoter

incorporated in its sequence is generated. This is used as a template to produce multiple single stranded biotin-labelled complementary RNA (aRNA). The aRNA is purified using RNA binding beads to remove enzymes, salts, and unincorporated nucleotides.

Following elution of the purified aRNA from the RNA binding beads, the quantity of aRNA generated was analysed on the NanoDrop spectrophotometer (as described in section 2.2.1.1.3). To assess RNA quality before running the GeneChips, 1µl aRNA was loaded on to Nano LabChip 6000 and run on the Agilent 2100 Bioanalyser.

The efficiency of the amplification process was assessed by the poly-A RNA control kit (Affymetrix), which involves the addition of serial concentrations of *in vitro* synthesised polyadenylated transcripts corresponding to the *B.subtilis* genes*, lys, phe, thr* and *dap*, to the RNA during the first amplification cycle. The amplification and target labelling process is monitored based on the expression levels of probe sets corresponding to the poly-A exogenous controls genes on the Eukaryotic GeneChip array.

2.2.1.2 Microarray analysis to detect differential gene expression in oxidised versus non-oxidised RNA fractions

2.2.1.2.1 GeneChip Microarrays

Mouse Genome 430 2.0 GeneChip Microarrays (Affymetrix) are high-density oligonucleotide arrays produced by using *in situ* light directed chemical synthesis of short oligonucleotide sequences on to a glass slide. A known gene or potentially expressed sequence tag (EST) are represented on the GeneChip by a 25-mer oligonucleotide probe. Multiple copies of a probe sequence with a perfect match (PM) to a region of an expressed transcript are synthesised in discrete cells on the GeneChip. Each PM sequence is paired to a mismatch (MM) probe sequence containing a single monomeric base substitution at the 13^{th} nucleotide of the probe. The MM probes serve as a control for non-specific hybridisation. The difference in signal contributed by PM and MM probes for a specific probe pair is a measure of the interfering background signal and non-specific hybridisation. This is used downstream to determine the true signal contributed by a given probe set. The Mouse Genome 430 2.0 GeneChips have 45,000 probe sets that are able to analyse the expression level of over 39,000 transcripts.

2.2.1.2.2 RNA fragmentation, RNA hybridisation and GeneChip scanning

15µg of biotin labelled aRNA (section 2.2.1.1.6) was fragmented using array fragmentation buffer containing Mg^{2+} ions (as part of the 3' IVT express kit, Affymetrix) by heating to 94°C for 35 minutes. Longer RNA molecules form secondary structures causing non-specific cross hybridisation. This step is carried out to generate smaller RNA molecules, which increases specificity and overall intensity of the array. 1µl of fragmented aRNA was loaded on to Nano LabChip 6000 and run on the 2100 Bioanalyser (Agilent, Palo Ato, CA) to analyse the size of fragmentation reaction products (figure 3.2).

12.5µg fragmented and labelled aRNA was mixed with control oligonucleotide B2 and hybridisation controls (Affymetrix). The arrays were prepared with 200µl pre-hybridisation mix for ten minutes at 45°C. After removing the pre-hybridisation mix the hybridisation mix containing the aRNA and the controls was added to the GeneChip. Hybridisation was carried out for 16 hours at 45°C, 60 RPM in a hybridisation oven (Affymetrix). The hybridisation controls in the hybridisation mix are biotinylated oligonucletotides corresponding to B2, E coli genes *bioB*, *bioC* and *bioD* and P1 bacteriophage gene *cre*. The oligo B2 hybridises to features along the outer edge and is used to provide alignment signals for each array during image analysis. The *bioB, bioC, bioD* and *cre* oligos are present in serial concentrations and the signal intensities of these genes on the GeneChip are a measure of efficiency of hybridisation, washing and staining steps during GeneChip scanning.

Following hybridisation for 16 hours, the GeneChips were vented through insertion of a pipette tip in to one of the septa. The hydridisation mix was removed through the remaining septa. The GeneChips were stained with streptavidin phycoerythrin (SAPE) and underwent stringency washes in a GeneChip Fluidics Station 400 (Affymetrix), before another round of staining with a biotinylated anti-streptavidin antibody. The fluorescent intensity of hybridised transcripts was determined with the high-resolution laser of the GeneChip 3000 Scanner (Affymetrix)

2.2.1.2.3 GeneChip normalisation

The oligo B2 control hybridises to features along the outer edge and is used to provide alignment signals to identify relative position of the probes and the probe set they belong to, for each array during image analysis. The scanning software acquires a raw image of probe fluorescent intensity, which is relative to the amount of hybridisation and presented

in the form of a DAT file. Normalisation acts to equalise the overall signal intensity across all the GeneChips to be compared. The signal intensity for each probe is generated and then normalised to the same probe set across the array to generate a single value. Using the smoothing algorithm the CEL file computes the intensity calculation on the DAT file pixel values to assign a present or absent state, based on the perfect match (PM) to mismatch (MM) fluorescent ratios. Signals from MM probes are thought to represent cross-hybridisation and these serve as a biological background correction for the PM signals.

2.2.1.2.4 Statistical analysis to investigate genes selectively expressed in an oxidised fraction of RNA

Statistical analysis of microarray data was performed using the Partek Genomics Suite version 6.5 (Partek Inc., St. Louis, MO, USA) for microarray technology. Data was imported into the software as a CEL file with Partek default settings selected. Interrogating, but not control, probes were imported, GC content of transcripts was adjusted for, an RMA background correction was performed, and quantile normalisation of GeneChips to assume a normal distribution was carried out. For the analysis, only known well-characterised genes were included. An expression signal for all genes in all cases is established as part of quality control. The raw data for the probes is presented in log scale; this was transformed into fold-change values using base 2. The oxidised and non-oxidised fractions from the same animal were paired and identified by age. One-way ANOVA was performed between individual genes in the oxidised and non-oxidised fraction from the same mouse spinal cord. The analysis uses oxidation as the factor and performs a within subjects analysis to identify differentially oxidised genes. Genes are identified as been up-regulated or down-regulated in the oxidised fraction in comparison to the non-oxidised fraction. The difference in expression for each gene was calculated for each animal at each age investigated, then an average of this difference calculated across the three subjects in each group. This increases statistical power, reduces the effect of individual animals, and enables differences due to oxidation to be identified. The data was then manually categorised as differentially oxidised if the gene had a fold-change greater or less than +/-1.5, and a p-value of less than 0.01.

2.2.1.2.5 Enrichment analysis

The Database for Annotation, Visualisation, and Integrated Discovery (DAVID) is a bioinformatics resource 6.7 (National Institute of Allergy and Infectious Diseases (NIAID), NIH) that can assign biological meaning to a large list of genes (Huang da*, et al.* 2009b). This tool was used to identify enriched classes of genes from the differentially oxidised and non-oxidised gene lists at each age. DAVID identifies enriched annotation terms associated with a gene list and clusters them by function. A gene list from six, twelve, and eighteen months was up-loaded and classes of differentially oxidised genes identified by functional annotation clustering. A threshold of three genes belonging to an annotation term was required before the term is used. A p-value or EASE score, which is a one-tail Fisher Extract Probability value, is calculated for each annotation term. The geometric mean of all the enrichment p-values for each annotation term that is associated with the genes within the enriched group is used to rank overall importance of gene groups to identify ones to investigate further. An enrichment score of 1.3 is equivalent to a non-log scale score of 0.05; therefore, groups with an enrichment score of ≥1.3 have greater significance.

2.2.1.3 Validation of differentially oxidised genes identified from GeneChips

2.2.1.3.1 cDNA Synthesis

RNA was extracted using the RNeasy kit (Qiagen), as outlined in section 2.2.3.6. The concentration of RNA was determined using the Nanodrop Spectrophotometer (section 2.2.1.1.3). 5µl of oxidised or non-oxidised RNA was synthesised into cDNA using the Quantitect Reverse Transcriptase kit (Qiagen), as according to manufacturer's instructions, on a Peltier Thermal Cycler PTC-200 (MJ Research, USA). The RNA sample was incubated in gDNA Wipeout Buffer at 42°C for two minutes to effectively remove contaminating genomic DNA. Genomic DNA contamination can be co-amplified during the PCR reaction, producing erroneous results. Primers designed to anneal to sequences spanning exon boundaries also reduce the possibility of genomic DNA amplification. After genomic DNA elimination, the RNA sample is reversely transcribed using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The samples are incubated at 42°C for 15 minutes, and the reaction is then inactivated by heating to 95°C for 15 minutes. The samples are placed on ice for one minute and stored at 4°C.

2.2.1.3.2 Primer design

RT-qPCR primers were designed using Primer-BLAST (basic local alignment search tool) software (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were designed to span an exon-exon boundary where possible to avoid potential genomic DNA contamination, with an amplicon length of 50-150 base pairs, a melting temperature (Tm) between 58-60°C, and 20-80% GC content. Probeset IDs of genes to be validated were searched for using NetAffx (http://www.affymetrix.com/). This provided details of the sequence used to build the probe. A standard nucleotide BLAST using the sequence was performed to identify which part of the transcript the sequence is from. Where possible the primers were designed to span the exons from which the probe was designed.

2.2.1.3.3 Primer optimisation

The optimum combination of primer concentrations was determined by carrying out RT-qPCR reactions with different concentration combinations of forward and reverse primers. For primer optimisation, 1µg of total RNA extracted from the spinal cord of a normal six-month mouse was reversed transcribed to cDNA as outlined in section 2.2.1.3.1. RT-qPCR was performed with 12.5ng universal cDNA, 1X Brilliant II SYBR Green PCR Master Mix (Stratagene), along with appropriate volumes of forward and reverse primers (table 2.4), to a final volume of 20µl. The assay was run on an MX3000P Real-Time PCR system (Stratagene). The optimum forward and reverse primer concentration was chosen based on the lowest threshold cycle (Ct), the amplification of a single product, and the absence of primer dimers. Using the optimal primer concentrations for each gene of interest (table 2.5), a standard curve was prepared from serial dilutions of control cDNA, to test the efficiency of the PCR over a range of template concentrations.

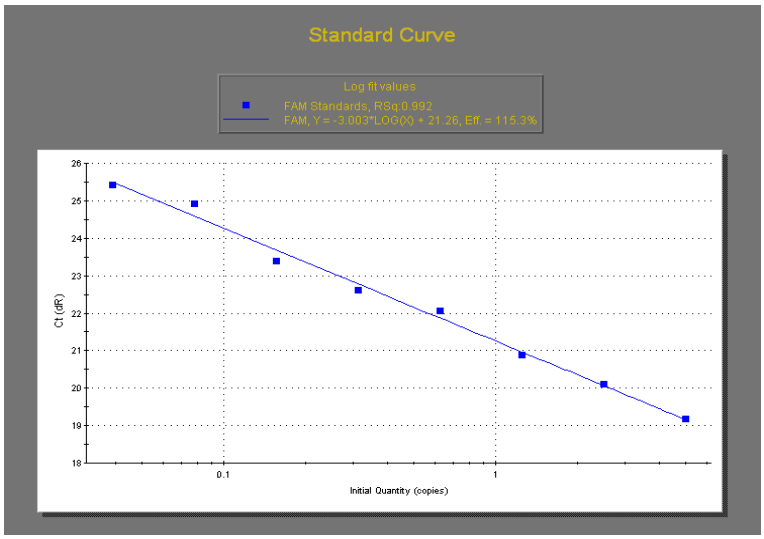


Figure 2.1: Assessing the efficiency of the primers over a range of concentrations

Table 2.4: *Reactions for primer optimisation*

Using starting stock concentrations of 5pmol/µl, appropriate volume of forward and reverse primers were added to test range of primer concentrations.

Forward primer concentration [nM] (volume added)	*Reverse primer concentration [nM] (volume added)*	*SYBR Green master mix (2X)*	*Template RNA (stock 50 ng/µl)*	*Water*
900 (3.6µl)	900 (3.6µl)	10µl	1µl	1.8µl
600 (2.4µl)	600 (2.4µl)	10µl	1µl	4.2µl
300 (1.2µl)	300 (1.2µl)	10µl	1µl	6.6µl
150 (0.8µl)	150 (0.8µl)	10µl	1µl	8.4µl

2.2.1.3.4 RT-qPCR

For each gene of interest, RT-qPCR was performed using the optimal concentration of primers (table 2.5). The fluorescent signal intensity was analysed using the MxPro software (Stratagene) and its gene expression value normalised to housekeeping gene *GAPDH* using the ddCT method (ABI PRISM 7700 Sequence Detection System protocol, Applied Biosystems). One-way ANOVA with Bonferroni post-test was performed using GraphPad Prism version 5.0d for Mac (GraphPad Software, La Jolla California USA) to determine statistical significance in differential expression.

Table 2.5: *RT-qPCR primer sequences and optimised concentrations for microarray validation*

Gene Name	*Gene*	*Primer Sequences*	*Concentration*
Poly(A) polymerase alpha	*Papola*	F 5' TTCACAGAAACCAGAATGCCA GTA 3'	600nM
		R 5' TTCTAGCCATGGGACCAAAGTT 3'	600nM
Apoptotic chromatin condensation inducer 1	*Acin1*	F 5' ACTTGGTCCTGGGAGGTCAAA 3'	300nM
		R 5' CAGCACTGAGCAATGGTGTGA 3'	600nM
ATP-dependent helicase	*Atrx*	F 5' CCCAAGTCCAAGCACTAGCATTA 3'	150nM
		R 5' GGAGCCGTCTATTCATAAGTATT CG 3'	150nM
DEAD (Asp-Glu-Ala-Asp) box helicase 6	*Ddx6*	F 5' TGTCCATGCTGCAGAAAGCT 3'	300nM
		R 5' AGCGATTTACAATGCAAAACGA 3'	600nM
18s rRNA	*18s*	F 5' GCAATTATTCCCCATGAACGA 3'	300nM
		R 5' CAAAGGGCAGGGACTTAATCAA 3'	300nM
Suppressor of G2 allele of SKP1 (S. cerevisiae)	*Sugt1*	F 5' GAAACGTGCCATGAACAAGTCA 3'	150nM
		R 5' TCCTTTTACCTACATCAGACCAG TTG 3'	150nM
CREB-binding protein	*Crebbp*	F 5' AAATTGGGGTATGTGACAGGA CA 3'	300nM
		R 5' GGGGATTTTCTGGTCAGGGG 3'	300nM
Glutamate receptor, ionotrophic, AMPA4	*Gria4*	F 5' CAAGCCCTGTGACACCATGA 3'	300nM
		R 5' GGTTTACGGGACCTCTCAGG 3'	300nM
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a,	*Smarca2*	F 5' AAGTCATCCAGCCACGAGC 3'	300nM
		R 5' CTGGTACCTCATCTTCTTCCTCA TT 3'	300nM
Serine/arginine-rich splicing factor 18	*Sfrs18*	F 5' TCCGAATCCCCCGGAAGTAG 3'	300nM
		R 5' AGGGGTTGTTGATCTTGATCGG 3'	300nM

2.2.2 Immunohistochemistry

2.2.2.1 Tissue Preparation

Immunostaining was carried out on formalin-fixed paraffin-embedded spinal cord tissue taken from G93A mutant *SOD1* transgenic mice and littermate controls at presymptomatic (60 day), symptomatic (90 day), and end-stage (140 day) disease. 8µm sections were cut on to positively charged slides (Leica microsystems, UK) and dried overnight at 37°C. Prior to staining sections were de-waxed in two changes of xylene and re-hydrated to water through a graded series of alcohols, then transferred to distilled water for further work.

2.2.2.2 Cresyl Violet stain

Cresyl violet staining was used to determine morphology of the spinal cord of G93A mutant *SOD1* mice and littermate controls. Sections were incubated in 0.1% Cresyl violet solution (Raymond A. Lamb, now part of Fisher Scientific, UK) at room temperature for five minutes. They were then rinsed quickly in distilled water and differentiated in 95% ethanol for 15-30 minutes, checking under the microscope for optimal staining. Sections were dehydrated in two changes of 100% ethanol for five minutes each, then cleared in two changes of xylene for five minutes each before being mounted in p-xylene-bis(pyridinium bromine) (DPX).

2.2.2.3 Haematoxylin and Eosin stain

To provide direct visualisation of cytoarchitecture, sections of spinal cord of G93A mutant *SOD1* mice and littermate controls were stained with Haematoxylin and Eosin dyes. Sections were mounted on to positively charged slides and dried overnight at 37°C. Sections were de-waxed in two changes of xylene for five minutes, then rehydrated through a graded series of alcohols. Sections were stained with Haematoxylin for 30 seconds, rinsed in water, washed in Scott's tap water for 30 seconds, rinsed in water, stained with Eosin for 30 seconds, rinsed in water, dehydrated through a graded series of alcohols before clearing in xylene and mounting in DPX.

2.2.2.4 Immunohistochemical staining

To assess nucleic acid oxidation and DNA damage repair response in G93A mutant *SOD1* transgenic mice and littermate controls, a series of sections were immunohistochemically stained for 8-hydroxyguanosine, 8-oxoguanine DNA glycosylase, DNA-dependent protein kinase, and H2A histone family member X (table 2.1).

Sections were prepared as described in section 2.2.6.1. Prior to staining sections were de-waxed in two changes of xylene and re-hydrated to water through a graded series of alcohols. Antigen retrieval was optimised, and final conditions for retrieval were using an auto-retriever (Labvision, UK) and TSC, pH 6. Sections were incubated in 30% hydrogen peroxide in methanol for 20 minutes to quench endogenous peroxidase activity, followed by three five-minute washes in TBS. To block non-specific antibody binding, sections were incubated in 15% (v/v) normal serum in TBS-T for 30 minutes. Sections were incubated in the appropriate primary antibody diluted in IHC antibody incubation solution 15% (v/v) normal serum in TBS-T, for one hour at ambient temperature. Negative controls were incubated with IHC antibody incubation solution with the antibody omitted. After three five-minute washes in TBS-T sections were incubated with the biotinylated secondary antibody diluted in TBS-T, for one hour at ambient temperature. Sections were washed and incubated with an avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector laboratories) for 30 minutes at ambient temperature. Sections were rinsed in TBS and immunoreactivity visualised by incubation in a DAB solution (Vector Laboratories) for five minutes. The reaction was quenched in distilled water, and sections were dehydrated through a graded series of alcohols and cleared in xylene before being cover slipped with DPX.

2.2.3 Cell Culture Methods

2.2.3.1 Maintenance of cell lines

Murine neuroblastoma spinal cord hybrid cells (NSC34) were produced through somatic fusion between the N18TG2 aminopterin sensitive neuroblastoma and motor neuron enriched embryonic day 12-14 spinal cord cells (Cashman*, et al.* 1992). NSC34 cells were transfected with pIRESneo (Clontech, Saint-Germain, France) using Lipofectamine 2000 (Invitrogen). Cells were transfected with empty vector (pIRES cells) or pIRESneo

containing the cDNA of wild-type human *SOD1* (WTSOD1) or the human *SOD1* mutants G93A, G37R, H48Q. NSC34's were maintained in DMEM containing 4.5g/l glucose, L-glutamine without Na pyruvate, supplemented with 10% fetal calf serum (FCS, BioSera Ltd), at 37°C in a humidified atmosphere of 5% CO_2/95% air. 50mg/ml neomycin (G418) was used for the selection of stably transfected NSC's. Media was changed every 2-3 days with addition of fresh G418. Cellular morphology was assessed daily using a microscope to ensure consistency between cell lines and passages.

Table 2.6: *Details of control and transfected NSC34 cells*

Cell line	*Transfection Information*
NSC34	Non-transfected
NSC34 transfected with pIRES-6	Mammalian expression vector only control
NSC34 transfected with WTSOD1-11	Wild-type human *SOD1*
NSC34 transfected with G93A-5	Mutant human *SOD1*-single amino acid substitution of glycine to alanine at codon 93
NSC34 transfected with G37R-2	Mutant human *SOD1*-single amino acid substitution of glycine to arginine at codon 37
NSC34 transfected with H48Q-9	Mutant human *SOD1*-single amino acid substitution of histidine to glutamine at codon 48

2.2.3.2 Cryo-preservation of control and transfected NSC34 cells

NSC34s were harvested and centrifuged at 400 RCF for four minutes to pellet. The media was removed and the pellet re-suspended in 500µl FCS with 10% Dimethyl sulfoxide (DMSO, Sigma). This cell suspension was transferred to a cryovial and placed in to the Cell Freezing System containing isopropanol, which lowers the temperature by 1°C/minute. The cryovials were initially frozen at -80°C, and transferred to liquid nitrogen for long-term storage.

2.2.3.3 Harvesting of NSC34 cells

NSC34 cells were cultured to reach 70-80% confluency. Media was aspirated and the cell layer washed with 5mls of PBS. A fresh 5mls of media was added to the cell layer and

cells were removed from the plate by pipetting. The suspension of cells was transferred to a 15ml falcon on ice, before centrifuging at 400 RCF for four minutes. The supernatant was discarded and the pellet taken forward.

2.2.3.4 Hydrogen Peroxide treatment of NSC34 cells

Control and transfected NSC34 cells were cultured to 70-80% confluency in a 10cm diameter petri dish. The cells were stressed by adding 30% (v/v) hydrogen peroxide (Sigma) at a final concentration of 50µM, 100µM, 250µM, 500µM, or 1mM. Treatments were carried out for two, six, and ten hours. Conditions were selected and a further four-hour time point incorporated. Following treatment cells were harvested and cell viability was measured by trypan blue exclusion. 10µl of Trypan blue was mixed with 10µl of cell suspension, and 10µl loaded on to a Countess chamber slide (Invitrogen, UK), this was placed into the Countess® Automated Cell Counter (Invitrogen, UK). Readout of the number of live, dead, and total cells is provided. After stressing the cells with hydrogen peroxide, the cells were harvested and conditions selected to take forward for analysis.

2.2.3.5 Preparation of cell lysates

Cells were harvested as described in section 2.2.3.3, and the pellet re-suspended in 200µl extra strong lysis buffer. Following 15 minutes incubation on ice the lysates were sonicated then centrifuged at 4°C for 25 minutes at 14,000 RPM. The supernatant was collected in fresh tubes and taken forward for analysis. Protein extracts were stored at -20°C.

2.2.3.6 RNA Isolation

Total RNA was extracted from Control and Transfected NSC34 cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Briefly, cells were lysed and homogenised in lysis buffer using a 25-gauge needle and 5ml syringe. An equivalent volume of ethanol was added to the lysate and this was transferred to a spin column. The RNA is bound to the column by centrifugation. The column is subsequently washed three times and the RNA eluted in RNase free water. RNA concentration was determined using the Nanodrop Spectrophotometer (section 2.2.1.1.3). All steps were performed at ambient temperature.

2.2.3.7 Immunoprecipitation and quantification of oxidised RNA

The immunoprecipitation of oxidised RNA was carried out as described in section 2.2.1.1.4. The concentration of RNA in the oxidised fraction was too low to be accurately quantified using the Nanodrop spectrophotometer, so the oxidised and non-oxidised fractions were quantified by RT-qPCR. cDNA was prepared as described in 2.2.1.3.1. A standard curve was set up using serial dilutions of total RNA from pIRES vector only NSC34 cells with 18S rRNA used as the reference gene. The Ct values for the respective fractions were compared against the Ct values of known RNA concentrations on the standard curve to determine the relative percentage of oxidised to non-oxidised RNA in each sample.

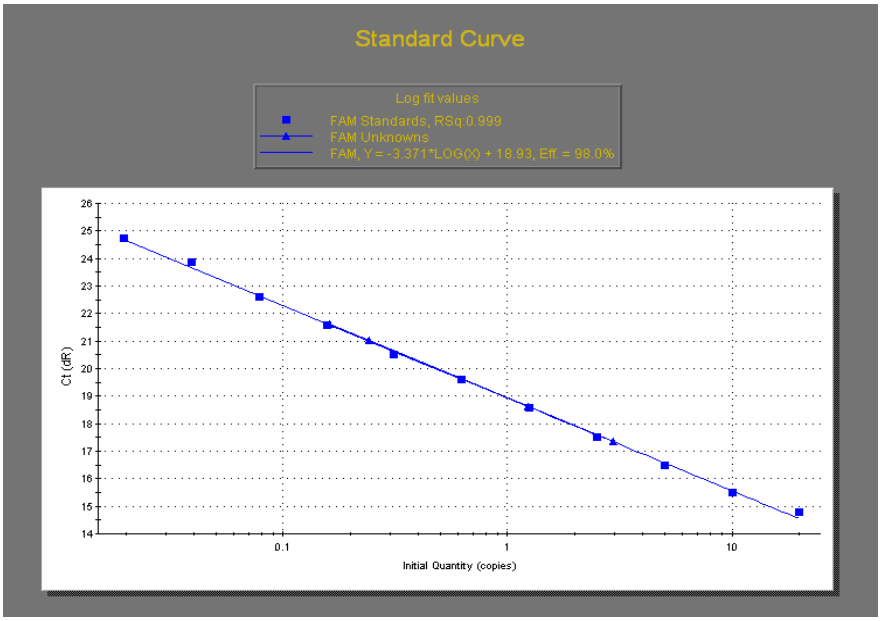


Figure 2.2: *Standard curve to investigate expression levels in an oxidised versus non-oxidised fraction of RNA*

2.2.3.8 Oxidative stress response analysis following exposure to hydrogen peroxide

Following RNA isolation, cDNA was prepared as outlined in section 2.2.1.3.1. For each gene of interest, RT- qPCR was performed as described in section 2.2.1.3.4 using the optimal concentration of primers (table 2.7).

Table 2.7: *RT-qPCR primer sequences and optimised concentrations for investigating oxidative stress response*

Gene Name	*Gene*	*Primer Sequences*	*Concentration*
Haem oxygenase	*HOX-1*	F 5' CACTTCGTCAGAGGCCTGCTA 3'	900nM
		R 5' GCGGTGTCTGGGATGAGCTA 3'	900nM
Nuclear erythroid-2-related factor2	*NRF2*	F 5' TGGAGGCAGCCATGACTGA 3'	100nM
		R 5' CTGCTTGTTTTCGGTATTAAG ACACT 3'	100nM
NAD(P)H dehydrogenase, quinone 1	*NQO1*	F 5' CGCCTGAGCCCAGATATTGT 3'	600nM
		R 5' ACTGCAATGGGAACTGAAATA TCA 3'	600nM
Cu/Zn Superoxide dismutase 1	*mmSOD1*	F 5' ATGGCGTAGAAACCGGTG 3'	500nM
		R 5' TGTCCTGACAACACAACTGGT 3'	500nM
Oxoguanine DNA glycosylase	*OGG1*	F 5' ATCCCAGGCTAAGGGCCCGA 3'	150nM
		R 5' GGTGGCTCCCGAGACAGGCT 3'	150nM

2.2.3.9 Statistical analysis of oxidative stress response of NSC34 cells

The fluorescent signal intensity was analysed using the MxPro software (Stratagene) and its gene expression value normalised to housekeeping gene GAPDH using the ddCT method (ABI PRISM 7700 Sequence Detection System protocol, Applied Biosystems). One-way ANOVA with Bonferroni post-test was performed using GraphPad Prism version 5.0d for Mac (GraphPad Software, La Jolla California USA) to determine statistical significance in differential expression.

2.2.3.10 Evaluating expression levels of human SOD1 transgenes in NSC34 cells

RT-qPCR and western blotting was used to evaluate the expression of human mutant SOD1 in the NSC34 cells. RT-qPCR was carried out as described in section 2.2.1.3.4 and western blotting was detailed in section 2.24. Following RT-qPCR the difference in the

threshold cycle (Ct) between human *SOD1* and endogenous mouse *Sod1* and housekeeping gene *GAPDH* was used to determine the relative expression of the human *SOD1* at the mRNA level (Pan, *et al.* 2012).

2.2.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.4.1 Determination of protein concentration

Protein concentrations of cell lysates were determined using a bicinchoninic acid (BCA) protein assay. A series of BSA standards in extra strong lysis buffer were freshly prepared at concentrations ranging from 0-2mg/ml each time the assay was performed, and plated in duplicate on a 96-well plate. This assay combines the known reduction of Cu^{2+} to Cu by protein in an alkaline medium (the biuret reaction), with the highly sensitive and selective colorimetric detection of Cu by BCA (Smith, *et al.* 1985). The protein samples were diluted 1:5 in extra strong lysis buffer and plated in duplicate on to a 96-well plate. The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1). 200µl of working reagent was added to each standard and all unknown samples. The plate was covered and incubated at 37°C for 30 minutes. Absorbance readings were recorded at a wavelength of 550nm using a Fluorostar Omega plate reader (BMG Labtech). Values obtained were compared to a standard curve to determine protein concentration in µg/µl. Before blotting samples were diluted in an appropriate volume of extra strong lysis buffer to ensure equal protein concentrations for loading.

2.2.4.2 SDS-PAGE preparation

The percentage resolving gel required depends upon the size of the protein of interest. Polyacrylamide gels were prepared as detailed in table 2.8 with volumes adjusted accordingly to the number of gels prepared. A 4% stacking gel was used for all gels. (table 2.9) Protein samples were mixed with an appropriate volume of either 6x or 2x Laemmli sample buffer. Samples were heated at 95°C for five minutes before centrifugation at 13,000 RPM for 15 seconds. Molecular weight standards and proteins were loaded on to the polyacrylamide gel. Gels were electrophoresed at 50 volts (V) for 30 minutes, and then for 90-120 V depending on size of protein for detection, until the dye front reached the bottom of the gel.

Table 2.8: *5mls resolving gel preparation*

Solution	*8% (mls)*	*10% (mls)*	*12% (mls)*	*15% (mls)*
Water	2.3	2.0	1.7	1.2
30% Acrylamide	1.3	1.7	2.0	2.5
1.5M Tris	1.3	1.3	1.3	1.3
10% SDS	0.05	0.05	0.05	0.05
APS	0.05	0.05	0.05	0.05
TEMED	0.003	0.002	0.002	0.002

Table 2.9: *2mls stacking gel preparation*

Solution	*4% (mls)*
Water	1.35
30% Acrylamide	0.67
1.5M Tris	0.5
10% SDS	0.04
APS	0.04
TEMED	0.004

2.2.4.3 Immunoblotting

PVDF was activated by immersing in methanol for 15 seconds and then soaked in 1x transfer buffer before use. Gels were placed on to PVDF membrane and sandwiched between Grade 1A filter papers (Whatman Laboratories) and sponges soaked in transfer buffer in a trans-blot cell transfer cassette (Bio-rad, UK), immersed in transfer buffer. Proteins were transferred onto PVDF at 250mA for 60 minutes. Non-specific binding of primary antibodies was blocked by incubating membranes in blocking solution, 5% (w/v) skimmed milk powder in PBS-T for 60 minutes at ambient temperature, and membranes were incubated overnight at 4°C with primary antibodies diluted in blocking solution.

Following incubation with primary antibodies, membranes were washed three times for ten minutes in PBS-T prior to the addition of the species specific HRP-conjugated secondary antibodies diluted in 5% (w/v) skimmed milk powder in PBS-T. Membranes were incubated for 60 minutes at ambient temperature, followed by an additional three washes for ten minutes in PBS-T. Proteins were detected using ECL chemiluminescence.

Equal volumes of EZ-ECL Reagent A and EZ-ECL Reagent B (to give sufficient coverage of the membrane) were mixed for two minutes prior to addition to the membrane.

2.2.4.4 Development of membrane and densitometric analysis

The membrane was scanned for an automatically calculated period of time and an image captured using the Intelli Chemi setting in the GeneSnap software and the G:BOX (Syngene). Intelli Chemi works through patented technology to detect chemiluminecence and automatically capture a sub-saturated image of the membrane. A histogram is generated which presents the raw data of the image, and enables detection of saturation. Densitometric analysis was carried out in GeneTools (Syngene). Bands were selected manually, using equal size boxes for each band. Background correction was performed automatically using the software. Raw data of the pixel intensity and the intensity of the bands in relative to a defined control is calculated. The intensity for the sample of interest is normalised to the loading control.

2.2.5 Seahorse Metabolic Assay

2.2.5.1 Preparation of cell culture plate

NSC34 cells transfected with either pIRES vector control, WTSOD1, G37R mutant *SOD1*, H48Q mutant *SOD1*, or G93A mutant *SOD1* were counted using the Countess Automated Cell Counter (Invitrogen, UK) and seeded at 60-70,000 cells/well in a 24 well Seahorse cell culture plate (Seahorse Bioscience) in 250µl DMEM supplemented with 10% Biosera fetal calf serum.

2.2.5.2 Preparation of plate for assay

The cells were incubated at 37°C/5% CO_2 overnight. The following day the cells were washed with 1ml supplemented XF Assay Media pH 7.4 (section 2.1.1), and incubated at 37°C for 60 minutes. Meanwhile, a 24 well, 4-port XF microplate (Seahorse Bioscience) was loaded with 5.0µg/ml Oligomycin (Sigma, UK), 2.50µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma, UK) and 5.0µM Rotenone (Sigma, UK). All were diluted in supplemented XF assay media. The XF microplate was

calibrated in a Seahorse XF24 analyzer prior to addition of the cell culture plate. Three basal measurements were recorded (three minutes each) prior to addition of Oligomycin, FCCP and finally Rotenone. The effect on oxygen consumption and pH by the drugs were measured for three minutes each.

2.2.5.3 Determining cell viability following assay

Cell number was normalised by addition of 4.0μM Calcein (Invitrogen, UK) to each well of the cell culture plate, which was incubated at 37°C for 30 minutes. Fluorescence was measured on a Fluorostar Omega plate reader (BMG Labtech) at Ex485nm/Em530nm every ten minutes for 40 minutes, with the reading at 30 minutes taken for normalisation.

2.2.5.4 Oxidative stress assay

For the stress assays, the cells were seeded as described in section 2.2.5.1. The following day the media was aspirated and cells were incubated with 50, 100, or 200μM H_2O_2 diluted in supplemented DMEM for one hour at 37°C/5% CO_2. The cells were washed with 1ml supplemented XF Assay Media pH 7.4 (section 2.1.1), and incubated at 37°C for 60 minutes before running the assay.

2.2.5.5 Statistical analysis

For each cell line, the metabolic assay was carried out three times under both basal and stress conditions, with three technical replicates generated for each cell line per experiment. One-way ANOVA with Bonferroni post-test was performed using GraphPad Prism version 5.0d for Mac (GraphPad Software, La Jolla California USA) for statistical analysis under basal conditions. A two-way ANOVA by H_2O_2 dose was performed to investigate the effect of the stress on oxygen consumption and extracellular acidification rate across the mutations.

2.2.6 Mitochondrial Morphology

2.2.6.1 Plating NSC34 cells

Control and transfected NSC34 cells (table 2.6) were cultured as described in section 2.2.3.1. 30,000 cells were seeded into an 8-well cell culture plate (ibidi, UK) designed for *in vitro* microscopy. Prior to imaging, the media was aspirated from the cells that were subsequently washed in PBS. 200µl rhodamine 123 (Invitrogen, UK) was added to cells and incubated at 37°C/5% CO_2 for five minutes. The cells were washed twice in PBS and 200µl PBS was added to cover the cells whilst imaging. For investigation of mitochondrial morphology following exposure to oxidative stress, cells were exposed to 100µM H_2O_2 for one hour subsequent to rhodamine staining.

2.2.6.2 Live cell imaging

Mitochondrial morphology was imaged using Leica TCS SPSII (Leica, Germany). For three-dimensional reconstruction of mitochondria, 3µm thick z-stacks were acquired for at least 10-15 cells under both basal and oxidative stress conditions. Three-dimensional projections were generated using Image J version 1.46.

2.2.6.3 Statistical analysis of mitochondrial morphology

Analysis of mitochondrial morphology was performed using an Image J macro (Dagda*, et al.* 2009). The green channel of cells stained with rhodamine 123 was extracted to grayscale, inverted to show mitochondria-specific fluorescence as red pixels. The mean area/perimeter ratio was employed as an index of mitochondrial interconnectivity, with mitochondrial elongation measured as inverse circularity.

Chapter 3
Gene expression profiling to identify genes differentially oxidised during ageing

3.1 Introduction

The selective oxidation of certain classes of RNA species have been identified in various neurodegenerative disorders, with an accumulation of oxidative modification to nucleic acids reported in ageing and neurological pathologies. Transcriptional profiling of the anterior spinal cord from ageing mice revealed differential oxidation of certain classes of RNA at each of the ages investigated. This novel study highlights transcriptome changes in an oxidised fraction of RNA during ageing and identifies the potential impact this might have on cellular function. This study builds on the methodology and findings reported in a recent study by Chang et al. (2008).

A complex combination of biological processes lead to the general decline in cognitive and motor function associated with ageing (section 1.2). The progressive structural and functional deterioration of biological systems with age leads to an increased risk of disease. Understanding the molecular changes underpinning this functional decline will potentially lead to therapeutics aimed at age-associated diseases, and address the subsequent social and economic connections. Gene expression profiling of ageing tissue has revealed genes whose expression changes with advancing age, and studies have identified shared characteristics of ageing across species. However, the rate of ageing and the nature and magnitude of gene expression changes differ between tissues and across species, adding further complexity to defining the causes of ageing (Fraser*, et al.* 2005, Miller*, et al.* 2008, Zahn and Kim 2007). Also to be considered when investigating the mechanism of ageing is whether candidate genes identified in the laboratory are genetically variable and affect lifespan in the natural population (Flatt 2004, Schmidt*, et al.* 2000).

Microarray technology facilitates the study of genome-wide patterns of transcriptional changes in model organisms and human tissue. Thousands of mRNA transcripts within a sample are quantified simultaneously, which can then be compared across samples. The principle of a DNA microarray is the hybridisation of an mRNA molecule in the test

sample to a cDNA probe, designed from the oligonucleotide sequence that it originated, which is immobilised to a glass slide (Courtney*, et al.* 2010). This type of microarray is most commonly seen in Affymetrix platform GeneChips, which is what were used in our study (figure 3.1). The RNA is labelled with a fluorescent tag and hybridises to its complimentary probe sequences on the GeneChip. The fluorescent signal generated by the hybridisation is proportional to the amount of transcript in the sample. The distribution of probe intensities across an array can be identified and converted to expression values to calculate the amount of transcript present for each probe set. By profiling the global RNA content of a sample in this manner it is possible to monitor in parallel all expressed genes, which can then be compared across samples (Schena*, et al.* 1998). Analysis of the gene expression levels can be used to identify pathways and regulatory networks for further investigation (Lyons 2002). Generating transcriptional profiles of ageing across species has been used to understand the evolution of ageing and identify potential biological changes that affect the physiological decline of an organism (Butler*, et al.* 2004, de Magalhaes*, et al.* 2009, Flatt and Schmidt 2009). Identifying gene expression changes during non-pathological ageing is important for understanding the relation to pathological neurodegeneration, and identifying features that may distinguish between the two. Many factors and pathways associated with ageing appear to be conserved, but it remains to be established whether evolutionary changes lie at the level of gene expression (Fraser*, et al.* 2005).

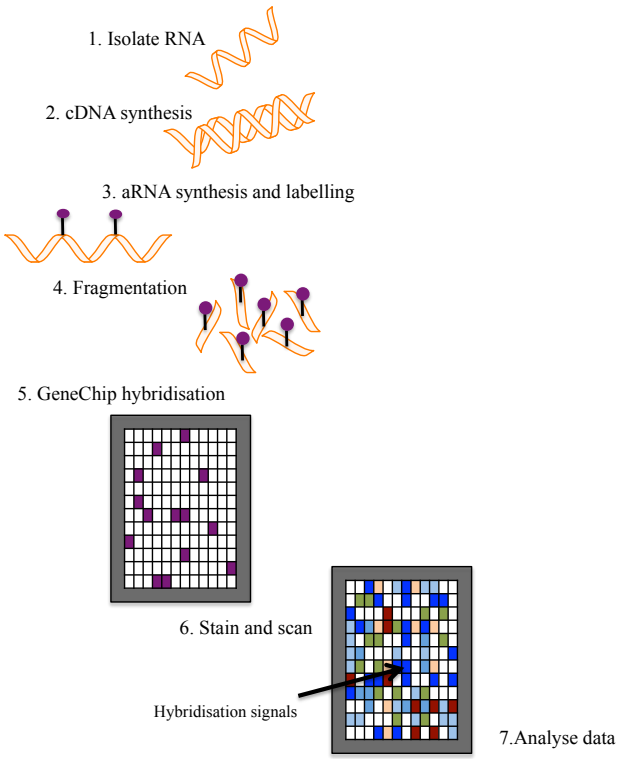


Figure 3.1: Outline of a microarray experiment

RNA is isolated from the target sample and a single stranded DNA molecule is produced by reverse transcription. Second strand synthesis generates a double stranded cDNA molecule, which serves as template for the production of multiple single stranded biotin-labelled complementary RNA (aRNA). The aRNA is purified and fragmented before hybridisation to the array. Subsequent staining and scanning of the array generates a signal where the aRNA has bound to the array. Statistical analysis reveals genes present on a particular array, which can then be compared between samples.

Multiple studies have identified large numbers of genes that display age-related changes in expression. Although these changes offer insight into what is happening at the transcriptome level, how they interact at a mechanistic level to drive ageing remains unknown. The changes identified during ageing are also open to different interpretations, for example rather than producing a detrimental effect, the gene expression changes seen may represent compensatory mechanisms or reflect a response to other changes (de Magalhaes and Toussaint 2004). Transcriptional profiling of the human frontal cortex identified a set of genes whose expression was variably reduced over the age of forty demonstrating specific transcriptome changes with age (Lu, *et al.* 2004). The inter-individual variation in gene expression in the middle-aged population (between 40-70 years of age) was a main finding of this study. This highlights how individuals diverge in their rates of ageing, which may be defined during development or in young adult life and

may be a potential factor in the susceptibility to developing an age-associated disease. Genes with reduced expression were found to have roles in synaptic plasticity, mitochondrial function, stress response, and DNA repair. However, whether these changes are due to reduced activation patterns, or are a consequence of increased activation of other genes in response to cellular changes remains unknown. In comparison, younger adults exhibited relatively homogeneous expression patterns.

Comparison of microarray studies across model organisms of ageing revealed that specific biological pathways are altered during the ageing process, and the age-induction of stress response genes is common across C.elegans, Drosophila, rodents, chimpanzees, and humans (Fraser*, et al.* 2005, Lee*, et al.* 2000, Lund*, et al.* 2002, McCarroll*, et al.* 2004, Pletcher*, et al.* 2002). Other similarities in gene expression changes across these species were also identified as described in section 1.2.1.3. Numerous studies have shown the ability to manipulate longevity through altering the expression of a small number of genes (Clancy*, et al.* 2001, Kimura*, et al.* 1997, Sun*, et al.* 2004, Tatar*, et al.* 2001, L. Wang*, et al.* 2007). However, the multifactorial nature of ageing, the insights from gene expression profiling, plus the increasing importance of RNA processing and metabolism, suggests this approach is too simplistic to understand physiological decline.

The free radical/oxidative stress theory of ageing has been extensively studied and a conserved feature of ageing appears to be an increase in expression of genes involved in stress-response pathways, which is associated with an increase in products of oxidative damage (Bishop*, et al.* 2010, Droge 2002, Haigis and Yankner 2010, Yankner*, et al.* 2008). An increase in cellular stress is exhibited by an increase in oxidative damage to nucleic acids, proteins, and lipids (Poon*, et al.* 2004), and has been linked to numerous other mechanisms proposed to underlie ageing and neurodegeneration (figure 1.2) (Boveris and Navarro 2008, Douglas and Dillin 2010). Oxidative modifications to nucleic acids have been shown to increase during ageing and neurodegenerative diseases, with 8-hydroxydeoxyguanosine and 8-hydroxyguanosine, modifications in DNA and RNA respectively, gaining the majority of attention because guanine is the most abundant among the oxidised bases (Bregeon and Sarasin 2005, Kasai 2002, Nunomura*, et al.* 1999, Nunomura*, et al.* 2012, Shan*, et al.* 2007). Evidence from post-mortem tissue and experimental models has demonstrated RNA oxidation to be a feature of ageing neurons with damage predominantly observed in vulnerable neurons at early stages of age-associated neurodegenerative disorders (section 1.6.3). Further investigations have been aimed at understanding the processes, mechanisms and consequences related to oxidative damage (section 1.6.4). Oxidised mRNA species have been implicated directly in

contributing towards the pathogenesis of neurodegenerative disorders. In 2008, Chang et al. used microarray technology to investigate mRNA oxidation in the spinal cord of the G93A human mutant *SOD1* murine model of ALS (Chang*, et al.* 2008). The array results indicated certain classes of mRNA transcripts are more susceptible to oxidative damage, with some oxidised mRNAs identified previously linked to ALS, which was also consistent with other studies in AD (Shan*, et al.* 2003). Modification of RNA transcripts may not only affect translational regulation, with damage to non-coding RNA (ncRNAs) potentially disrupting multiple cellular and metabolic processes simultaneously, however, this remains to be investigated.

Although evidence supports the preferential oxidation of certain classes of RNA during neurodegenerative diseases (Chang*, et al.* 2008, Shan*, et al.* 2003), it remains unknown whether similar classes are targeted for modification during ageing. In this study, we aimed to build on the methodology and research by Chang et al. (2008) to identify whether oxidative modification of RNA transcripts is a non-selective phenomenon or whether specific classes of transcripts are targeted for damage during ageing. Modifications of transcripts are potentially one mechanism influencing the gene expression changes identified in global genetic profiling, or may contribute towards the physiological decline of cells through downstream disruption to metabolic/homeostatic processes. Using Affymetrix GeneChips we sought to identify differentially oxidised genes by comparing an oxidised and non-oxidised fraction of RNA extracted from the anterior spinal cord of normal mice aged six, twelve, and eighteen months.

3.2 Results

This study aimed to identify whether transcripts are differentially oxidised during ageing by comparing an oxidised and non-oxidised fraction of RNA extracted from the anterior spinal cord of normal mice aged six, twelve, and eighteen months. The oxidised fraction of RNA was immunoprecipitated with an anti-8-OHG antibody. Subsequently the oxidised and non-oxidised fractions were analysed on Affymetrix GeneChips. Having identified differentially oxidised genes at six, twelve, and eighteen months of age using Genespring and Partek analysis software, gene ontology analysis was used to identify enrichment of gene classes at each age. RNA processing genes were found enriched within the oxidised fraction at all ages. The genes identified in this ontology group were subject to further functional and pathway analysis, and a select few taken forward for validation. We identified the majority differentially oxidised transcripts changed during

ageing, and few of these over-lapped with those found to be differentially oxidised in a transgenic mouse model of ALS (Chang et.al 2008).

3.2.1 Gene expression profiling and quality control

Gene expression profiling of the anterior thoracic spinal cord of normal mice aged six, twelve, and eighteen months identified selectively oxidised genes. Immunoprecipitation using an antibody against 8-Hydroxyguanosine was used to separate the oxidised fraction from the non-oxidised fraction of RNA. The method for RNA immunoprecipitation was previously described (Chang, *et al.* 2008), and optimised for our study using RNA extracted from NSC34 cells (described in chapter 4). 15µg of linearly amplified oxidised or non-oxidised RNA was fragmented to yield smaller molecules and hybridised onto microarray chips. Prior to hybridisation a series of quality control steps were carried out (figure 3.2).

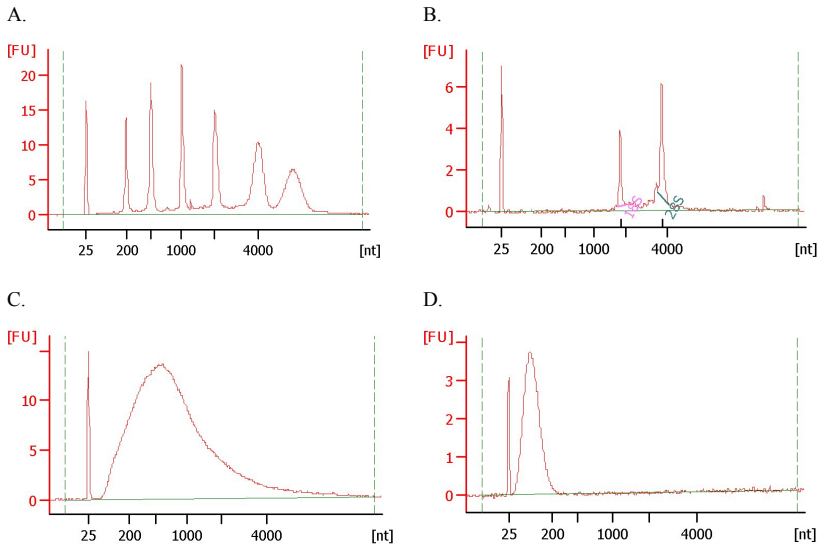


Figure 3.2: Representative electropherograms of RNA quality control obtained using the Agilent Bioanalyser

A) Ladder B) Total RNA extracted from anterior horn C) Linearly amplified fraction of RNA D) Fragmented fraction of RNA

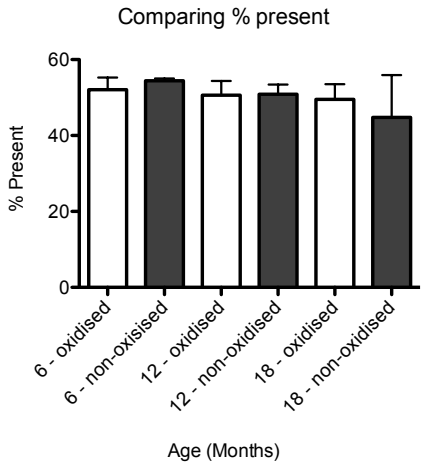
The Affymetrix software generates data files containing information from the GeneChips (section 2.2.1.2.3). Quality control reports based on the transcriptional profile of the oxidised and non-oxidised samples at each age were produced using the microarray

analysis software Expression Console (Mas5.0) (table 3.1). The numbers of probe sets called present are expressed as a percentage of the total number of probe sets on the array, calculated by the Affymetrix detection algorithm. Approximately 50% of the 45,000 probe sets arrayed on the GeneChip were detected as present for each of the oxidised and non-oxidised samples, with no significant difference between these or the average background signal across the arrays at each condition (figure 3.3). The percentage present should be similar across all samples, and an average background signal of 20-100 is typical for a good quality array (McCall 2011). Both of these are similar across the arrays in our study. Other parameters measured include the RawQ, which is the noise level and is measured from pixel-pixel variation across probe cells. The scale factor, used for the normalisation of arrays, provides a constant factor for every gene on the array. This allows adjustment of the average signal value for each array to an equal value, which enables the arrays to be compared. Within an experiment, arrays are expected to have a scale factor within three-fold of each other (McCall 2011). The 3'-5' ratio is the signal intensity ratio of the 3' probe set over the 5' probe set, which provides qualitative information on sample quality and amplification efficiencies.

Table 3.1: *Quality control parameters determined following hybridisation of samples to GeneChips*

Sample	*% present*	*Background signal*	*RawQ*	*Scale-factor*	*GAPDH 3'-5' ratio*
6 month oxidised	50.42	27.88	0.68	2.13	62.36
6 month non-oxidised	54.20	35.16	1.04	0.70	99.47
6 month oxidised	55.78	30.04	0.76	1.28	13.03
6 month non-oxidised	55.11	32.53	0.93	0.82	49.32
6 month oxidised	49.97	29.14	0.75	1.97	42.02
6 month non-oxidised	53.94	30.54	0.84	1.12	48.38
12 month oxidised	50.65	26.83	0.72	1.75	23.08
12 month non-oxidised	50.97	29.82	0.77	1.54	97.82
12 month oxidised	54.36	30.83	0.86	0.99	36.89
12 month non-oxidised	53.37	31.06	0.85	0.89	38.37
12 month oxidised	46.79	28.31	0.70	2.40	43.48
12 month non-oxidised	48.31	30.44	0.83	1.12	46.75
18 month oxidised	50.07	28.94	0.75	2.08	32.94
18 month non-oxidised	49.34	28.97	0.77	1.99	110.37
18 month oxidised	45.35	29.48	0.76	2.44	150.66
18 month non-oxidised	32.06	29.20	0.75	5.45	103.28
18 month oxidised	53.22	29.73	0.79	1.30	58.41
18 month non-oxidised	52.92	37.53	1.06	0.73	30.74

A.



B.

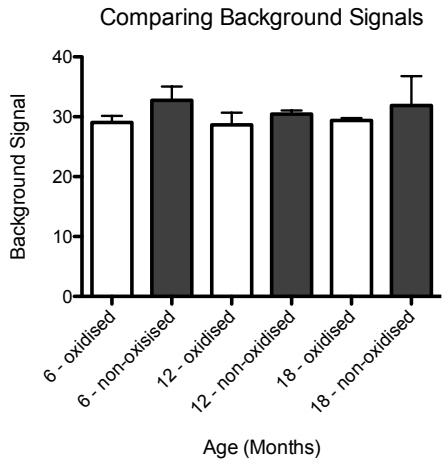


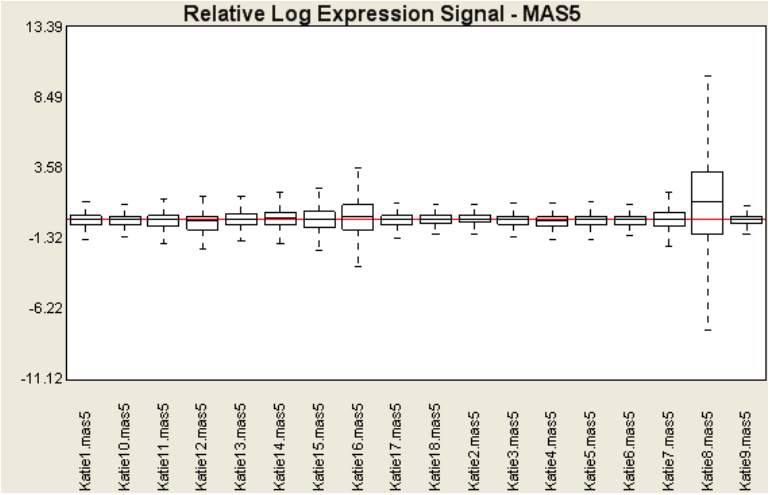
Figure 3.3: The average percentage of transcripts present and the background signal for the transcripts in each group

There was no significant difference between the percentage of transcripts deemed present by the Affymetrix detection algorithm (A), and the background signal of transcripts between the oxidised and non-oxidised fraction at each age (B). Data presented as mean with standard deviation (n=3), statistical analyses by one-way ANOVA with Bonferroni post-test.

The signal values produced from the image file (DAT file) represent quantification of transcript abundance for each probe set, which is generated based on the fluorescent ratio of present match (PM) to mismatch (MM) signal (section 2.2.1.2.3). The distribution of probe set intensities for each array is calculated from the median signal intensity across all arrays, which are transformed on to a logarithmic scale. This allows visualisation of

the variation of transcript abundance for an array and between a set of arrays (figure 3.4). Discrepancies between samples can be identified, which may represent low quality data possibly caused by differences in amplification or labelling. In this study, an outlier identified was an oxidised sample from a twelve-month-old mouse (figure 3.2 (A)). This dataset and the corresponding non-oxidised dataset were removed from the experiment and additional oxidised and non-oxidised sample were run on GeneChips. The distribution of probe set intensity for these samples were then compared to the rest of the arrays (figure 3.2 (B)). The data in table 3.1 and in figure 3.2 represent the data from figure 3.2 B.

A.



B.

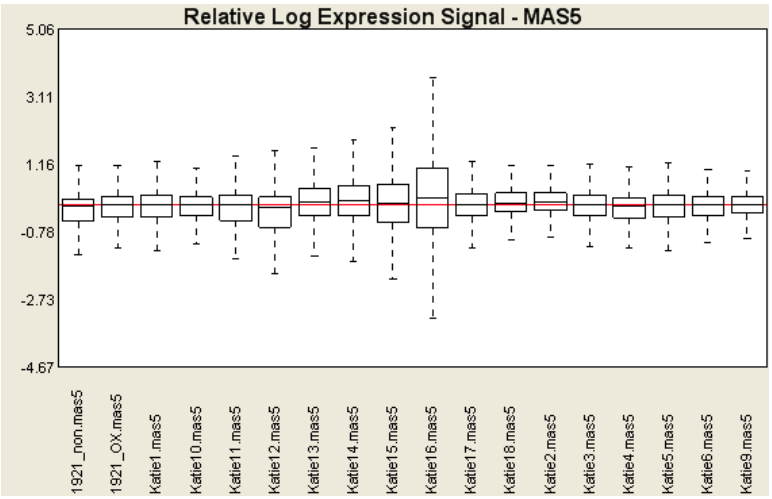


Figure 3.4: The relative expression signal for each array generated by microarray analysis software (MAS5.0)

This represents, for each GeneChip, the overall deviation from the corresponding median gene expression levels across all arrays. The Y-axis is the relative log expression signal for all samples which are represented along the X-axis. This demonstrates the overall deviation of probe set intensities across all arrays. The relative log expression values are calculated by subtracting the median gene expression estimate across arrays from each gene expression estimate.

3.2.2 Identifying differentially oxidised RNA transcripts during ageing

The transcription profiles of the oxidised and non-oxidised fractions from mice aged six, twelve, and eighteen months were analysed using Genespring and Partek software

(section 2.2.1.2.4). The genes differentially oxidised at each age are enriched in the oxidised fraction in comparison to the non-oxidised fraction. Transcripts were identified as significantly differentially oxidised if the expression level in the oxidised fraction was altered with a fold change of ≥1.5, and a *p*-value ≤0.01 when compared to the non-oxidised fraction (table 3.2 and 3.3). Genes were identified as significantly enriched in the non-oxidised fraction in comparison to the oxidised fraction, if their fold change and p-value was ≤-1.5 and 0.01 respectively. The probe set IDs of differentially oxidised genes from each age were compared using genevenn (http://genevenn.sourceforge.net/) to identify the number of genes that were differentially oxidised at each age, and how many were expressed at multiple ages (figure 3.5) identified by both Partek and Genespring analysis software.

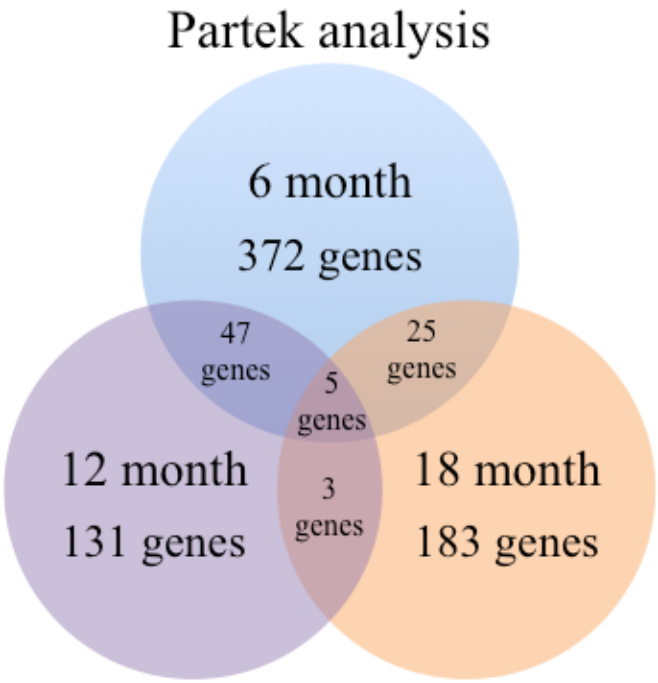
Table 3.2: *Summary of total genes identified and classified as differentially oxidised from Genesping and Partek analyses*

	6 months	*12 months*	*18 months*
GeneSpring –Total number of genes	6133	4883	1698
GeneSpring FC>1.5	973 up 115 down	831 up 210 down	322 up 87 down
GeneSpring FC>2	284 up 7 down	300 up 12 down	172 up 2 down
Partek –Total number of genes	7038	5049	4559
Partek FC>1.5	1014 up 180 down	660 up 179 down	703 up 118 down
Partek FC>2	296 up 7 down	265 up 12 down	184 up 0 down

Table 3.3: *Summary of number of differentially oxidised genes taken forward from both analyses for further investigation*

	6 months	*12 months*	*18 months*
GeneSpring FC>1.5 P<0.01	595 up 62 down	394 up 99 down	68 up 17 down
Partek FC>1.5 P<0.01	395 up 54 down	141 up 45 down	188 up 28 down

A.



B.

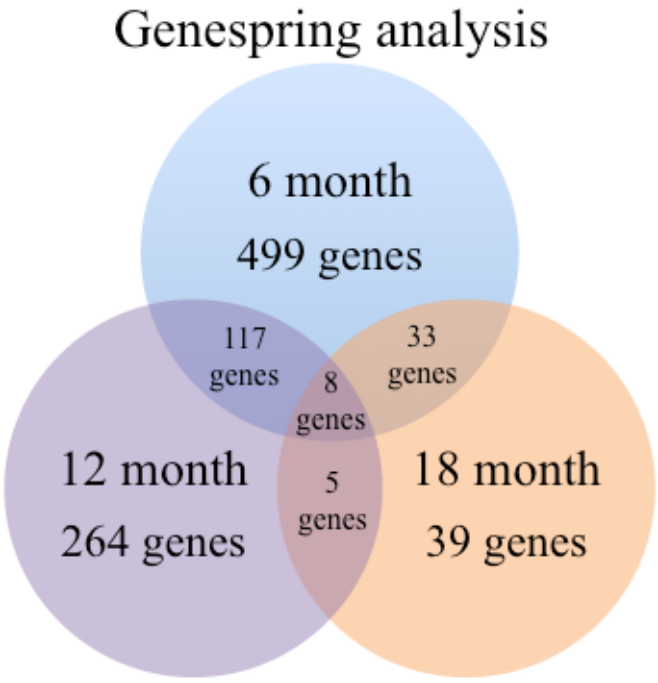


Figure 3.5: Differentially oxidised genes identified by Genespring and Partek analyses

Comparison of genes differentially oxidised at each age identified those differentially oxidised at a single age or at multiple ages. Both Partek (A) and Genespring (B) analysis was performed to determine which genes are differentially oxidised

Of the total number of genes identified at each age i.e. with no fold change or p-value exclusion (highlighted in table 3.2), only 88 genes were present on the arrays at all ages investigated and identified by both Partek and Genespring analyses (figure 3.6). Differentially oxidised genes at six, twelve, and eighteen months were then compared between the two analyses to determine the degree of overlap (figure 3.7).

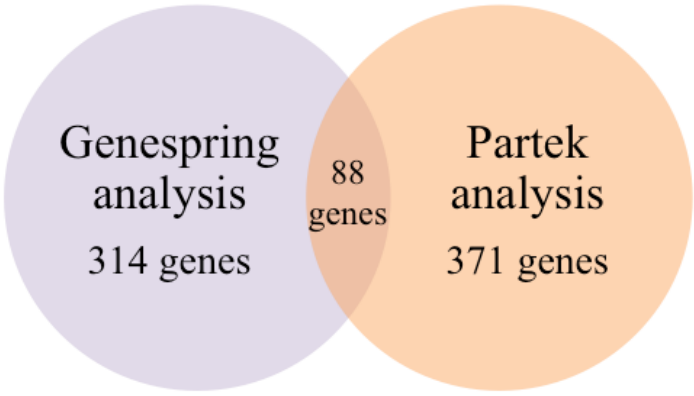
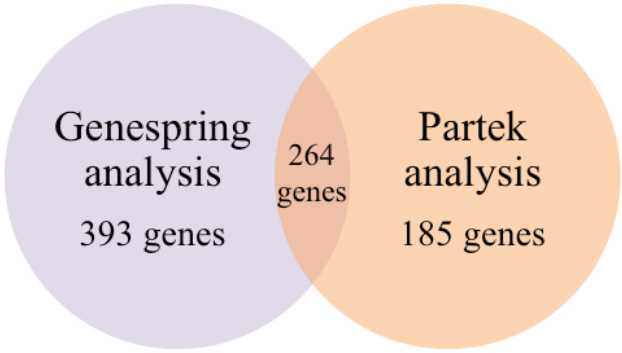


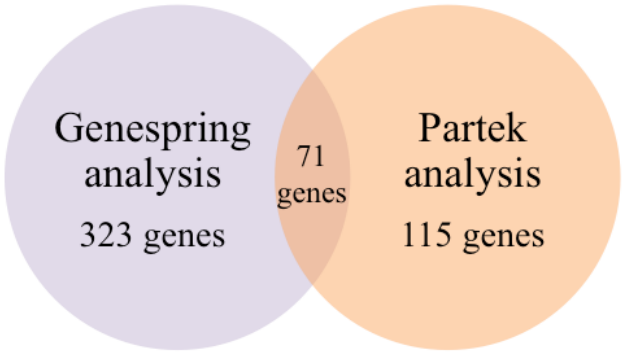
Figure 3.6: Genes identified as oxidised at six, twelve, and eighteen months using Genespring and Partek analysis

From the total number of genes identified at each age (highlighted in table 3.2) only 314 genes were expressed at all three ages using Genespring analysis and 371 genes using Partek analysis. A comparison of these two lists of genes found 88 genes in common were identified by both analyses and present at all the ages investigated.

A.



B.



C.

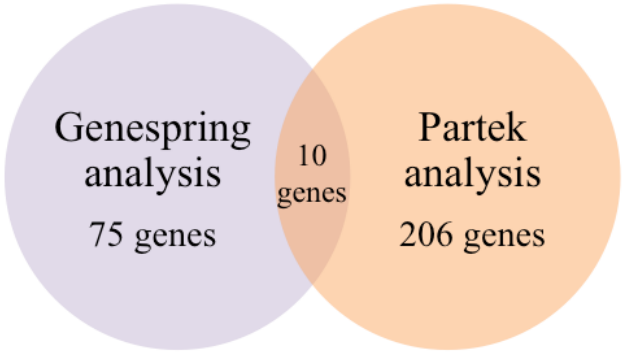


Figure 3.7: Differentially expressed genes identified by Partek and Genespring analysis at each age

Differentially oxidised genes identified at six months (A), twelve months (B), and eighteen months (C) by Genespring and Partek analysis were compared by Gene Venn to identify which genes were identified by both analyses.

3.2.2.1 Investigating differential RNA oxidation by Partek Genomics Suite analysis

Further analysis of the differentially oxidised genes was carried out based on the data generated from Partek analysis only. Partek analysis applies a robust multi-array analysis (RMA), which consists of background correction, quantile normalisation, and summarisation (Bolstad*, et al.* 2003, Irizarry*, et al.* 2003). RMA expression measure is calculated for all GeneChips, and are based upon an average of $\log_2$(B(PM), where B(PM) are background corrected present match (PM) intensities. The background correction is non-linear, and performed for each individual chip. Quantile normalisation makes the distribution of probe intensities identical across a set of arrays, and normalisation is carried out for each individual probe before summarisation. Summarisation converts these probe-level values in to probe set intensity values, generating a single expression measure per gene per chip. This is presented as a box plot of the log expression signal for each array, in which the deviation of gene expression levels from the corresponding median expression level is generated across all arrays (figure 3.8); this is similar to that generated using microarray software MAS5.0.

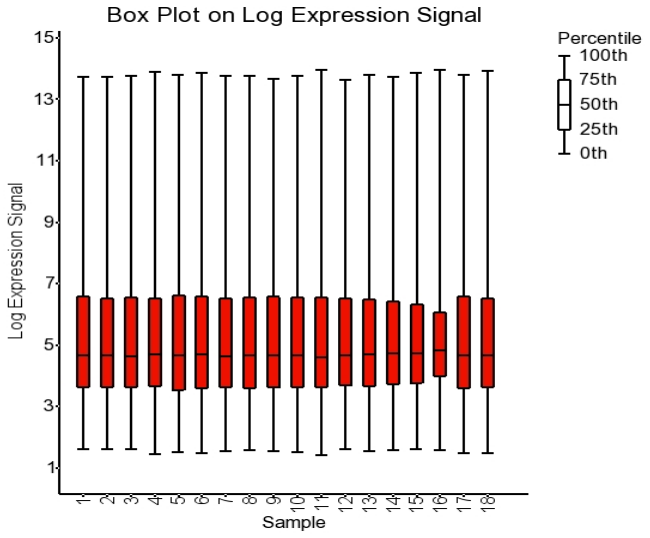


Figure 3.8: Representation of the log expression signal generated by Partek Genomics Suite

This box plot was generated from the probe set signal values of each array that have subsequently been normalised and summarised.

The list of differentially oxidised genes for each age was compared, and genes differentially oxidised at more than one age identified. One of the aims was to identify whether transcripts are randomly oxidised or whether specific classes of genes are targeted for modification. We were also interested to discover whether oxidation was a function of age; for example, does the set of genes that are oxidised at six months, twelve months, and eighteen months differ.

3.2.2.2 Investigating enrichment of gene classes present in an oxidised fraction of RNA during ageing

The Database for Annotation, Visualisation and Integrated Discovery (DAVID) was used to identify any enrichment of gene classes at each age (table 3.4, 3.5, and 3.7) (Huang da*, et al.* 2009b, Huang da*, et al.* 2009a). Probeset IDs of differentially oxidised and non-oxidised genes at six, twelve and eighteen months were investigated. DAVID condenses a large list of genes into biologically meaningful terms, enabling functional annotation clustering based on biological definition. The analysis identified a change in enrichment terms across the ages investigated. Enrichment of specific groups of genes that appear preferentially oxidised is greatest at six months, however genes involved in transcriptional regulation and RNA processing remain enriched in the oxidised fraction at twelve and eighteen months. The overall number of differentially oxidised genes at six months is 50% greater than at twelve and eighteen months, and the enrichment scores for the classes of genes oxidised at six months are greater, indicating that these genes are in a group of greater significance. Enrichment scores are based on the overall EASE score (p-value) for each enriched annotation term (section 2.2.1.2.5).

Table 3.4: *Processes enriched within the oxidised fraction at 6 months*

Biological Process	*Enrichment score*	*Number of genes*	*%*	*p-value*
RNA Binding	5.04	38	9.31	1.93E-07
RNA Processing	3.79	28	6.86	1.26E-06
Intracellular non-membrane bound organelle	3.48	65	15.93	3.40E-05
Nucleoplasm	3.21	25	6.13	0.001
Cell projection	3.18	15	3.68	5.25E-04
Regulation of transcription	2.75	68	16.67	0.003

Table 3.5: *Processes enriched within the oxidised fraction at 12 months*

Biological Process	*Enrichment score*	*Number of genes*	*%*	*p-value*
Regulation of transcription	3.54	21	12.28	5.59E-04
Cell projection	2.22	11	6.43	1.65E-02
Glucose regulation	2.12	3	1.75	3.23E-03
Cytoskeleton	1.82	8	4.68	1.45E-02
RNA processing	1.73	10	5.85	6.26E-04

Table 3.6: *Processes enriched within the oxidised fraction at 18 months*

Biological Process	*Enrichment score*	*Number of genes*	*%*	*p-value*
RNA Binding	4.96	23	11.06	7.33E-09
Ribonucleoprotein complex	3.32	11	5.29	5.53E-05
Regulation of transcription	2.68	22	10.58	7.31E-04
GTPase regulation	1.83	12	5.77	1.52E-03
Ribosome	1.63	8	3.85	1.08E-03

Of the 88 transcripts identified at all three ages by Partek and Genespring analyses (figure 3.6), six differentially oxidised genes were selected for validation based on their function. These genes are interesting not only functionally but because they are in the small group of differentially oxidised genes present at six, twelve, and eighteen months. The list of probeset IDs for the 88 genes were entered into DAVID and functional categorisation of transcripts revealed an enrichment of processes involving RNA metabolism and regulation of gene expression. Because of the reduced number of genes for classification, further manual analysis of genes enriched within the specific groups was used to identify potentially interesting genes. Fold change information for the genes was gathered from the Partek analysis and genes were selected based on their molecular function ascertained from the gene ontology (GO) classification using DAVID and through literature searches. Genes for validation were selected based on the fold-change and functional information gathered (table 3.7).

Table 3.7: *Genes selected for validation*

Fold change and p-values from Partek analysis

Gene Title	*Gene Symbol*	*Function*		*6 Months*	*12 Months*	*18 Months*
Poly (A) polymerase alpha	*Papola*	Polyadenylation	*Fold-change*	2.83	3.04	2.56
			p-value	0.009	0.006	0.04
Sgt1, suppressor of G2 allele of SKP1 (S. cerevisiae)	*Sugt1*	Kinetochore function and required for the G1/S and G2/M transitions	*Fold-change*	2.64	2.72	4.04
			p-value	0.003	0.027	0.03
Apoptotic chromatin condensation inducer 1	*Acin1*	Splicing complex, apoptotic chromatin condensation	*Fold-change*	4.64	5.22	4.36
			p-value	0.007	0.01	0.0008
Alpha thalassemia/mental retardation syndrome X-linked homolog (human)	*Atrx*	Transcriptional regulator	*Fold-change*	4.77	4.41	4.04
			p-value	0.006	0.006	0.008
DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	*Ddx6*	RNA helicase, translation suppression, mRNA degradation,	*Fold-change*	3.71	4.26	3.94
			p-value	0.001	0.008	0.01

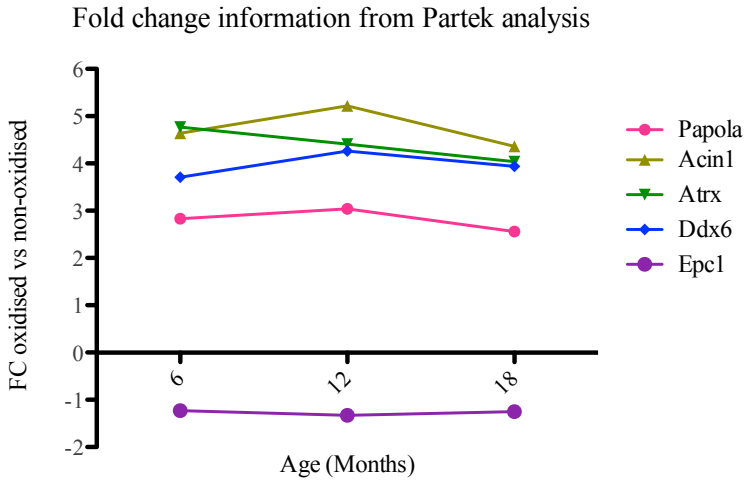
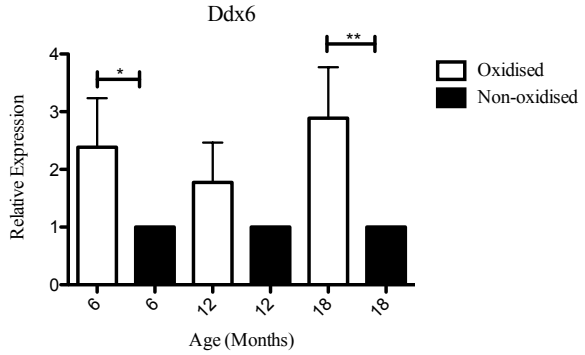


Figure 3.9: Fold change data for each gene selected for validation

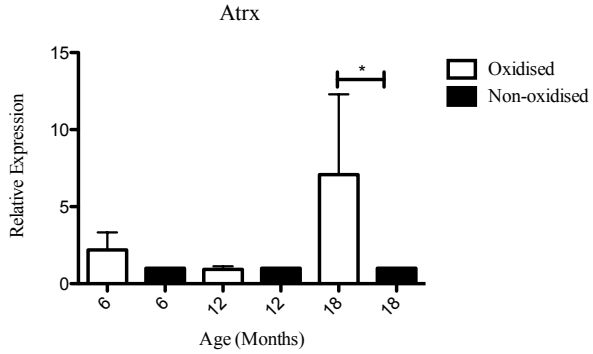
Genes selected for initial validation were differentially oxidised at all ages investigated and were identified in both Partek and Genespring analysis. They represent the few genes whose expression did not substantially change between six, twelve, and eighteen months.

Although the majority of genes differentially oxidised were different across the ages, a few were found to be differentially oxidised at all three ages. These genes were selected for the initial validation, as the minority they are also interesting to study. The optimum primer concentration for each gene was determined (section 2.2.1.3.3) and the genes were validated by RT-qPCR. The Epc1 gene primers were optimised, however standard curve analysis to determine their efficiency revealed discrepancies between samples, with the slope efficiency exceeding 100%. Consequently, this gene was removed from the validation. For the remaining five genes, standard curve efficiency was consistently high with few outliers. The selected genes were validated using additional oxidised and non-oxidised samples prepared from the ageing series (figure 3.10). The relative expression level of each gene in the oxidised and non-oxidised fraction at each age was calculated using the delta-delta Ct (ddCt) method. No significant differences between the oxidised and non-oxidised fraction were identified for each gene. Expression was greater in the oxidised fraction in comparison to the non-oxidised fraction in most cases, with the fold change generally showing the right direction, corresponding to the array findings.

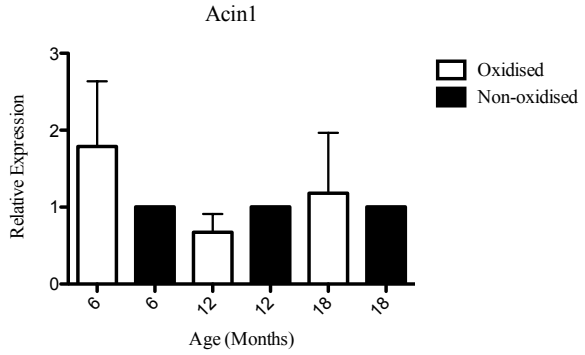
A.


Ddx6
Relative Expression
Age (Months)
Oxidised
Non-oxidised
*
**
0
1
2
3
4
6
6
12
12
18
18

B.


Atrx
Relative Expression
Age (Months)
Oxidised
Non-oxidised
*
0
5
10
15
6
6
12
12
18
18

C.


Acin1
Relative Expression
Age (Months)
Oxidised
Non-oxidised
0
1
2
3
6
6
12
12
18
18

D.

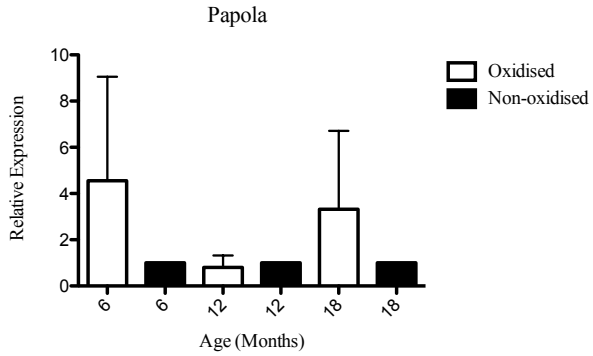

Papola
Relative Expression
Age (Months)
Oxidised
Non-oxidised
0
2
4
6
8
10
6
6
12
12
18
18

Figure 3.10: Results of RT-qPCR validation for selected genes at six, twelve, and eighteen months

The oxdised and non-oxidised fraction for the selected genes were normalised to house-keeping gene *Actin* before the relative concentration was determined by comparing the ddCt of the oxidised fraction to the ddCt of the non-oxidised fraction. Data presented as mean with SD (n=3), statistical analysis by one-way ANOVA with Bonferroni post-test.

3.2.2.3 Further enrichment and pathway analysis of genes in an oxidised fraction of RNA

Following the initial validation, we aimed to further characterise the classes of genes selectively targeted for oxidation and use pathway analysis tools to investigate how specific pathways might be affected as a result of oxidative modification to RNA. The DAVID analysis described previously showed enrichment for genes involved in RNA processing and metabolism. Further analysis of the data was relatively subjective in that we targeted our investigation to focus on RNA processing genes, as these were enriched within the oxidised fraction at six, twelve, and eighteen months, and these mechanism have been associated with neurodegenerative disease. Identifying which genes are oxidised during ageing, and how expression changes for genes involved in these processes may change during ageing as result of oxidative modification to the transcript is of interest.

The analysis of differentially expressed genes revealed differences not only in the number of genes differentially oxidised during ageing, but also differences in the functional categories these genes are classified into. This reveals a change in transcripts oxidatively modified with age, which could represent age-associated changes in the transcriptome, with genes selectively exhibiting oxidation due to their availability within the cell. In gene ontology terms, RNA processing is defined as any process involved in the modification of primary transcripts into mature RNA molecules, and RNA binding refers to interacting selectively with an RNA molecule or a protein thereof. Some genes were found to be over-lapped between the ontology terms relating to RNA processing/metabolism. Genes classified into the ontology terms in table 3.8 were grouped at each age as genes related to RNA processing. In order to identify whether genes were oxidised at more than one age, the transcripts from RNA gene ontology analysis were manually categorised at each age. This generated a single list of RNA

processing/metabolism genes that could be compared between the ages. Analysis demonstrated a change in the number of differentially expressed genes in the oxidised fraction enriched in RNA processing during ageing (figure 3.11).

Table 3.8: *The ontology terms grouped under RNA processing*

DAVID enrichment term	*Ontology*	*Definition*
Ribonucleoprotein complex	Cellular component	A macromolecular complex containing both protein and RNA molecules
Ribosome	Cellular component	Intracellular organelle, site of protein biosynthesis
RNA processing	Biological process	Any process involved in the conversion of one or more primary RNA transcripts into one or more mature RNA molecules
Regulation of transcription, DNA-dependent	Biological process	Any process that modulates the frequency, rate or extent of cellular DNA-dependent transcription
RNA binding	Molecular fun ction	Interacting selectively and non-covalently with an RNA molecule or a portion thereof
Nucleoplasm	Cellular component	Part of the nuclear content other than the chromosomes or the nucleolus
Histone modification	Biological process	The covalent alteration of one or more amino acid residues within a histone protein

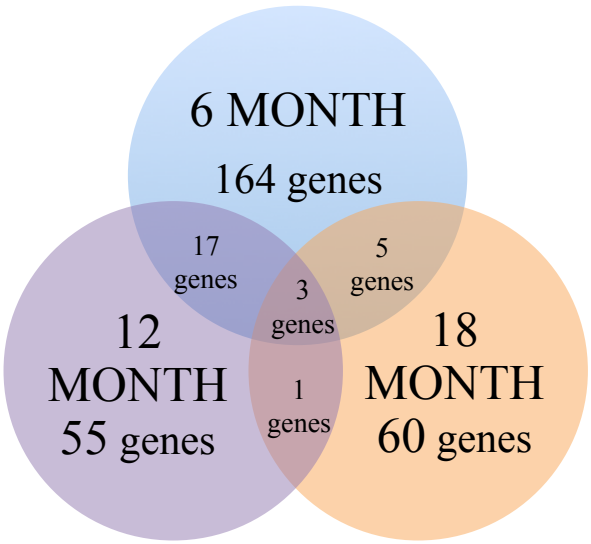


Figure 3.11: Functional annotation of genes enriched in RNA processing mechanism

Partek analysis identified a number of genes differentially oxidised at six, twelve, and eighteen months that have known roles in RNA processing. This venn diagram represents the number of genes involved in these processes at each age and how many were identified at multiple ages

Further investigation of genes, involved analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis tool. This allows identification of pathways/processes in which the genes in the oxidised fraction of RNA are involved and the other genes they interact with, which is discussed below for a few select genes. This allows speculation on how disruption to these pathways may occur if genes within them are selectively oxidised, providing further routes for investigation into cellular decline. We were also interested in genes that were enriched within the cellular homeostasis ontology group, as disruption to these regulatory processes could have widespread effects on cellular function.

3.2.3 Differential oxidation of transcripts during ageing

3.2.3.1 Transcriptional Regulation

Gene ontology analysis revealed genes involved in RNA processing and transcriptional regulation were significantly enriched within the oxidised fraction of RNA at all ages (figure 3.11). Identifying transcription factors, transcription cofactors, and components of the transcriptional machinery that are differentially oxidised is of interest, as disruption to their activity would have major implications for cellular function. Epigenetic changes can modify the temporal and spatial pattern of gene expression without altering the DNA sequence. Age-related changes in gene expression are influenced by cumulative insults from endogenous and exogenous factors, which accompanied by pathological damage to a select group of transcripts, adds further complexity to studying the genetics of the ageing process. Previous work has shown that oxidative modification of mRNA leads to a reduction in functional protein and may consequently impact other metabolic processes downstream. The control of chromatin organisation and sequence specific interaction of transcription factors facilitates transcriptional regulation. Erroneous transcription due to disturbances in the regulatory steps could potentially cause widespread alterations to the transcriptome, affecting cellular function and susceptibility to degeneration. We identified multiple transcription factors, cofactors, and epigenetic modifiers to be differentially oxidised during our study, some of which are described below. Pathway analysis enabled identification of possible downstream consequences due to oxidative modification of these select genes.

Table 3.9: *Differentially oxidised RNA processing genes*

Gene Name	*Function*		*6 Month*	*12 Month*	*18 Month*
CREB-binding protein (*Crebbp*)	Transcriptional coactivator, histone acetyltransferase	Fold-change	3.04	2.77	3.66
		p-value	0.05	0.01	0.01
GATA zinc finger domain containing 2 (*Gata2*)	Transcription factor	Fold-change	2.03	1.89	1.68
		p-value	0.003	0.008	0.0009
SNW domain containing 1 (*Snw1*)	Transcriptional coactivator, splicing	Fold-change	2.04	2.32	3.26
		p-value	0.02	0.02	0.04
Myeloid/lymphoid or mixed-lineage leukemia (*Mll*)	Transcriptional coactivator, histone methyltransferase	Fold-change	1.6	1.5	Not expressed
		p-value	0.03	0.05	
Peroxisome proliferator-activated receptor coactivator (*Pgc-1* α)	Transcriptional coactivator	Fold-change	2.26	2.22	Not expressed
		p-value	0.01	0.04	
Drosophila absent, small, or homeotic discs 1 (*Ash-1*)	Histone methyltransferase	Fold-change	1.56	-1.1	Not expressed
		p-value	0.0004	0.002	
Methyl CpG binding protein 2 (*Mecp2*)	Transcriptional regulation	Fold-change	1.8	Not expressed	Not expressed
		p-value	0.001		

Further analysis of these genes was carried out to identify their molecular function and how disruption to their function due to oxidative modification may impact cellular processes.

cAMP responsive binding element protein (CREB) is a transcription factor that binds to cAMP response elements in DNA to regulate transcription. CREB is differentially oxidised at six months, but not at twelve or eighteen months. However, CREB-binding protein (Crebbp), a transcriptional co-activator, is differentially oxidised at six, twelve, and eighteen months. CREB dependent gene expression is reliant upon the presence and enzymatic activity of Crebbp, establishing this protein as functionally important for the activation of metabolic processes. Selective oxidation of this transcript may lead to changes of the transcriptome as less Crebbp protein is available for transcriptional activation of target genes.

Crebbp is a ubiquitously expressed, global transcriptional co-activator, which shares regions of very high sequence similarity with protein E1A binding protein p300, including in its bromodomain, cysteine-histidine-rich regions, and histone acetyltransferase domain. Crebbp acts a transcriptional co-activator through facilitating the recruitment of translational machinery and as a histone acetyltransferase (HAT) by modifying chromatin structure thereby making DNA more accessible to transcription factors and activators (Bannister and Kouzarides 1996, Goodman and Smolik 2000) (figure 3.12). One protein Crebbp has been shown to interact with is the GATA zinc finger domain containing 2 (Gata-2) transcription factor. Gata-2 is selectively expressed in endothelial cells (Minami*, et al.* 2004), and Gata-2 null mice were shown to have a profound reduction in hematopoietic stem cell differentiation and neurodevelopmental defects including axon path finding and fasciculation (El Wakil*, et al.* 2006, Tsai*, et al.* 1994). Findings support a crucial role of Gata-2 for gene expression in vascular endothelial cells, however the molecular mechanism by which Gata-2 brings about differential expression remains largely unknown (Minami*, et al.* 2001, Minami*, et al.* 2009, Wozniak*, et al.* 2007). Our study involved gene expression profiling of RNA extracted from the anterior spinal cord of normal mice, and although this was to enrich for a MN population, genes specifically expressed in other cell types would be present/identified. We discovered Gata-2 to be up-regulated in the oxidised fraction at six, twelve, and eighteen months. This gene is not constitutively expressed in all cell types, so it is interesting that it is selectively oxidised, most likely in endothelial cells. A reduction in transcriptional fidelity within these cells would also leads to changes at the

transcriptome, which may have a wider impact on the surrounding cells which may require their support.

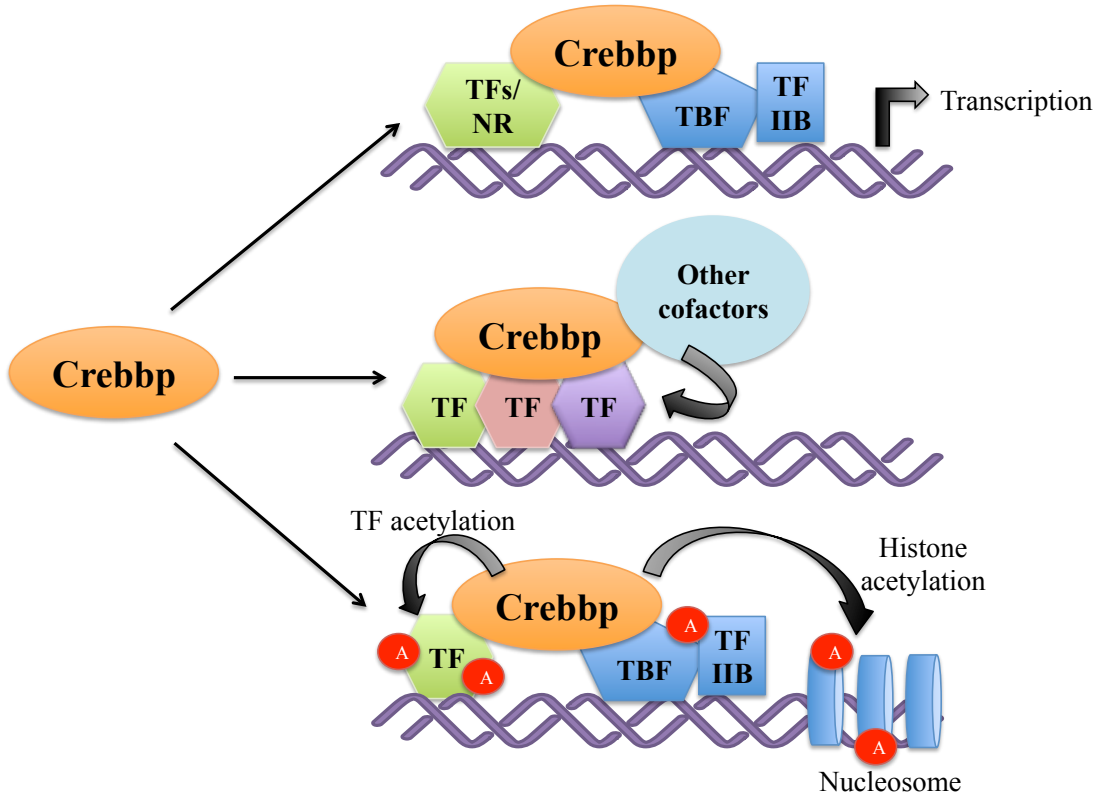


Figure 3.12: CREB-binding protein transcriptional control mechanisms. Crebbp can regulate transcription through the bridging of various transcription factors, nuclear receptors, and other basal transcription machinery. Crebbp can act as a scaffolding protein for the formation of multi-protein complexes including transcription factors and co-factor proteins. The modification of core histones and non-histone transcription factors by post-translational chromatin modifications can modulate the activity of genes. Crebbp facilitates this through modification by acetylation. Abbreviations: Crebbp, CREB binding protein; NR, nuclear receptors; TBF, TATA box binding protein; TF, transcription factor; TFIIB, transcription factor II B (Adapted from Karamouzis, *et al.* 2007)

ATP-dependent helicase Atrx encodes an ATP-dependent chromatin-remodelling protein chromodomain and helicase-like domains subclass of SNF-2 like proteins. These proteins are involved in transcriptional regulation, replication, and DNA repair (Carlson and Laurent 1994, Matson, *et al.* 1994, Picketts, *et al.* 1996). Atrx was found differentially oxidised at six, twelve, and eighteen months (table 3.7).

SNW domain containing 1 (SNW1), also known as SKIP and NCoA62, is a transcriptional co-activator of nuclear receptors and functions as part of the splicosome, providing regulatory coupling between transcriptional activation and RNA processing. SNW1 has been reported to interact with a number of putative transcriptional co-activators and co-repressors, including Crebbp, glutamate receptor interacting protein 1 (Grip1), and steroid receptor co-activator 1 (Src1). The SNW1 homologs in *Saccharomyces cerevisiae* (Prp45) and *Drosophila* (BX42) are essential for cell viability, splicing (Ambrozkova*, et al.* 2001, Gahura*, et al.* 2009, Valinluck*, et al.* 2004), and nuclear export of spliced mRNAs (Farny 2008). SNW1 is differentially oxidised at six months, and is present in the oxidised fraction at twelve and eighteen months with a significant fold change but with a p-value of >0.01. SNW1 functions as part of the spicing machinery as another dimension of gene expression regulation (Zhang*, et al.* 2003). SKIP has been demonstrated as a critical for the splicing and expression of *p21*, but not other investigated p53 target genes (Chen*, et al.* 2011) This study revealed SNW1 and associated factors are critical for cell survival upon DNA damage, through specific regulation of p21 splicing. SNW1 was found to be dispensable for p21 transcription under stress conditions, but the absence of SKIP reduced splicing capability. Although this study was conducted in cancer cells, the results suggest a DNA damage response mechanism of SNW1, which can be modulated depending on the availability of the protein.

Peroxisome proliferator-activated receptor coactivator (PGC-1α) is a transcriptional co-activator with known roles in the regulation of cellular energy metabolism, gluconeogenesis, and mitochondrial biogenesis (Kressler*, et al.* 2002, Liang and Ward 2006, Mootha*, et al.* 2003). The gene was identified as differentially oxidised at 6 months and 12 months, however it was not expressed at 18 months of age in our study.

3.2.3.2 Epigenetic regulation of transcription

Changes to the transcriptome during development and ageing may be regulated in part by epigenetic changes, which determine the phenotypic traits of cells and may support cellular function, particularly in differentiated neurons (Dulac 2010, Riccio 2010). We identified genes involved in epigenetic modification to be differentially oxidised in our study, some of which are highlighted and discussed below. Histone modifications can be covalently modified to influence the structure and function of chromatin, thus allowing or

preventing the access of transcriptional machinery to DNA (Turner 2007). A bromodomain is a conserved structural module that specifically recognises and binds acetylated lysine residues on histone tails, facilitating the binding of protein complexes to chromatin. The bromodomain is present in a large number of diverse proteins, including histone acetlytransferases, methyltransferases, helicases, transcriptional co-activators, chromatin-remodelling complex proteins, and nuclear scaffolding proteins (Sanchez and Zhou 2009). These proteins modulate chromatin structure and have important influence over transcriptional regulation.

Epigenetic effects can alter the phenotype of a cell by selectively modifying which genes are expressed. We identified multiple bromodomain-containing genes to be differentially oxidised. The oxidative modification of these transcripts and potential subsequent reduction in protein availability may affect the transcriptional regulation and subsequent spatio-temporal expression of genes. This potentially has downstream effects in the activation/repression of certain genes for specific functions, processes, or signalling pathways. Bromodomain containing proteins are considered as functionally independent and non-redundant as shown by gene knockout studies (Basu*, et al.* 2008, Basu*, et al.* 2009, Daniel*, et al.* 2010, Glaser*, et al.* 2009, Yu*, et al.* 1995), suggesting an array of cellular disruption due to modification of various transcripts.

The histone methyltransferase (HMT) myeloid/lymphoid or mixed-lineage leukemia (MLL) protein family members contain a bromodomain and function as transcriptional co-activators. We identified the MLL3 gene, which encodes a nuclear protein involved in histone methylation, to be differentially oxidised six months and twelve months, but is not present at 18 months.

The Droshophila absent, small, or homeotic discs 1 (Ash-1) is a trithorax group histone methyltransferase, and the mammalian homolog, Ash-1 like (Ash1L) was identified as differentially oxidised at 6 months and 12 months, but was not expressed at 18 months in our study. Ash1L has histone methyltansferase activity and been shown to associate with the transcribed regions of active gene. It appears to have a general role in transcription as demonstrated through its association with both housekeeping and tissue-specific genes (Gregory*, et al.* 2007). Immunofluorescence demonstrated Ash1L localisation to intranuclear speckles and tight junctions (Nakamura*, et al.* 2000), however the relevance of this to gene expression and chromatin remodelling remains unknown (Balda and Matter 2009).

Methyl-CpG-binding protein 2 (Mecp2) is a chromosomal protein that binds to methylated DNA, recruiting chromatin-remodelling proteins. Mecp2 was found to be differentially oxidised at 6 months, but was not expressed at 12 or 18 months in our study. Mecp2 mediates transcriptional repression through interaction with histone deacetylases, however studies also suggest a role of Mecp2 in transcriptional activation and splicing regulation (Bogdanovic and Veenstra 2009, Young*, et al.* 2005). Mecp2 interacting proteins include Atrx, CREB, and Smarca2, which have also been identified in this study. Evidence has shown the presence of 8-hydroxydeoxyguanosine at a hemimethylated CpG dinucleotide reduces methyl binding protein association (Valinluck*, et al.* 2004), suggesting oxidative DNA damage also impacts transcriptional regulation.

3.2.3.3 Cellular homeostasis

Genes involved in maintaining cellular homeostasis and involved in normal cellular functions were identified in the oxidised fraction of RNA. Changes in cellular homeostasis and genes involved in regulatory functions have previously been shown to change expression during ageing (Brink*, et al.* 2009). Disruption to normal cellular metabolism has also been highlighted in ALS, with disturbances to mitochondrial function, neurotransmission, and protein homeostasis linked to the underlying pathology. Here we discuss some of the genes identified in the oxidised fraction, which are involved in important cellular processes.

3.2.3.3.1 Glutamatergic system

A number of genes implicated in glutamatergic neurotransmission were identified in this study. This highly regulated system maintains precise physiological concentration of glutamate in the CNS; dysregulated excitatory neurotransmission leading to increased levels of extracellular glutamate leads to cellular damage (reviewed in Niciu*, et al.* 2012). Some of the interesting genes identified here are listed in table 3.9, and although some of the fold changes are not significant, a slight change in neurotransmission regulation could lead to excitotoxicity.

Table 3.10: *Differentially oxidised genes involved in glutamatergic neurotransmission*

Gene name	*Function*		*6 month*	*12 month*	*18 month*
Glutamate receptor, ionotropic, AMPA4 (alpha 4) (*Gria4*)	Glutamatergic neurotransmission	*Fold-change*	1.88	1.5	1.96
		p-value	0.0003	0.0003	0.013
Solute carrier family 1 Glial high affinity glutamate transporter, member 3 (*Slc1a3*)	Removal of glutamate from the synaptic cleft	*Fold-change*	No expression	No expression	1.3
		p-value			0.04
Solute carrier family 1 (glial high affinity glutamate transporter), member 2 (*Slc1a2*)	Removal of glutamate from the synaptic cleft	*Fold-change*	1.8	No expression	-1.5
		p-value	0.002		0.02
Glutamate receptor, metabotropic 5 (*Grm5*)	Glutamatergic neurotransmission	*Fold-change*	1.59	1.63	1.32
		p-value	0.004	0.048	0.048

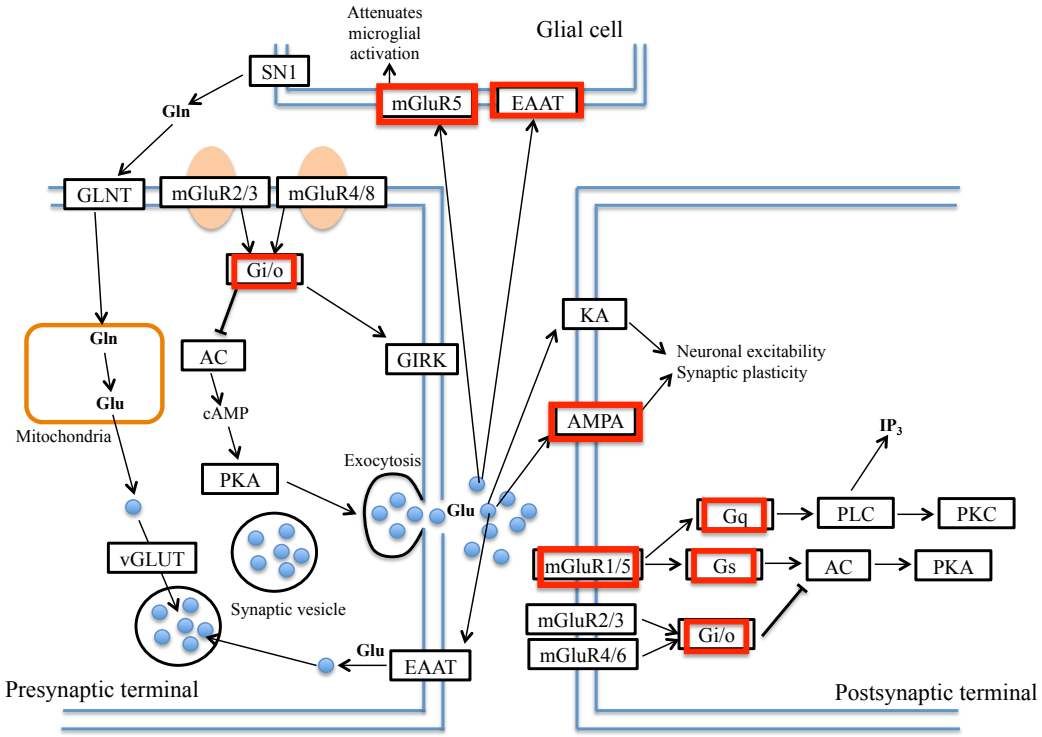


Figure 3.13: Glutamatergic neurotransmission

The proteins whose transcripts were identified to be differentially expressed as result of oxidative modification to their RNA transcript are highlighted in red.

3.2.3.4 Signal Transduction

Multiple genes enriched within the gene ontology category of signal transduction were identified as differentially expressed in the oxidised fraction during ageing, a few of which are shown in table 3.11. Signal-transduction cascades mediate the sensing and processing of stimuli. The identification of differentially oxidised genes enriched in signal transduction suggests these transcripts may be targeted for oxidation as a mechanism of cellular regulation. However, if oxidative modification to the RNA is detrimental the lack of proteins available for these transduction cascades could lead to widespread cellular dysfunction.

Table 3.11: *Genes involved in signal transduction*

Gene name	*Function*		*6 month*	*12 month*	*18 month*
Guanine nucleotide binding protein, alpha stimulating complex locus (GNAS)	Signal transduction	*Fold-change*	1.94	1.73	1.74
		P-value	0.05	0.02	0.02
Guanine nucleotide binding protein, alpha inhibiting 1 (Gnai1)	Signal transduction	*Fold-change*	4.64	3.69	3.69
		P-value	0.02	0.02	0.02
Glycogen Synthase Kinase 3-β (GSK3β)	Signal transduction	*Fold-change*	2.1	Not expressed	1.4
		P-value	0.01		0.002

3.2.4. Further validation from functional annotation analysis

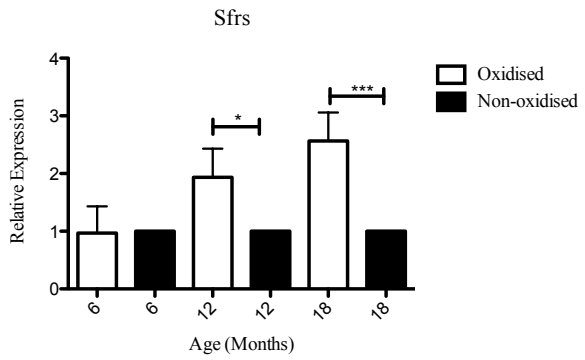
Further to the enrichment and pathway analysis, additional genes were selected for validation (table 3.12). These were selected based on their functional association with mechanisms identified as dysregulated in MND. The levels of transcript show a general up-regulation in the oxidised versus non-oxidised fraction, however they are significantly greater in the oxidised fraction for *Sfrs* and *Gnai* (figure 3.14). The fold change generally show a trend in the right direction for all, corresponding to the array findings, however the variability between samples meant this did not reach significance in some cases.

Table 3.12: *Genes selected for further validation*

Fold change information from Partek analysis

Gene Title	*Gene Symbol*	*Function*		6 Months	12 Months	18 Months
CREB-binding protein	*Crebbp*	Transcription	*Fold-Change*	3.0	2.8	3.7
			P-value	0.008	0.01	0.01
SWI/SNF related, matrix associated, actin dependent	*Smarca2*	Transcription	*Fold-Change*	4.8	4.4	6.2
			P-value	0.0009	0.003	0.02
Glutamate receptor, ionotrophic, AMPA4	*Gria4*	Glutamate signalling	*Fold-Change*	1.9	1.5	2.0
			P-value	0.0003	0.0004	0.01
Serine/arginine-rich splicing factor 18	*Sfrs18*	Alternative splicing factor	*Fold-Change*	2.6	2.5	3.5
			P-value	0.01	0.0005	0.01
Guanine nucleotide binding protein, alpha inhibiting polypeptide 1	*Gnai*	Inhibition of adenylate cyclase	*Fold-Change*	3.2	3.7	3.9
			P-value	0.007	0.02	0.01

A.


Sfrs
Relative Expression
Age (Months)
Oxidised
Non-oxidised
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2
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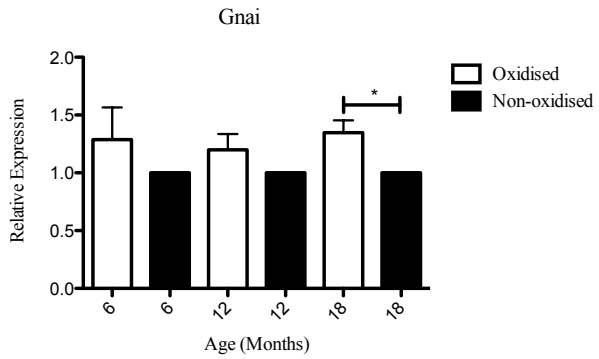
18

B.


Gnai
Relative Expression
Age (Months)
Oxidised
Non-oxidised
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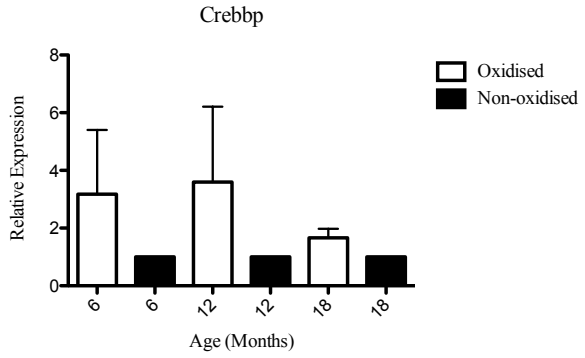
1.0
1.5
2.0
6
6
12
12
18
18

C.


Crebbp
Relative Expression
Age (Months)
Oxidised
Non-oxidised
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2
4
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12
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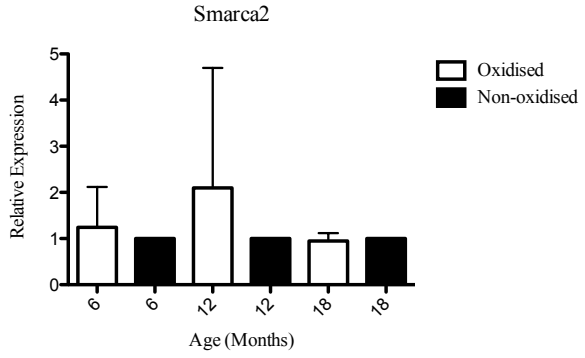
18

D.


Smarca2
Relative Expression
Age (Months)
Oxidised
Non-oxidised
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1
2
3
4
5
6
6
12
12
18
18

Figure 3.14: Results of second RT-qPCR validation for selected genes at six, twelve, and eighteen months

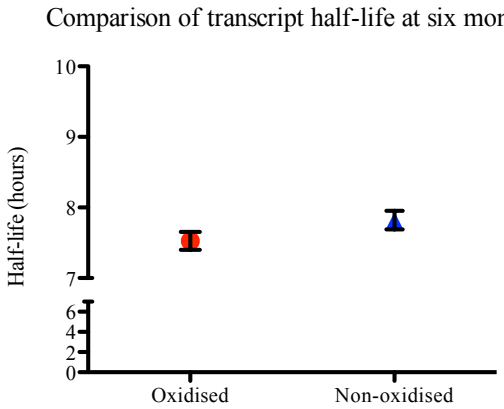
The oxdised and non-oxidised fraction for the selected genes were normalised to house-keeping gene Actin before the relative concentration was determined by comparing the ddCt of the oxidised fraction to the ddCt of the non-oxidised fraction. Data presented as mean with SD (n=3), statistical analysis by one-way ANOVA with Bonferroni post-test

3.2.5 Selective targeting of transcripts for oxidation

Functional annotation and pathway analysis revealed specific classes of RNA transcripts that are targeted for oxidative modification, and identified how this could affect the pathways they are involved in. To determine whether specific properties of the differentially oxidised transcripts explain in part their increased susceptibility to oxidative modification, our lists of differentially oxidised genes at six, twelve, and eighteen months were compared to a list of 19,000 genes with known information on mRNA length, number of exons, mRNA half-life, and decay rate. This information was available for a select number of genes identified in our study; a total of 4214 genes at six months, 3024 genes at twelve months, and 2727 genes at eighteen months.

For analysis of transcript features genes with a fold change of ≥1.5 were classified as oxidised and those <1.5 (1-1.49) were classed as non-oxidised. mRNA length, number of exons, mRNA half-life, and decay rate were analysed, with the mean and standard error of the mean for each fraction plotted to determine a difference between them. Analysis revealed no significant difference in the mRNA half-life of oxidised and non-oxidised transcripts at six and eighteen months. However there is a significant increase in the mRNA half-life of non-oxidised transcripts at twelve months (figure 3.15). The mean half-life for the oxidised transcripts is 7.5 hours at six, twelve and eighteen months. The average mRNA length, measured in base pairs (bp), of transcripts in the oxidised fraction at six and twelve months was significantly greater in comparison to the non-oxidised transcripts. No significant differences in mRNA length were seen for transcripts at eighteen months (figure 3.16). The mean mRNA length of transcripts in the oxidised fraction was about 4400bp for those expressed at six and twelve months, and 3308bp for those expressed at eighteen months. The number of exons was significantly greater for the oxidised transcripts at six and twelve months in comparison to the non-oxidised transcripts, however no significant differences were seen at eighteen months (figure 3.17).

A.



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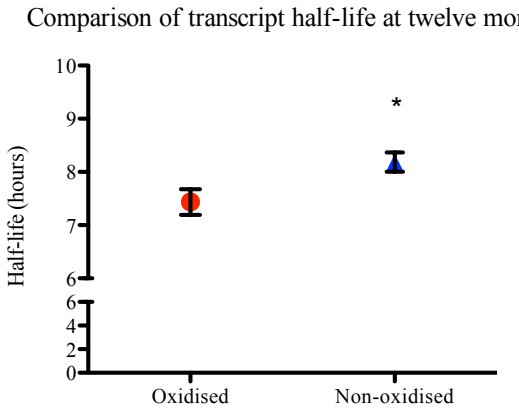

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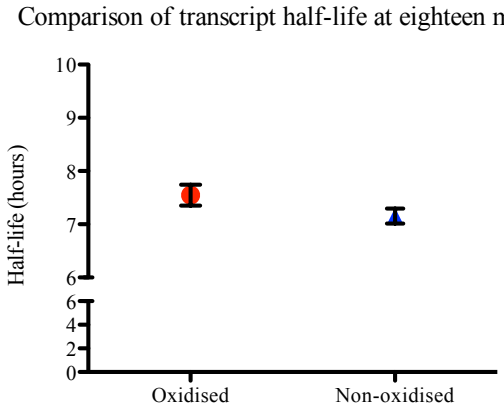
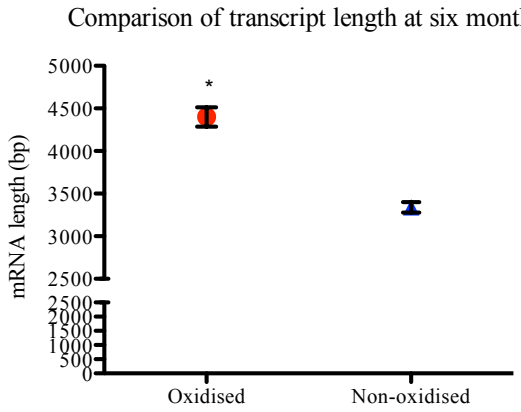


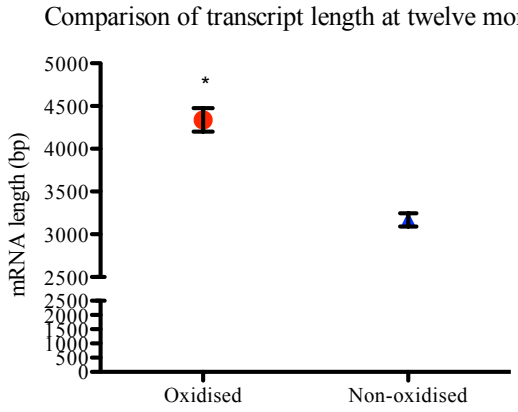
Figure 3.15: Comparison of transcript half-life of genes differentially oxidised at six, twelve, and eighteen months

Genes in the oxidised fraction have a FC>1.5, genes in the non-oxidised fraction have a FC<1.5. Data presented as mean and standard error, statistical analysis by two-tailed t-test *=P≤0.05

A.



B.



C.

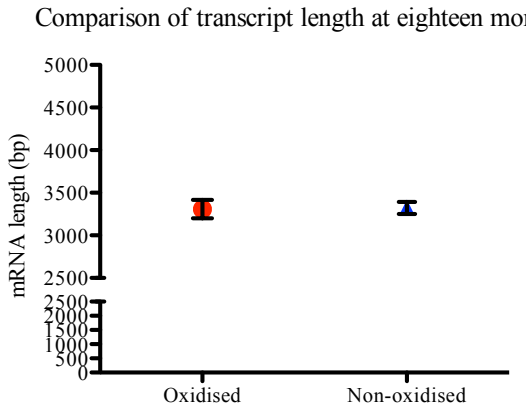
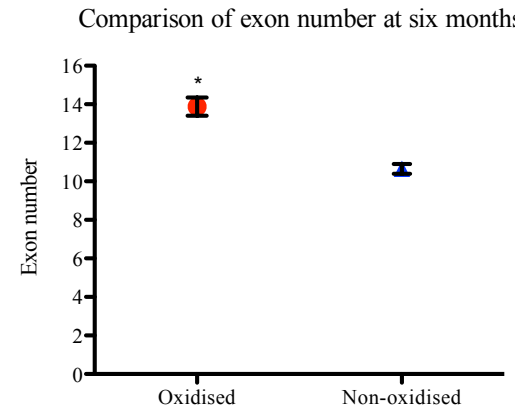


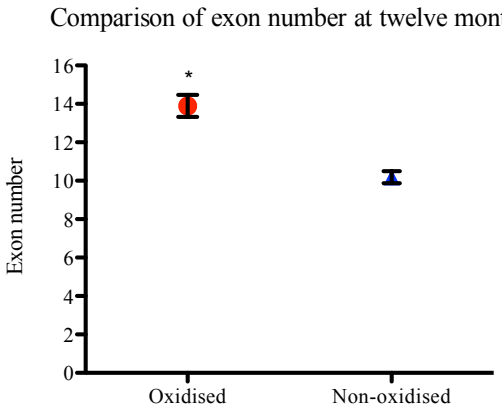
Figure 3.16: Comparison of mRNA length of genes differentially oxidised at six, twelve, and eighteen months

Genes in the oxidised fraction have a FC>1.5, genes in the non-oxidised fraction have a FC<1.5. Data presented as mean and standard error, statistical analysis by two-tailed t-test *=P≤0.001

A.



B.



C.

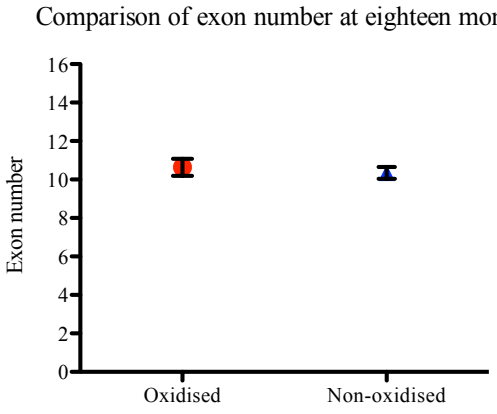


Figure 3.17: Comparison of exon number of genes differentially oxidised at six, twelve, and eighteen months

Genes in the oxidised fraction have a FC>1.5, genes in the non-oxidised fraction have a FC<1.5. Data presented as mean and standard error, statistical analysis by two-tailed t-test *=P≤0.001

3.3 Discussion

The development of microarray and RNA sequencing technology has enabled investigation of the transcriptome of cells and tissues under various physiological and pathological conditions (Malone and Oliver 2011, Schena*, et al.* 1998). Genome-wide patterns of transcriptional changes associated with ageing have been studied in both model organisms and various human tissues. In our study we used microarray technology to identify genes that are differentially oxidised in RNA extracted from the anterior thoracic spinal cord of normal mice aged six, twelve, and eighteen months. We revealed oxidative modification affects specific transcripts, and which transcripts are modified largely changes with age. Functional annotation and pathway analysis revealed the classes of RNA targeted for modification also changes during ageing. Age-related expression changes may either be due to gene expression changes, or changes in cell heterogeneity within a tissue (Zahn and Kim 2007). The genes differentially oxidised may be a result of the increased availability of these transcripts for modification. It is also plausible that some oxidised transcripts may not have been successfully precipitated and are either present in the non-oxidised fraction, or were lost during the immunoprecipitation process. As RNA technology improves the immunoprecipitation of RNA will hopefully be as efficient and sophisticated as that for proteins.

This study was based around an investigation by Chang et al. 2008, which identified oxidation of specific transcripts in a murine model of ALS (Chang*, et al.* 2008). Our aim was to investigate whether specific classes of transcripts are targeted for oxidative modification during ageing, and whether this recapitulates what is seen in a neurodegenerative disorder. This study built on the work by Chang et al. through optimisation of the immunoprecipitation procedure (section 4.2.2.2) and improvements to the methodology. In the Chang et al. study, RNA pooled from two whole cords dissected from G93A mutant *SOD1* transgenic mice was used for the immunoprecipitation and subsequent microarray analysis. We originally aimed to use laser capture microdissection (LCM) to isolate MNs from the spinal cord tissue of normal mice aged six, twelve, and eighteen months, to investigate selective oxidation of transcripts in an individual cell population. LCM uses a high-energy laser source to isolate individual cells from the rest of the tissue (Bonner*, et al.* 1997, Emmert-Buck*, et al.* 1996). LCM of astrocytes from post-mortem tissue with different Braak classification identified changes in the astrocyte transcriptome during ageing (Simpson*, et al.* 2011). The use of LCM in gene expression studies has identified different gene expression profiles within the grey and white matter

of the hippocampus during early AD, and shown an up-regulation of cell-death associated genes with a corresponding reduction in the expression of cytoskeleton and transcription related genes in post-mortem tissue from ALS patients (Blalock*, et al.* 2011, Jiang*, et al.* 2005, Simpson*, et al.* 2011). It is also a useful tool for investigating changes in the transcriptome of select cell types during disease progression, through their isolation from disease models such as the familial-ALS mutant *SOD1* mouse model (Ferraiuolo*, et al.* 2007). In our study, the quantity of RNA obtained through this method was insufficient for use in downstream applications such as the isolation of the oxidised fraction of RNA. Therefore, we chose to use the anterior spinal cord, which is a MN enriched region of the spinal cord, and a method widely used in gene expression profiling due to the quantity and quality of RNA obtained from extraction (Ginsberg*, et al.* 2004). Whilst this is a clear improvement on the samples used in the Chang et al. study, the heterogeneous cell population within the samples must be considered during analysis.

3.3.1 Identification of oxidised transcripts during ageing

Genespring and Partek analysis software was used to identify genes selectively present in an oxidised fraction of RNA. Further functional annotation and pathway analysis revealed specific classes of genes to be oxidatively modified during ageing. Further to this, the individual transcripts identified to be modified largely changed during ageing. The Database for Annotation, Visualisation, and Integrated Discovery (DAVID) was used to identify any enrichment of gene classes at each age. The functional annotation algorithm measures relationships between the annotation terms based on the degree of their co-association genes. DAVID is a good tool for initially categorising large list of genes into manageable data sets, and provides an overview of whether select classes of transcripts are enriched in the analysis. For greater in-depth analysis manual categorisation and further functional annotation using other bioinoformatic tools and manual methods is required. In our study, we identified chromatin-remodelling, transcriptional co-activators, histone modification, and DNA methylation genes to be enriched in the oxidised fraction, which are further discussed below. Some of the genes identified have also been linked to neurological disorders.

3.3.1.1 Oxidative modification of transcriptional regulators

Maintaining gene expression patterns is essential for specific gene activation in specific cell types under certain conditions. A slight change in the expression or function of these proteins could have widespread effects on gene expression and genomic stability. The identification of an enrichment of genes involved in transcriptional regulation in the oxidised fraction at six, twelve, and eighteen months highlights these particular species may be more susceptible to oxidative modification. Some of the key genes identified in this study are discussed here.

MeCP2, which specifically recognises and binds methylated DNA, andcan promote the activation or repression of gene transcription depending on the methylation state of the promoter region (Chahrour*, et al.* 2008, Cohen*, et al.* 2008), was enriched in the oxidised fraction at six months in our study. Significant changes in DNA methylation have been demonstrated in normal and pathological ageing, which was associated with changes in mRNA levels suggesting modification of the transcript has downstream effects of transcriptional regulation (Siegmund*, et al.* 2007). PGC-1α, a transcriptional activator, was identified as differentially oxidised at six and twelve months in our study. An increased expression of PGC-1α is found in tissues with abundant mitochondria, and PGC-1α promotes its effects through the expression and activation of various transcription factors. Its importance in metabolism has been demonstrated through *in vivo* studies, where knockout of PGC-1α led to reduced mitochondrial function and oxidative capacity (Leone*, et al.* 2005, Lin*, et al.* 2002). PGC-1α is a transcriptional co-activator that interacts with a broad range of transcription factors to regulate a variety of biological processes, including mitochondrial biogenesis, antioxidant defense, fatty acid metabolism and fibre type alteration in skeletal muscle. PGC-1α has a CREB response element in its promoter region, rendering CREB responsible for its activation (Herzig*, et al.* 2001). Other cellular signals known to control energy and nutrient homeostasis have also been shown to activate PCG-1α (Puigserver and Spiegelman 2003), and its antioxidant properties suggest a role in protecting neurons against ROS induced damage (St-Pierre*, et al.* 2006). This is accompanied by *in vitro* studies, which demonstrate an increase in mitochondrial dysfunction and oxidative stress following PGC-1α silencing. Reduced neuronal PGC-1α levels have also been associated with neurodegenerative disorders including HD, PD, and MS, which is thought to be due to reduced activation of its target genes and subsequent mitochondrial redox imbalance (Witte*, et al.* 2013). This evidence provides a link between mitochondrial and transcriptional regulation as mechanisms of

neurodegeneration at the molecular level. Under homeostatic conditions, PGC-1α may maintain a balance between metabolic requirements and protection from ROS. The evidence suggests the absence of PGC-1α, due to oxidative modification of the RNA transcript for example, may render cells vulnerable to oxidative stress related dysfunction and oxidative damage leading to neurodegeneration.

Crebbp is also a transcriptional co-activator through association with CREB, a transcription factor that has been implicated in neuroprotection (Chan and La Thangue 2001, Lonze and Ginty 2002). Crebbp was differentially oxidised at six, twelve, and eighteen months, and a number of known Crebbp interaction partners were subsequently identified within this fraction. Crebbp acts as a transcriptional activator for multiple target genes. Its HAT activity enables fine-tuning of gene expression through coordinating with histone deacetylases (HDACs) to control transcriptional activation (Verdone*, et al.* 2005). Specific patterns of acetylation at specific lysines within the N-terminus of histones allow targeted transcriptional activation. Different activators induce different acetylation patterns *in vivo*, suggesting selectivity of HATs and co-activators (Deckert and Struhl 2001). The epigenetic regulation creates another opportunity for disruption to transcription due to oxidation, by reducing the modification of chromatin and subsequently restricting transcriptional activation. Many other non-histone proteins, including transcription factors have been shown to be substrates for Crebbp, which expands the possible mechanisms of transcriptional activators in gene expression regulation (Glozak*, et al.* 2005, Yang and Seto 2008).

The CREB signalling pathway is a prominent regulatory pathway, with roles in cell cycle, differentiation, and apoptosis, which may in part explain why this transcript was identified in the oxidised fraction at all the ages investigated. Oxidative modification of this transcript and the potentially subsequent erroneous proteins produced may reduce the activation of critical genes involved in normal cellular processes. Acetylation of the C-terminal regulatory domain of p53 by Crebbp/p300 has been demonstrated to be critical for its regulation. p53 interacts directly with Crebbp/p300 and links Crebbp activity to cell cycle regulation and genome stability (Gu*, et al.* 1997, Lill*, et al.* 1997, Scolnick*, et al.* 1997).

Crebbp has also been shown to interact with Nrf-2 and as part of the ARE-binding complex (Katoh*, et al.* 2001, Zhu and Fahl 2001) to enhance Nrf-2 dependent gene activity. Further to this Crebbp/p300 was found to directly acetylate Nrf-2 *in vivo* in response to arsenite-induced stress (Sun*, et al.* 2009). This establishes acetylation as

novel regulatory mechanism that functions together with Keap-1 in modulating Nrf-2 antioxidant response, which is pivotal for cellular response and protection to insult. A reduction in antioxidant response during periods of oxidative stress due to a reduction in the availability of Crebbp for transcriptional activation would increase cellular vulnerability to damage. NF-κB is another transcription factor that depends on Crebbp/p300 as transcriptional co-activators (Hassa*, et al.* 2003, Zhong*, et al.* 1998). It also requires interaction of poly(ADP-ribose) polymerase-1 (PARP-1) which is acetylated by Crebbp (Perkins 1997). Acetylation of specific lysine residues is required for its interaction with NF-κB and subsequently for transcriptional activation. This provides two mechanisms whereby a loss of Crebbp could lead to a redundancy in the inflammatory response. Although none of these genes were identified as being differentially expressed due to oxidation, the removal of Crebbp upstream would reduce the activation of these targets.

Atrx, a protein involved in transcriptional regulation, replication, and DNA repair (Carlson and Laurent 1994, Matson*, et al.* 1994, Picketts*, et al.* 1996) was identified as differentially oxidised in our study. Atrx has been reported to interact with the death domain-associated protein DAXX chaperone to regulate the incorporation of histone variant H3 at highly repetitive regions, supporting the role of Atrx in chromatin remodelling (Lewis*, et al.* 2010). Atrx has also been shown to interact with the murine homologue of Drosophilla HP-1 (Chromobox homologue 5, Cbx5), a structural adapter, which may facilitate its assembly into chromatin-remodelling complexes. Interesting Cbx5 was enriched in the oxidised fraction at six months in our study. Mutations in Atrx have been shown to cause aberrant methylation of repetitive DNA elements (Berube*, et al.* 2000, Gibbons*, et al.* 2000). Methylation of DNA is an epigenetic signalling mechanism used to silence genes.

3.3.1.2 Oxidative modification of epigenetic factors

Epigenetic changes can include chemical modifications at the level of the nucleotides, modifications at the histone level, and nucleosome remodelling. Post-translational histone modifications are capable of altering the condensation of the chromatin and, as a consequence, the accessibility of the DNA to the transcriptional machinery. Genes involved in epigenetic modification were identified as differentially oxidised at all three ages, some of which are identified in the results and discussed in further detail here.

MLL3, a member of the mixed-lineage leukemia family of proteins was identified as differentially oxidised in our study. The histone H3, lysine 4 (H3K4) methylation mark is broadly correlated with the presence of RNA polymerase II at sites of active gene expression (Guenther*, et al.* 2005). The MLL histone methyltransferases act to maintain these active chromatin domains (Patel*, et al.* 2007). MLL3 gene knockout studies in rodents indicate MLL3 is involved in regulating genes associated with metabolic homeostasis, and in turn revealing a specific role for these genes in cellular metabolism (Lee*, et al.* 2008). The gene knockout studies highlight the importance of the normal activity of genes, and determine the consequences of a loss of function, as a result for example of oxidative modification. MLL3 is also found in complexes with other transcriptional co-activators involved in nuclear receptor transactivation. In particular it is found in the activating signal cointegrator-2 complex (ASCOM), which colocalises in the nucleus with ATPase-dependent chromatin remodelling complex Swi/Snf for efficient binding to target genes (S. Lee*, et al.* 2009). Interestingly some Swi/Snf proteins were also differentially oxidised in our study.

Wide changes in gene expression during ageing could be attributed to DNA damage. The redistribution of chromatin modifiers may be a protective response to DNA damage and/or occur during normal ageing, however this may lead to epigenetic changes affecting genomic integrity and gene expression (Oberdoerffer and Sinclair 2007). Yankner and colleagues demonstrated a link between global age-related gene repression and oxidative damage to the promoters of repressed genes (Lu*, et al.* 2004). Oxidative modification to RNA transcripts and subsequent reduction in functional protein complexes may also impact transcriptional activation/repression.

3.3.1.3 Oxidative modification of genes involved in glutamatergic neurotransmission

3.3.1.3.1 Glutamate Receptors

The ionotropic glutamate receptor AMPA 4 (Gria4/GluR4) and the glutamate receptor, metabotropic 5 (Grm5/mGluR5) were present in the oxidised fraction at six, twelve, and eighteen months. A reduction in Gria4 or Grm5 protein due to oxidative modification of the RNA transcript may lead to an alteration in their signal transduction properties and excitatory synaptic transmission. Ionotropic receptors for ion channels depend on cation flux, whereas metabotropic receptors are linked to G-proteins and exert their effects

through downstream signal transduction cascades (Heath and Shaw 2002). GluR4 is an AMPA receptor; these undergo RNA editing which is fundamental for determining the calcium permeability of receptor complexes containing GluR2. GluR4 exhibits a more restricted spatial and temporal distribution in comparison to GluR1, GluR2, and GluR3 (Monyer*, et al.* 1991).

mGluR5 couple to the Gq family of G-proteins, leading to the activation of phospholipase C, the production of diacylglycerol and inositol 1,4,5-trisphosphate and release of intracellular calcium (Niswender and Conn 2010). Activation of these pathways modulates synaptic activity and plasticity. Stimulation of mGluR5 through agonist binding activates Gs proteins (stimulatory G-proteins), which activate adenylate cyclase (AC) leading to an increase in intracellular cAMP. An increase in cAMP activates protein kinase A (PKA), a serine/threonine kinase, which phosphorylates and in turn regulates other kinases and transcription factors. Interestingly we identified multiple G-proteins to be differentially oxidised in our study. A reduction in translational efficiency of mGluR5 could lead to reduced expression of the receptor at the cell surface, or aberrant protein expression, affecting the structural and signalling properties of the receptor, with downstream functional consequences. This receptor is present in glial cells, and the activation of this receptor attenuates their activation. Reduced agonist interaction due to conformational changes may lead to widespread activation of glial cells. Studies have demonstrated mGluR5 involvement in learning, memory, and synaptic plasticity (Balschun and Wetzel 2002). mGluR5 modulators have been shown to have a wide range of often controversial effects in animal models of neurological disorders, making this a key area of active drug discovery (Bird and Lawrence 2009, Carroll 2008, Cook 2010, Gasparini*, et al.* 2008, Krystal*, et al.* 2010).

3.3.1.3.2 Glutamate Transporters

The solute carrier family includes high affinity glutamate transporters with distinct functional properties (Kanai and Hediger 2004). In our study, we identified the solute carrier family 1 (glial high affinity glutamate transporter), member 2 (Slc1a2) to be differentially oxidised at six months of age. Interestingly this gene was not expressed at twelve months, and was present in the non-oxidised fraction at eighteen months of age. This gene encodes a membrane-bound glutamate transporter, which acts to clear glutamate from the extracellular space to regulate neurotransmission and prevent neuronal damage from excessive activation of receptors. This protein has been demonstrated to be highly vulnerable to oxidative stress (Pedersen*, et al.* 1998), which

could account for its up-regulation in the oxidised fraction seen here at six months. Oxidative modification of the transcript may lead to the production of aberrant proteins, which are more likely to aggregate and be exposed to damage/sequestration. Reduced expression of Scl1a2 in the spinal cord and motor cortex of ALS patients has been demonstrated, which is accompanied by studies showing a reduction in glutamate transport in human mutant *SOD1* transgenic mice (Canton*, et al.* 1998, Guo*, et al.* 2000). The uptake of glutamate in to astrocytes is essential for its conversion to glutamine. A reduction in glutamate uptake due to reduced or aberrant protein expression as a consequence of oxidative modification to the transcript could lead to an energy deficit, with neurons requiring alternative routes to sustain glutamate production (Sala*, et al.* 2005). The changes in Scl1a2 expression observed here may reflect the changes to the transcriptome with age. These proteins regulated by neuronal activation (Benediktsson*, et al.* 2012), and during development and adolescence, increased neuronal and synaptic activity and changes in metabolic conditions may result in increased expression of these transporters, making them susceptible to oxidative modification. Abnormal splicing of the Scl1a2 mRNAs have been reported in the affected areas of ALS patients, which correlates with reduced protein expression (Lin*, et al.* 1998). Oxidative modification to the transcript may affect its recognition by the spicing machinery, leading to abnormal splice variants which are unable to produce the proper protein.

3.3.1.4 Oxidative modification of genes involved in signal transduction

G-protein coupled receptors (GPCRs) transduce extracellular stimuli in to intracellular signals through interaction of their intracellular domains with heterotrimeric guanine nucleotide binding proteins (G-proteins). We identified the guanine nucleotide binding protein, alpha stimulating complex locus (GNAS) gene, which encodes the stimulatory G-protein-α subunit to be differentially expressed in the oxidised fraction at six months, twelve months, and eighteen months. Alternative splicing of this transcript results in different forms of the stimulatory G-protein-α subunit, which is essential for the activation of adenylate cyclase (AC). A reduction in AC activation could potentially lead to widespread metabolic defects impacting multiple processes, including reduced phosphorylation of CREB and subsequent disruption to transcriptional activation. Interestingly guanine nucleotide binding protein, alpha inhibiting 1 (Gnai1) was also identified to be differentially oxidised at six, twelve, and eighteen months. In contrast to the alpha stimulating G-protein, the alpha inhibiting G-protein inhibits AC preventing

activation of the cAMP dependent pathway. This adds complexity when investigating oxidative modification of transcripts as a detrimental process.

In this study the serine/threonine protein kinase glycogen synthase kinase 3-β (GSK3β) was differentially oxidised at 6 months. GSK3β forms a scaffold complex with adenomatous polyposis coli (APC), AXIN1, and β-catenin as part of the Wnt signalling pathway. This APC-AXIN1-GSK3β complex can either direct β-catenin towards degradation through the phosphorylation by GSK3β (Aberle*, et al.* 1997), or allows its translocation to the nucleus where it associates with T-Cell Factor (TCF)/Lymphoid Enhancer Factor (LEF) transcription factors facilitating the transcriptional activation of Wnt-responsive genes (Eastman and Grosschedl 1999, Miller*, et al.* 1999).

In the presence of Wnt signalling the N-terminal of GSK3β is phosphorylated, leading to an accumulation of unphosphorylated β-catenin which translocates to the nucleus. An increase in β-catenin levels promotes changes in the transcriptional machinery facilitating transcriptional activation. An increase in oxidative modification to GSK3β, potentially leads to a reduction in GSK3β protein, meaning it cannot facilitate the activation of Wnt-responsive genes or phosphorylate its targets, including β-catenin. GSK3β phosphorylates a number of other substrates such as glycogen synthase and other metabolic enzymes, transcription factors CBP (CREB Binding Protein), c-Myc and c-Jun, and the translation initiation factors eIF2 and eIF2B. In addition, GSK3β is negatively regulated by PI3K (Phosphatidylinositol 3-Kinase)-mediated activation of Akt/PKB (Protein Kinase-B). As part of the insulin signalling pathway, phosphorylation of glycogen synthase 1 (GSY) stimulates glycogenesis to maintain glucose homeostasis. A reduction in GSK3 potentially leads to reduced activation of GSY, resulting subsequently in an energy deficit.

3.3.2 Selective vulnerability to oxidative modification

The number of genes identified at each age to be involved in a particular function, such as RNA processing, was small, suggesting changes in the transcriptome reflect cellular development and ageing. The cells that are present at eighteen months represent ones that have not already succumbed to degeneration or are a result of neuronal pruning during the ageing process. The transcripts that are expressed, and thus more likely to be targeted for oxidation, differ across the ages, and at eighteen months represent those that are maintaining homeostasis and cell survival. There is a consensus of programmed cell

death during development, accompanied by an increased efficiency in processing and function through elimination of unnecessary or inadequate cells.

Synaptic plasticity mechanisms change in the aged brain, which correlates with memory deficits in aged animals (Bach*, et al.* 1999, Barnes 1979). These changes in gene expression could explain why some transcripts are vulnerable to oxidation at different ages. The distinction between genomic malleability and instability is complex and subtle. Certain modifications may be essential for adaptation and function; for example, the methylation or oxidation of cytosine contributes towards genetic variability by altering gene expression in response to endogenous or environmental changes (Nabel*, et al.* 2012). The change in genes involved in epigenetic mechanisms identified in the oxidised faction with age may represent changes in epigenetic regulation with age.

The cytoplasmic level of mRNA within a cell is determined by the ratio of its synthesis rate and its degradation rate. A study concluded that mRNA abundance correlated best with mRNA half-life, and labile transcripts were rare, while those that were more stable were more abundant, independent of transcription rate (Sharova*, et al.* 2009, Yang*, et al.* 2003). This suggests transcripts with important physiological roles, which remain stably expressed within cells, may be vulnerable to oxidative modification. The comparison of our differentially expressed genes to a list of genes with known information on mRNA length, number of exons, mRNA half-life, and decay rate, revealed certain features of transcripts that may make them more vulnerable to oxidation. Interestingly in the genes identified, transcript half-life was not a factor influencing selective oxidation in our study. It may be hypothesised that transcripts with a longer half-life would be increasingly susceptible, due to the availability of the transcript for modification. Transcript length and number of exons however were significantly increased for those species identified in the oxidised fraction at six and twelve months, suggesting longer transcripts are more readily modified. For future work it would be interesting to compare the GC content of transcripts between the oxidised and non-oxidised transcripts, as it may be hypothesised an increase in GC content may increase the affinity of the transcript for oxidation. It is currently unknown whether guanine residues in specific sequences are more prone to oxidation; however as guanine is the most readily oxidised base there is reason to suspect this is a factor.

3.3.3 Relation to neurodegenerative disease

It would be interesting to investigate whether genes selectively oxidised during ageing, are also selectively targeted for oxidation during motor neuron disease and other neurological disorders. A similar study was performed in human G93A mutant *SOD1* transgenic mice to investigate selective oxidation of RNA species in ALS (Chang*, et al.* 2008). Few genes identified in the ALS study correlated with those identified in our study. However, H3 histone family 3A (H3f3a) was identified differentially oxidised in the ALS study and in our study at six and eighteen months, whereas Retinoblastoma binding protein 4 (Rbbp4) which was also identified as differentially oxidised in the ALS study was identified to be differentially non-oxidised at six and twelve months in our study. The lack of parallel findings could reflect the differences in study design. The ALS study used pooled RNA, extracted from whole cord from two mice aged 60 days (presymptomatic stage ALS) for the immunoprecipitation of the oxidised species, whereas we extracted RNA from the anterior spinal cord only and had three replicates per age. For a direct comparison, a similar study design would need to be applied, with three replicates per condition, with ideally the oxidised fraction analysed at different stages of disease progression and compared to a healthy control.

Crebbp is a transcriptional coactivator identified as differentially oxidised in our study. Evidence implicates Crebbp dysfunction in neurodegenerative disease. Rubinstein-Taybi syndrome type 1 (RSTS1) is an autosomal dominant disorder caused by defects in Crebbp, which is characterised by craniofacial abnormalities, mental retardation and a propensity for development of malignancies (Murata*, et al.* 2001, Roelfsema*, et al.* 2005). The underlying mutation in polyglutamine (PolyQ) expansion diseases is an expansion of a CAG trinucleotide repeat. Huntington's Disease (HD) is a PolyQ disorder, characterised by the expansion of a CAG repeat within the Huntingtin (htt) protein (Di Prospero and Fischbeck 2005, Gatchel and Zoghbi 2005) leading to motor dysfunction and cognitive decline. In a Drosophila model of polyglutamine disease, the binding of the polyglutamine-containing domain of the htt protein directly to the acetyltransferase domain of Crebbp led to disruption of its activity, thus blocking Crebbp-mediated transcription (Steffan*, et al.* 2000). That Crebbp can interact with htt, led to speculation that mutant huntingtin can cause cell toxicity by interfering with the function of Crebbp and disrupting gene expression (Nucifora*, et al.* 2001). Colocalisation of Crebbp with polyQ aggregates has also been observed in cells in culture, transgenic mice, and postmortem HD brain tissue (McCampbell*, et al.* 2000, Nucifora*, et al.* 2001). Crebbp dysfunction due to a reduction of Crebbp HAT activity is sufficient to cause hypo-

acetylation, which may contribute towards cell death in the cases of HD. In cells transfected with mutant htt, cell toxicity was accompanied by a loss of Crebbp and subsequent hypo-acetylation. Interestingly over-expression of Crebbp in these cells rescued histone acetylation, resulting in reduced cellular toxicity, which suggests Crebbp dysfunction and altered gene transcription are major contributors to neurotoxicity induced by mutant htt (Jiang*, et al.* 2006). This mechanism of dysfunction could be present in other neurological disorders as RNA processing including transcriptional regulation is becoming increasingly recognised to be associated with other neurological disorders.

A murine model of ALS over-expressing human G86R mutant *SOD1* (Ripps*, et al.* 1995) showed specific depletion of Crebbp in MNs (Rouaux*, et al.* 2003). Crebbp and H3 histone acetylation levels in G86R mutant mice in comparison to wild-type (WT) littermates were investigated at 3.5 months of age, which corresponds to the symptomatic stage of disease. Crebbp protein levels were reduced by >70%; reduced immunoreactivty confirmed this, along with a reduction in histone H3 acetylation reactivity in MN nuclei of G86R mutant mice (Rouaux*, et al.* 2003). Another study demonstrated a dose-dependent reduction in cell viability of NSC34 cells exposed to oxidative stress, which was accompanied by a progressive reduction in Crebbp protein and histone H3 acetylation levels. Combined these results raise the possibility that Crebbp/p300 loss of function could be a hallmark of neurodegeneration. Epigenetic modifications provide a link between the environment and gene expression changes that may lead to disease phenotypes. These modifications are readily responsive to pharmacological interventions and are potentially platforms for therapeutic approaches against neurological disorders.

Mutations in MeCP2 are the cause of most cases of Rett syndrome, a progressive neurological disorder, highlighting the importance of this protein in modulating chromatin structure and gene expression (Amir*, et al.* 1999, Wan*, et al.* 1999). MeCP2 binds methylated promoter sites and recruits a corepressor complex (Kriaucionis and Bird 2003), suggesting the pathology associated with mutations in MeCP2 results from aberrant gene expression patterns. Dysregulation of brain-derived neurotrophic factor (BDNF), a MeCP2 target gene, could also account for some of the neuropathology as BDNF has crucial roles in neuronal survival, development, and plasticity (W. G. Chen*, et al.* 2003, Martinowich*, et al.* 2003).

Mutations in Atrx cause X-linked mental retardation syndromes and α-thalassemia myelodysplasia syndrome (Gibbons*, et al.* 2000, Gibbons*, et al.* 2003, Steensma*, et al.* 2004). The Atrx chromatin-remodelling complex contains the transcriptional co-factor

Daxx, which may target Atrx to specific promoters. Mutation of Atrx may cause aberrant methylation of repetitive DNA elements and the affect the accessibility of DNA, impacting gene expression regulation.

PGC-1α plays a central role in mitochondrial metabolism, and alterations to PGC-1α expression and function have been reported in neurodegenerative diseases. In addition, the protective effects of PGC-1α have been demonstrated in *in vivo* models of ALS. PCG-1α expression in human G93A mutant *SOD1* transgenic mice prevented mitochondrial fragmentation and increased neuronal viability (Song*, et al.* 2013). Other studies have reported the induction of detoxifying enzymes is regulated by PGC-1α (St-Pierre*, et al.* 2006, St-Pierre*, et al.* 2003, Valle*, et al.* 2005), which in turn is regulated by CREB (Handschin*, et al.* 2003). Impaired expression and/or function of PGC-1α has been reported in HD, with relation to mitochondrial dysfunction (Chaturvedi*, et al.* 2009, Cui*, et al.* 2006). Microarray data from human HD and PD post-mortem tissue revealed reduced expression of PGC-1α target genes (Weydt*, et al.* 2006, Zheng*, et al.* 2010), suggesting aberrant transcriptional regulation due to PGC-1α impairment. The role of PGC-1α in ageing is supported by evidence of its involvement in telomere control, which is critical for the maintenance of chromosome integrity (Maser and DePinho 2002). A study in multiple sclerosis (MS) patients revealed a significant reduction of PGC-1α in the myelinated cingulate gyrus and frontal cortex, which correlated with reduced expression of mitochondrial antioxidant enzymes (Witte*, et al.* 2013).

Although some of the genes preferentially oxidised in this study have been identified to be associated with neurodegenerative disease, in some cases the modification of the transcript may have a protective role; for example, by regulating protein levels within a cell. Here we identified mGluR5 to be differentially oxidised at six, twelve, and eighteen months, however its over-expression has been reported in neurodegenerative pathologies. GSK-3, differentially oxidised in our study, has been linked with the pathogenesis and neuronal loss in neurodegenerative diseases, including PD, HD, and AD, in which elevated levels of GSK-3 have been reported. Regulation of levels during normal ageing through oxidative modification may mediate activation of signalling pathways. Increased cAMP levels promote survival of neuronal cells by inactivating GSK3 via a PKA–dependent mechanism. Oxidative modification may provide a similar mechanism. Oxidation of the transcript has been shown to affect normal protein production, which was hypothesised to contribute towards the underlying pathogenesis of neurodegenerative disease. In normal ageing an accumulation of oxidative modification as age advances has been reported (Nunomura*, et al.* 2012), which correlates with an increase in oxidative

stress. This increase in stress and accumulation of damage may be detrimental to cellular processes in a similar way as in neurodegenerative diseases, but the genes/mechanisms that are affected by this might be different, due to the gene expression changes accompanying disease might increase particular transcripts susceptibility to damage. The increased accumulation of oxidative modifications of particular transcripts may be a result of the availability of the transcripts for damage. Oxidative modification during ageing may be used as a regulatory mechanism by cells, or may be maintained at a level, which is not detrimental to cellular processes. It is likely that oxidative damage to RNA is less lethal than that of DNA, and RNA may represent a removal mechanism for ROS to prevent deleterious damage to DNA.

The processes that were identified as potentially being affected due to RNA oxidation in this study, are processes dysregulated in the pathogenesis of ALS, for example RNA processing. ALS is an age-related disorder, and the degradation of RNA processing and metabolism as a result of RNA oxidation during normal ageing may contribute towards the propensity of MNs to degenerate. The presence of RNA oxidation in MN has been demonstrated at presymptomatic stage of disease, suggesting it has an early involvement in pathogenesis (Chang*, et al.* 2008). It would be interesting to compare our data to the oxidised fraction of RNA extracted from the anterior horn of a transgenic model of ALS, to determine whether the selective modification of targets identified here during normal ageing is a feature of neurodegeneration.

3.3.4 Relation to other gene expression profiling studies of ageing

It is difficult to compare this study with previous ageing gene expression studies, as we were specifically interested in genes that were expressed in an oxidised fraction of RNA and whether these genes changed during ageing. We were not interested in transcriptome changes with age per se; however oxidative modification to the transcript may have an influence on those changes identified in other studies.

The Atlas of Gene Expression in Mouse Aging Project (AGEMAP) is a database containing gene expression changes during ageing in mice. It is a comprehensive standardised study of expression changes in sixteen tissues (Zahn*, et al.* 2007). Comparison of the expression profiles from mouse and human ageing revealed a small number of genetic pathways that age similarly; however no overall correlation in age-

related transcriptional changes were observed between mouse and human. Ageing has been associated with specific alterations at the mRNA level that may represent changes in gene expression, mRNA stability or turnover (Lee*, et al.* 2000). Oxidative modification of RNA may be one mechanism contributing towards these changes. To determine if what we see in our study matches the transcriptome changes identified previously the analysis needs to incorporate both the oxidised and non-oxidised fraction of each animal at each age and identify differential gene expression changes across the ages. In our study, the non-oxidised fraction was the control.

Genes identified in expression profiling studies may represent downstream markers of ageing, for example the increased expression of stress response genes due to accumulated oxidative damage. Identifying these age-related genes could provide important insight into mechanisms that drive these transcriptional changes during ageing. The AGEMAP work was used for a further study to investigate gene expression profiles in five regions of the CNS (Xu*, et al.* 2007). The number of age-associated genes was identified to be greatest in the spinal cord. Following functional classification, genes encoding proteins involved in transcriptional regulation, protein synthesis and degradation, and signal transduction were the most responsive to ageing. If a prerequisite for selective modification is the presence/abundance of the transcript at a particular age, we might expect to see an increase in oxidative modification to genes that are present during ageing. However, enrichment analysis did not reveal antioxidant enzymes/oxidative stress response genes to be enriched within the oxidised fraction, suggesting other factors may govern the selective modification of classes of transcripts.

3.3.5 Future work

For this study, the anterior thoracic spinal cord from normal mice aged six, twelve, and eighteen months were used for investigation of oxidative modification to RNA during ageing. For future work, isolating individual cell types from the spinal cord would enable a comprehensive study on selective RNA oxidation within specific cell types, which could be compared to the data from this study. The initial aim was to use laser capture microdissection to investigate RNA oxidation specifically within a MN population, however the quantity of RNA obtained through this method was insufficient for use in downstream applications. This may be made possible as improvements in technology, specifically isolation of RNA species, are improved. It would also be interesting to include a younger and an older time point to have data over an extensive time scale to

compare. Performing RT-qPCR for cell type specific genes in the RNA samples used in this study would enable us to have a better understanding of the contribution of different cell types towards the analysis, and which cell types are enriched for in our fractions.

In our study we were particularly interested in which genes are expressed at one time point only and ones that are expressed at multiple ages. Are these genes preferentially oxidised because they are involved in processes at these ages. Could the modification to these transcripts specifically affect cellular metabolism and processes, potentially predisposing the animal to age-related diseases? The fractions analysed at eighteen months were taken from animals that had survived to that age. Whether the animals that were used at six and twelve months would have lived as long, or developed an age-associated disease remains unknown, and a problem that must be factored for when using models for study. A general decline in physiological functioning characterises ageing. This deterioration of homeostasis leads to an increased risk of disease, thus age-related biological systems degradation may explain an increased incidence of many complex diseases with age. One mechanism predisposing an organism to age-related diseases might be the selective modification of RNA transcripts, which impacts cellular functioning, influences gene expression changes and ultimately leads to cellular decline. It would be interesting to determine whether the changes identified are region specific i.e. selective to the anterior spinal cord, or whether this is a general feature of CNS ageing.

Further work would involve investigation in to whether RNA oxidation affects the protein production for the modified transcripts. Initially this was attempted using *in vitro* translation to generate proteins from the transcripts within an oxidised and non-oxidised sample of RNA. Subsequent Western blot analysis was used to determine differences in protein concentration, however this was technically challenging. Further optimisation would hopefully eliminate this problem. Another approach would be to immunoprecipitate the proteins of transcripts selectively oxidised, from total protein lysate of the anterior spinal cord, to determine differences in concentration between samples. Downstream targets/processes of genes that were selectively oxidised could be investigated, to determine whether the modification of the transcripts affects cellular function.

Chapter 4
Investigating RNA oxidation in ageing and neurodegenerative disease

4.1 Nucleic acid oxidation in *in vitro* and *in vivo* models of ALS

4.1.1 Oxidative damage and neurodegenerative disease

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated as result of normal cellular metabolic processes. ROS and RNS have roles in cellular signalling and redox homeostasis; however, detrimental effects of these free radicals can occur during periods of oxidative or nitrosative stress. Oxidative stress has been extensively studied in relation to the pathogenesis of neurodegenerative diseases and the neurobiology of ageing, however, whether it is the primary cause of toxicity or the result of numerous dysfunctional mechanisms remains to be understood (Jellinger 2009, Koudinov*, et al.* 2009, Radak and Boldogh 2010). The direct measurement of oxidative stress is challenging and is mainly determined by investigating the degree of oxidative damage to cellular macromolecules, measuring antioxidant levels, studying expression levels of genes involved in the stress response, and investigating the activation of damage repair enzymes (DNA repair reviewed in section 1.6).

Pathological studies demonstrating increased levels of ROS and oxidative damage in affected brain regions of patients with neurodegenerative diseases including AD, PD, and ALS (Andersen 2004), accompanied by the use of *in vitro* and *in vivo* models of disease, have contributed much towards our understanding of oxidative stress in neurodegeneration. Oxidative stress appears to be an early and sustained event contributing towards cellular decline and selective MN degeneration in ALS (Bogdanov*, et al.* 1998, Liu*, et al.* 1999, Shaw and Eggett 2000). Investigation of post-mortem tissue from sporadic ALS (sALS) and familial ALS (fALS) patients has demonstrated an accumulation of oxidative damage to proteins, lipids, and DNA/RNA, providing support for the association of oxidative damage with underlying pathogenesis (Barber*, et al.* 2006, Ferrante*, et al.* 1997a, Sayre*, et al.* 2008). This includes elevated protein carbonyl

levels, lipid peroxidation, and nucleic acid oxidation (Ferrante, *et al.* 1997b, Fitzmaurice, *et al.* 1996, Shaw 2005, Shibata 2001). More recently, an increase in RNA oxidation in the brain and spinal cord of sALS patients and the spinal cord of the G93A mutant *SOD1* mouse model of fALS in comparison to controls has been reported (Chang, *et al.* 2008). RNA may be more susceptible to oxidative modification because of its abundance and intracellular location close to sites of ROS production, its single-stranded nature, and the lack of protective proteins in comparison to DNA. Increased RNA damage has been demonstrated in studies simultaneously comparing levels of oxidative damage to DNA and RNA (Hofer, *et al.* 2008, Wamer, *et al.* 1997). RNA oxidation changes have since been reported in studies of neurological disorders, diabetes, atherosclerosis, and ageing (Broedbaek, *et al.* 2011, Hoffman, *et al.* 2011, Martinet, *et al.* 2004, Martinet, *et al.* 2005, Shan, *et al.* 2007, Simpson, *et al.* 2010, Zhang, *et al.* 1999).

The degradation of damaged RNA was long thought to be the mechanism for its removal, rather than its repair, as this would be a more efficient process and RNA lacks a template strand for the correction of errors. However, the increasing importance of RNA with regards to the processing and metabolism of transcripts, the identification of multiple non-coding RNAs and their increasing functional roles, has led to the investigation of the consequences of RNA modification. The identification of at least one mechanism for RNA damage repair has led to speculation that other unidentified proteins and pathways might be involved in the RNA quality control process (Aas, *et al.* 2003, Barnes, *et al.* 2009, Vascotto, *et al.* 2009a).

Nucleic acid oxidation can reduce the fidelity of transcription, translation, and/or the processing of transcripts, affecting cellular processes downstream. Reduced levels of rRNA, tRNA, and protein synthesis have been identified due to RNA oxidation (Ding, *et al.* 2007, Ding, *et al.* 2005, Tanaka, *et al.* 2007), suggesting ribosome dysfunction as one consequence of increased modification to RNA. Although 8-hydroxyguanosine (8-OHG) is the oxidised base which will be focused on throughout the rest of the chapter, it must be noted that oxidative damage to other bases can occur, which may have equally damaging effects to cells. We have chosen to study this particular oxidative modification as guanine is the most frequently oxidised base, and previous studies support the investigation of this modification as a biomarker for oxidative damage. Studies revealing elevated RNA oxidation in neurodegenerative diseases have demonstrated the regional distribution of the damage to be consistent with selective neuronal vulnerability, and further investigation has addressed the consequences of oxidative RNA modifications, and how this may contribute to neurological decline (section 1.6).

The oxidative modification of macromolecules can alter their functions, resulting in downstream changes in cellular and metabolic mechanisms. For example, changes in redox-sensitive signalling accompany the structural damage when cells are exposed to oxidative stress. Understanding redox regulation and the cellular response to oxidative stress is essential for deciphering the impact of multiple factors that relate cellular dysfunction to disease pathology. This is being achieved partly through exploring cellular responses of *in vitro* models of disease to exogenous stresses, including investigation of the control of redox sensitive signalling cascades and induction of detoxifying enzymes (Gasch*, et al.* 2000, Xu*, et al.* 2011, Zheng*, et al.* 2001). Gene expression changes are also a major component of stress responses. Both general and stress specific adaptive responses function simultaneously, from post-translational effects providing immediate responses, to regulation of gene expression essential for slower long-term adaptation and recovery phases (de Nadal*, et al.* 2011). Gene expression profiling in the NSC34 cellular model for *SOD1*-associated fALS identified a reduction in the expression of detoxifying enzymes and antioxidant response proteins in cells carrying a human G93A mutant *SOD1* transgene in comparison to controls (Kirby*, et al.* 2005). A significant reduction in the expression of genes involved in antioxidant activity and stress response were also reported in the G93A mutant *SOD1* mouse model of fALS (Ferraiuolo*, et al.* 2007), which suggest a transcriptional repression of oxidative response genes is induced by mutant SOD1.

4.1.2 In vivo and in vitro models of ALS

Several models of fALS based on identified genetic mutations have been generated to study the pathogenic mechanisms leading to ALS. The human G93A mutant *SOD1* mouse was the first *in vivo* transgenic murine model of human fALS (Gurney*, et al.* 1994). Since then over ten different lines of transgenic mouse for select *SOD1* mutations have been generated, accompanied by other transgenic worm, fly and rodent models (Bruijn*, et al.* 1997, Guo*, et al.* 2011, Kato 2008, Nagai*, et al.* 2001, Turner and Talbot 2008, Wang*, et al.* 2003). Motor symptoms, cellular alterations, and loss of upper and lower MNs identified in these models makes them suitable for the study of ALS. *In vitro* models of ALS include cell lines, for example the neural hybrid cell line NSC34 used in this study, and primary neurons and glial cells isolated from the embryonic murine spinal cord (Avossa*, et al.* 2006, Cashman*, et al.* 1992, Gingras*, et al.* 2007). These have proved valuable in identifying causative factors for disease. For example, the role of astrocytes in MN degeneration in ALS was shown in co-culture studies, which identified increased cell

death of MNs cultured in the presence of mutant *SOD1*-expressing astrocytes (Marchetto*, et al.* 2008, Nagai*, et al.* 2007). However, despite these models, how mutant *SOD1* selectively impairs MN function leading to degeneration remains to be understood.

The familial ALS-linked G93A mutant *SOD1* murine model of ALS and NSC34 cells stably expressing the cDNA of human G93A mutant *SOD1*, human G37R mutant *SOD1*, human H48Q mutant *SOD1*, human wild-type *SOD1* were used for investigation in this study, so attention will be focused on these mutations (table 4.1). Human Cu/Zn SOD1 is a 32kDa homodimeric enzyme, responsible for the conversion of superoxide radicals to molecular oxygen and water. Each monomer folds as an eight-stranded β-barrel, binds one atom of copper and one atom of zinc, and contains an oxidised disulfide bond between cysteine 57 and cysteine 146 (Hartz and Deutsch 1972, Tainer*, et al.* 1982). This post-translational modification renders the protein enzymatically active, adopting the correct quaternary structure, achieved by the bound copper and zinc ions respectively (Arnesano*, et al.* 2004). Histidines 46, 48, 63, and 120 bind the copper ion, whilst the zinc ion is bound by Histidines 63, 71, 80, and Asparagine 83.

Table 4.1: *Human SOD1 mutations expressed in the NSC34 cell line*
Numbers refer to clone number

Cell line	*Transfection Information*
NSC34 transfected with pIRES-6	Mammalian expression vector only control
NSC34 transfected with WTSOD1-11	Wild-type human *SOD1*
NSC34 transfected with G93A-5	Mutant human *SOD1*-single amino acid substitution of glycine to alanine at codon 93
NSC34 transfected with G37R-2	Mutant human *SOD1*-single amino acid substitution of glycine to arginine at codon 37
NSC34 transfected with H48Q-9	Mutant human *SOD1*-single amino acid substitution of histidine to glutamine at codon 48

The mutations in *SOD1* associated with the development of ALS are scattered throughout the *SOD1* gene. The G93A, G37R, and H48Q human *SOD1* mutations were investigated in this study. Mutations in two of the four histidine residues that coordinate copper have been identified in fALS, histidine-46 to arginine (H46R) and histidine-48 to glutamine (H48Q). The proximity of the H48Q mutation to the copper-binding site reduces the protein affinity for copper, and the mutant protein is devoid of enzyme activity (Ratovitski*, et al.* 1999, J. Wang*, et al.* 2007). The G93A and G37R *SOD1* mutations are confined to the β-strands, distant from the copper-binding site. In comparison to the H48Q mutation, G93A has exhibited fully active bound copper in a coordination environment similar to that of WTSOD1 (Hayward*, et al.* 2002).

Although these models offer a system for the study of the pathogenic mechanisms of ALS, the severity of disease in fALS *SOD1* mice can differ depending on expression of the transgene (Bruijn*, et al.* 2004). The transgene copy number can produce genetic and phenotypic differences in terms of the level of protein expression, and the onset and duration of disease, which must be considered when using these models to investigate disease. Studies have established a toxic gain property for *SOD1* mutations as causative of ALS, as opposed to a loss of radical scavenging function. Differences exist between the mutations in terms of copper binding, aggregation, and redox state, and although the mutations are expressed ubiquitously the mechanisms by which they selectively damage MNs remains to be fully understood (Pardo*, et al.* 1995, Wong*, et al.* 1995). It has been suggested that aberrant copper redox chemistry and SOD1 misfolding are causally linked to mutant SOD1 toxicity in MNs (Milardi*, et al.* 2010).

4.1.3 Investigating the consequences of oxidative damage in in vivo and in vitro models of familial-ALS

Observations of an increase of oxidative base damage in the brains of AD and PD patients and in spinal cord tissue of ALS patients highlights the importance of determining the contribution of nucleic acid oxidation to the observed loss of neurons in various neurological disorders (Chang*, et al.* 2008, Shan*, et al.* 2003, Zhang*, et al.* 1999). The aim of this part of the study was to determine how oxidative damage to nucleic acids changes during normal ageing and ALS progression using *in vitro* and *in vivo* models of ALS. Using immunohistochemical techniques, the presence and distribution of oxidative damage to nucleic acids and the presence of DNA damage repair within the spinal cord of transgenic mice expressing familial ALS-linked

G93A mutant *SOD1* and age-matched controls was demonstrated. An antibody against 8-hydroxyguanosine (8-OHG) that recognises both DNA and RNA oxidative modification was used as a marker for oxidative damage. DNase/RNase pretreatment before 8-OHG staining was used to identify whether the cytoplasmic 8-OHG reactivity observed is predominantly RNA, or mtDNA damage. Markers of DNA damage repair investigated include, Ogg1, DNA-PK, and γ-H2AX. These markers were chosen for investigation due to their involvement in the DNA damage response (DDR), and their association with ageing and neurological disease. Ogg1 is a DNA glycosylase that initiates the base excision repair (BER) pathway for the removal of oxidative lesions, by catalysing the cleavage of an N-glycosidic bond between a deoxyribose sugar and the modified base (Robertson*, et al.* 2009). DNA-PK serves as both a sensor and transducer of DNA damage signals. It localises to double-stranded breaks in DNA that can be formed by ROS, and initiates repair. DNA-PK can also activate apoptotic pathways in response to severe DNA damage or critically shortened telomeres (reviewed in Burma and Chen 2004). DSB's can induce DNA-PK dependent phosphorylation of H2AX and evidence suggests phosphorylated H2AX (γ-H2AX, activated form) recruits repair/signaling proteins to foci of DNA damage to initiate repair.

In addition, NSC34 cells stably expressing the cDNA of human G93A mutant *SOD1*, human G37R mutant *SOD1*, human H48Q mutant *SOD1*, human wild-type *SOD1*, or the pIRES mammalian expression vector only, were used to investigate whether the presence of different *SOD1* mutations influences the amount of oxidative damage to RNA. The effect of hydrogen peroxide (H_2O_2) on cellular viability and differences in levels of oxidative damage to nucleic acids following exposure to stress were also investigated. We hypothesised that cells carrying a human *SOD1* mutation would be more susceptible to oxidative damage in terms of RNA oxidation, and the induction of stress would lead to a dose-dependent increase in oxidative damage and subsequent reduction in cell viability.

Further to this, we sought to determine whether the expression of oxidative stress response genes was consistent between the investigated cell lines by RT-qPCR. The activation of stress responses in these cells was also investigated to determine whether the presence of a mutation affects the cells ability to respond to a sub-lethal exposure of oxidative stress. We hypothesised the activation of stress response genes would be reduced in cells carrying the mutant *SOD1* transgene, as a result of them having impaired stress response capacity.

4.2 Results

Having established differentially oxidised genes during ageing, we proceeded to investigate RNA oxidation in model systems of ALS. RNA oxidation has previously been reported at presymptomatic stage of disease in ALS and other neurological diseases. Intense cytoplasmic reactivity of 8-OHG was identified in the anterior horn MNs of G93A mutant SOD1 transgenic mice at presymptomatic stage disease in comparison to age-matched controls. Intense nuclear and cytoplasmic reactivity was also identified for Ogg1, whilst DNA-PK and H2AX had variable levels of nuclear reactivity in the MNs of both transgenic mice and controls. Further to this, we used a cellular model of ALS to investigate oxidative stress and RNA oxidation. Three human SOD1 mutations were transfected in to NSC34 cells to investigate whether different mutations confer differential susceptibility to oxidative stress and subsequent cellular damage. G93A mutant SOD1 transfected NSC34 cells demonstrated increased susceptibility to oxidative stress induced cellular decline, and increased levels of RNA oxidation in comparison to both the H48Q and G37R mutations, and the vector only and WTSOD1 controls. Following this the gene expression levels of various oxidative stress markers was examined by RT-qPCR, to identify whether these mutations affected the cells ability to respond to oxidative stress.

4.2.1 Oxidative damage and DNA repair in the spinal cord of transgenic mice expressing familial ALS-linked G93A mutant SOD1

4.2.1.1 Spinal cord morphology of G93A mutant *SOD1* transgenic mice and age-matched controls

The histomorphology of the anterior horns of G93A mutant *SOD1* transgenic mice and the age-matched controls was examined using haemotoxylin and eosin stained tissue sections (figure 4.1). Haematoxylin stains the basophilic structures blue, and eosin stains the eosinophilic structures pink. Cresyl violet staining was also used to visualise the morphology of the sections and to observe the distribution of the Nissl substance, which is stained purple (figure 4.2). The Nissl substance is composed of rough endoplasmic reticulum and ribosomes, and therefore contains an abundance of RNA.

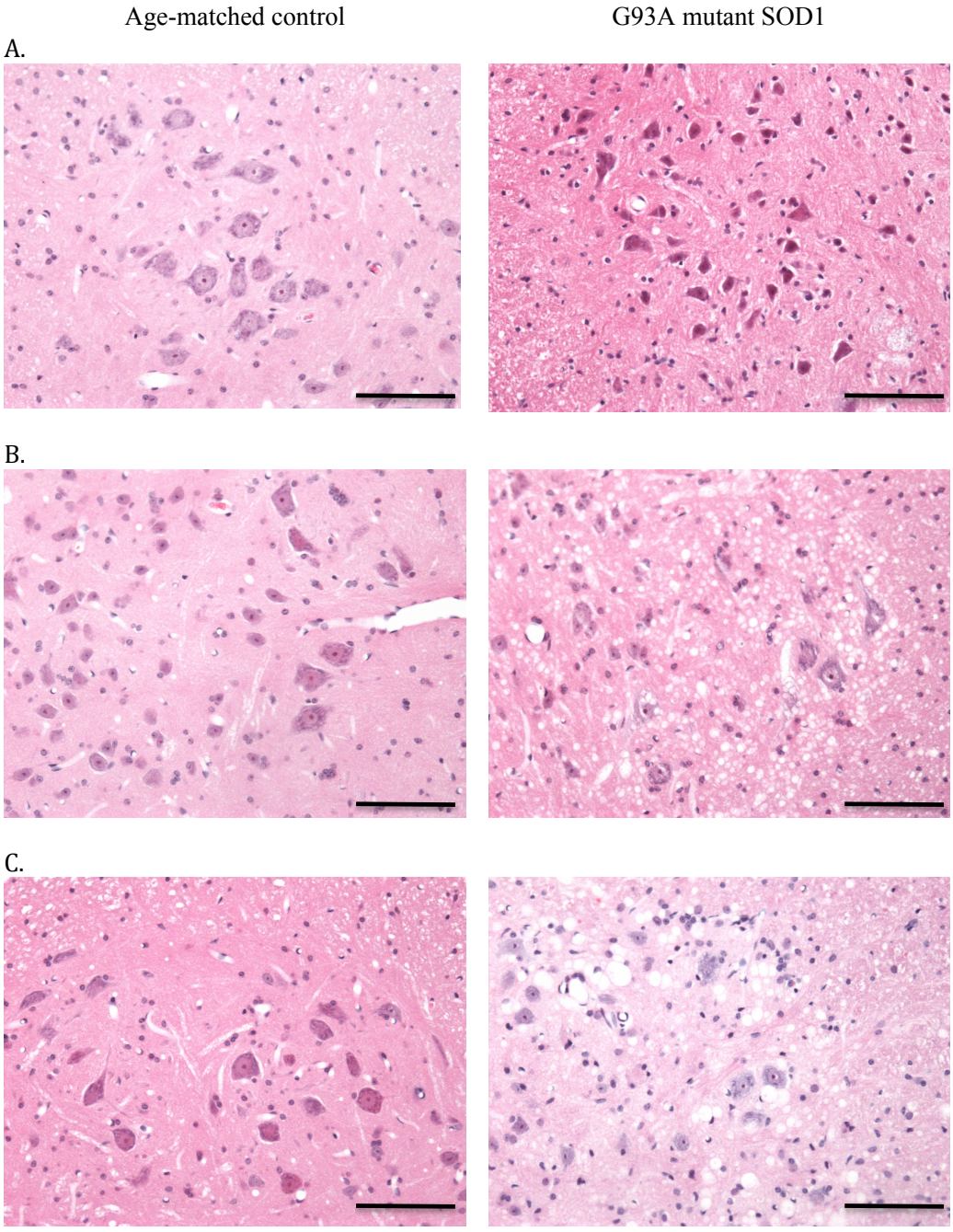


Figure 4.1: Haematoxylin and Eosin reactivity in the anterior horn of human G93A mutant *SOD1* transgenic mice

(A) Presymptomatic stage disease (B) Symptomatic stage disease (C) End-stage disease. Age-matched controls are represented in the first column. These images are a representation (n=3). Scale bar = 100µm.

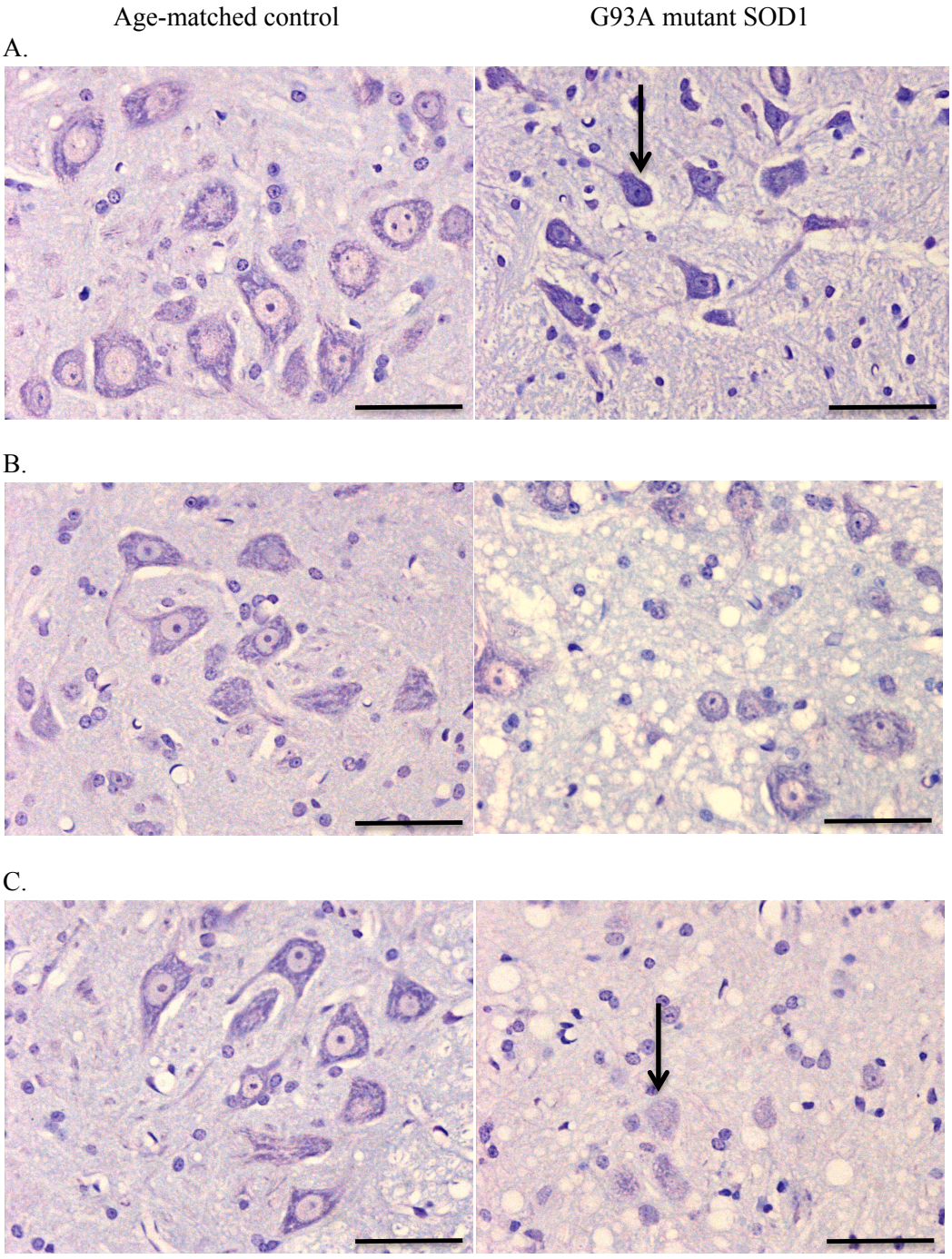


Figure 4.2: Nissl staining in the anterior spinal cord of human G93A mutant *SOD1* transgenic mice

(A) Presymptomatic stage disease (B) Symptomatic stage disease (C) End-stage disease. Age-matched controls are represented in the first column. Nissl staining identifies tissue histomorphology. The MNs in the transgenic cord display a shrunken appearance (arrow) in comparison to age matched controls at presymptomatic stage of disease (A). Towards end-stage disease (C) MNs in the transgenic mice display a loss of morphology (arrow). These images are a representation (n=3). Scale bar represents 100µm.

4.2.1.2 8-OHG reactivity displays cytoplasmic predominance in MNs

Nucleic acid oxidation within the spinal cord of transgenic mice expressing familial ALS-linked G93A mutant *SOD1* was investigated by immunohistochemical examination of 8-OHG (figure 4.3). The purpose of this work was to examine the cellular localization of nucleic acid oxidative damage. The presence of 8-OHG reactivity in MNs and small cells (glia, small neurons, endothelial cells) in the spinal cord of both transgenic and non-transgenic mice demonstrated the presence of nucleic acid oxidation during both normal ageing and neurodegenerative disease. MNs in the anterior horn of both G93A *SOD1* transgenic and non-transgenic spinal cord displayed granular cytoplasmic predominance of 8-OHG reactivity. At presymptomatic stages (60 days), the MNs in the transgenic cord displayed a shrunken appearance in comparison to the non-transgenic cord (figure 4.4, A, arrows). The staining pattern within the MNs appeared granular and clumpy which may represent the positive reactivity of the Nissl substance. At presymptomatic stages 8-OHG reactivity also extended in to neurites and was seen in the nucleus. In comparison, the nucleus of the MNs at symptomatic, end-stage disease, and in the age-matched controls displayed relatively little 8-OHG reactivity suggesting a lack of DNA oxidative damage in comparison to RNA at these stages of disease (figure 4.4, A, arrow). An intensely stained dot within the nucleus suggests perinucleolar staining, as these structures contain a plethora of ribonucleoproteins and nucleic acid.

As disease progresses there appeared to be a change in the staining pattern and a reduction in the amount of 8-OHG within the cells in the transgenic cord with a progressive loss of morphology. However, this was only assessed qualitatively and was not quantifiable from the immunohistochemical preparations. At symptomatic stages (90 days), reactivity appeared reduced in comparison to the presymptomatic cord, and there is prominent granular staining predominantly localised around the edge of the cell body (figure 4.4, B, arrow). The alteration in reactivity becomes particularly prominent towards end-stage (140 days) where perikaryon (cell body) staining was more diffuse (figure 4.4, C). The appearance of perinucleolar staining was lost, which was accompanied with a lack of distinguishable nuclear structures in some neurons. In the age-matched controls, the pattern of staining remains the same throughout with prominent perikaryon staining in the MNs.

A similar pattern and intensity of 8-OHG staining was observed in age-matched controls at each disease stage. The grainy distribution of cresyl violet reactivity supports the changes in morphology of the MNs during disease as seen with 8-OHG staining.

4.2.1.3 Nucleic acid oxidation in small cells

At presymptomatic stages, diffuse nuclear labelling with some cytoplasmic staining of 8-OHG was observed within small cells. However, the staining intensity differed across each cell, which persists at symptomatic and end-stage disease. This was also observed in the non-transgenic mouse spinal cord tissue. The intensity of staining seen within the small cells appeared darker than that for the MNs at each of the stages, which could be due to their size or their increased susceptibility to oxidative damage. Quantification of positive nuclear staining within the small cells displayed no significant change in reactivity or cell number at each time point and with disease progression. In some of the transgenic sections, small cells were seen in close proximity or at the edge of the MN at end-stage. Endothelial cells are characterised by a flat elongated nucleus, and some of these cells displayed positive 8-OHG reactivity in both disease cases and controls (figure 4.3F, open arrowheads).

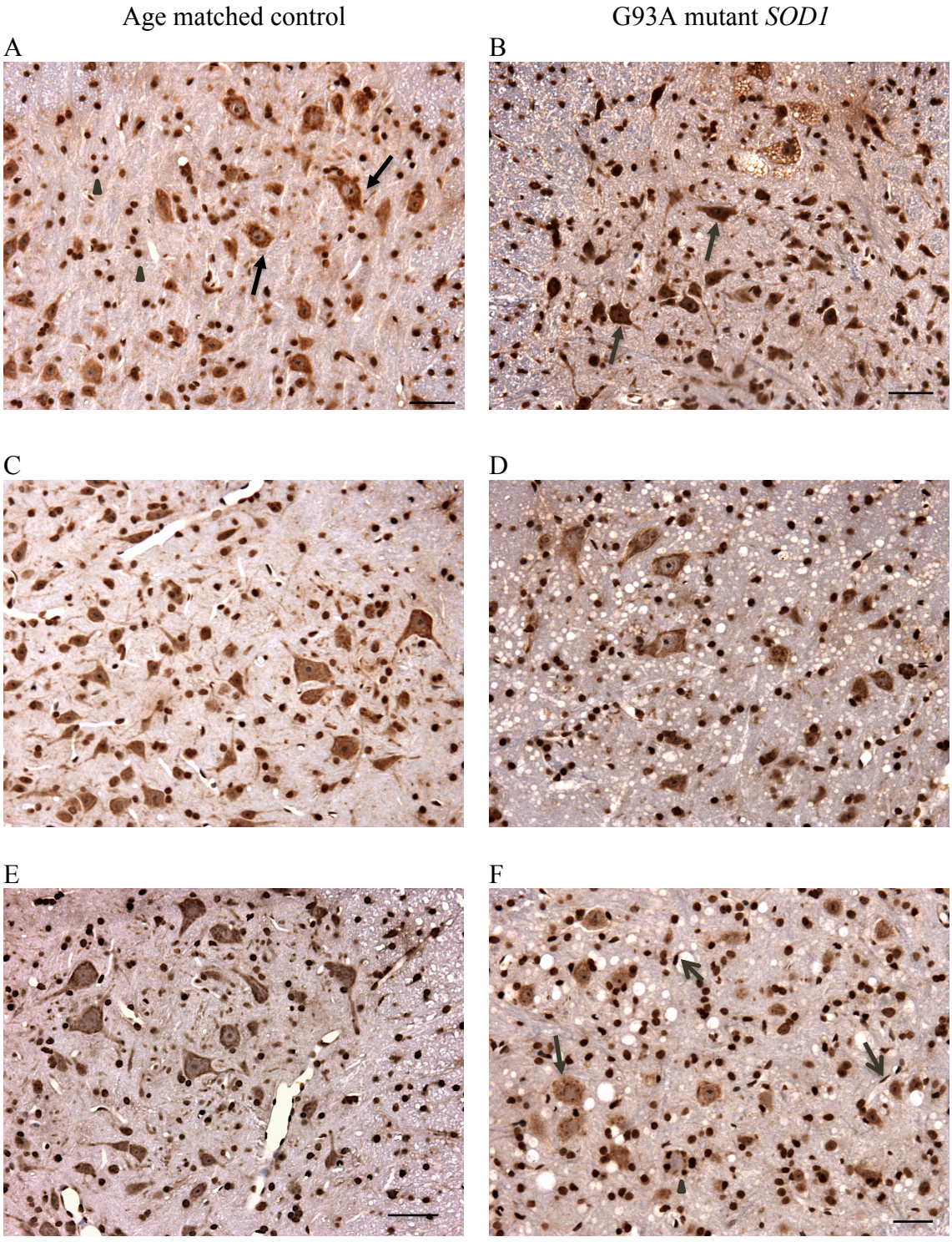


Figure 4.3: 8-OHG reactivity in the anterior spinal cord of G93A mutant *SOD1* transgenic mice

Presymptomatic, symptomatic, and end-stage disease (B, D, F respectively) in comparison to age-matched controls. Nuclear and cytoplasmic 8-OHG reactivity is prominent at presymptomatic stage of disease (A, arrows). The MNs also appear shrunken in morphology in comparison to the age-matched controls, which demonstrate a

lack of nuclear reactivity (A, arrow). Glial cells are intensely stained in both the mutant and controls (arrowhead). At end-stage disease (F), MNs appear shrunken with a loss of morphology (closed arrow). These are a representation of 8-OHG reactivity in the G93A *SOD1* transgenic mouse model of ALS. Three separate cords for each age were analysed by IHC. Scale bar represents 100μm.

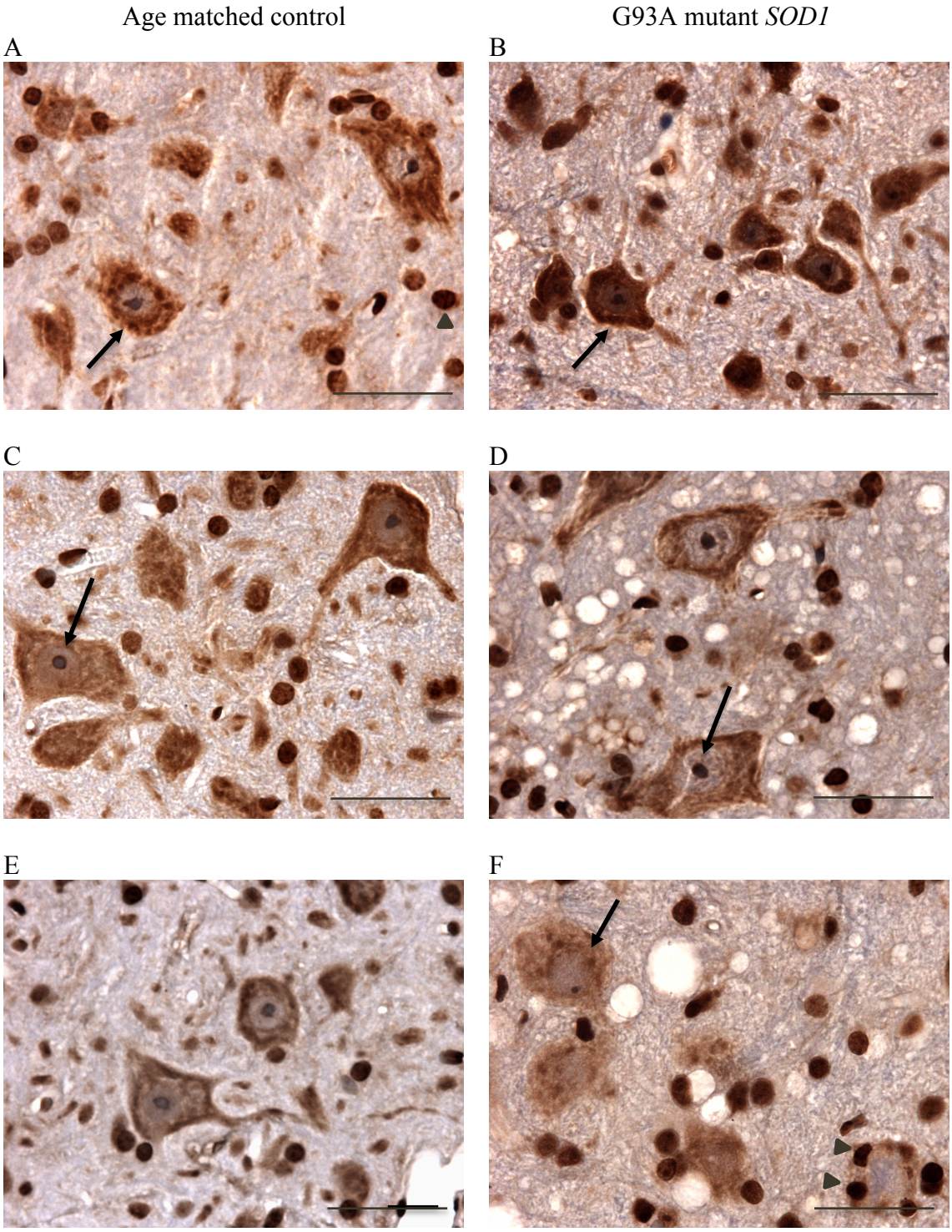


Figure 4.4: 8-OHG reactivity in the anterior spinal cord of G93A mutant *SOD1* transgenic mice

Presymptomatic, symptomatic, and end-stage disease (B, D, F respectively) in comparison to age-matched controls. Nuclear and cytoplasmic 8-OHG reactivity is prominent at presymptomatic stage of disease (A, arrow) morphology in comparison to the age-matched controls, which demonstrate a lack of nuclear reactivity (A, arrow). This

pattern continues at symptomatic stage disease (D). At end-stage disease (F), MNs appear shrunken with a loss of morphology (closed arrow), and glial cells cluster around degenerating neurons (F, arrowheads). These are a representation of 8-OHG reactivity in the G93A *SOD1* transgenic mouse model of ALS, and high power images of figure 4.3. Three separate cords for each age were analysed by IHC. Scale bar represents 100μm.

4.2.1.4 8-OHG immunoreactivity after DNase/RNase pre-treatment

After DNase treatment a clear ring of reactivity remained in some of the small cells in the control sections at 60 days, suggesting RNA oxidation either within the cytoplasm or in perinucleolar regions, with the same pattern displayed in the sections from the transgenic animals. The MNs showed prominent granular cytoplasmic and perinucleolar staining in the transgenic cord, similar to what is seen without DNase treatment at presymptomatic stage of disease. Towards end-stage disease, the MNs displayed diffuse staining accompanied by a loss of morphology. Following RNase treatment, few MNs were distinguished as the predominant cytoplasmic staining was diminished. The identifiable MNs showed a diffuse pattern of cytoplasmic staining, which appeared reduced in intensity compared to the sections without RNase treatment (figure 4.5). Variable reactivity was seen again across the small cells with both DNase and RNase treatment, suggesting the presence of both DNA and RNA damage within these cells during ALS.

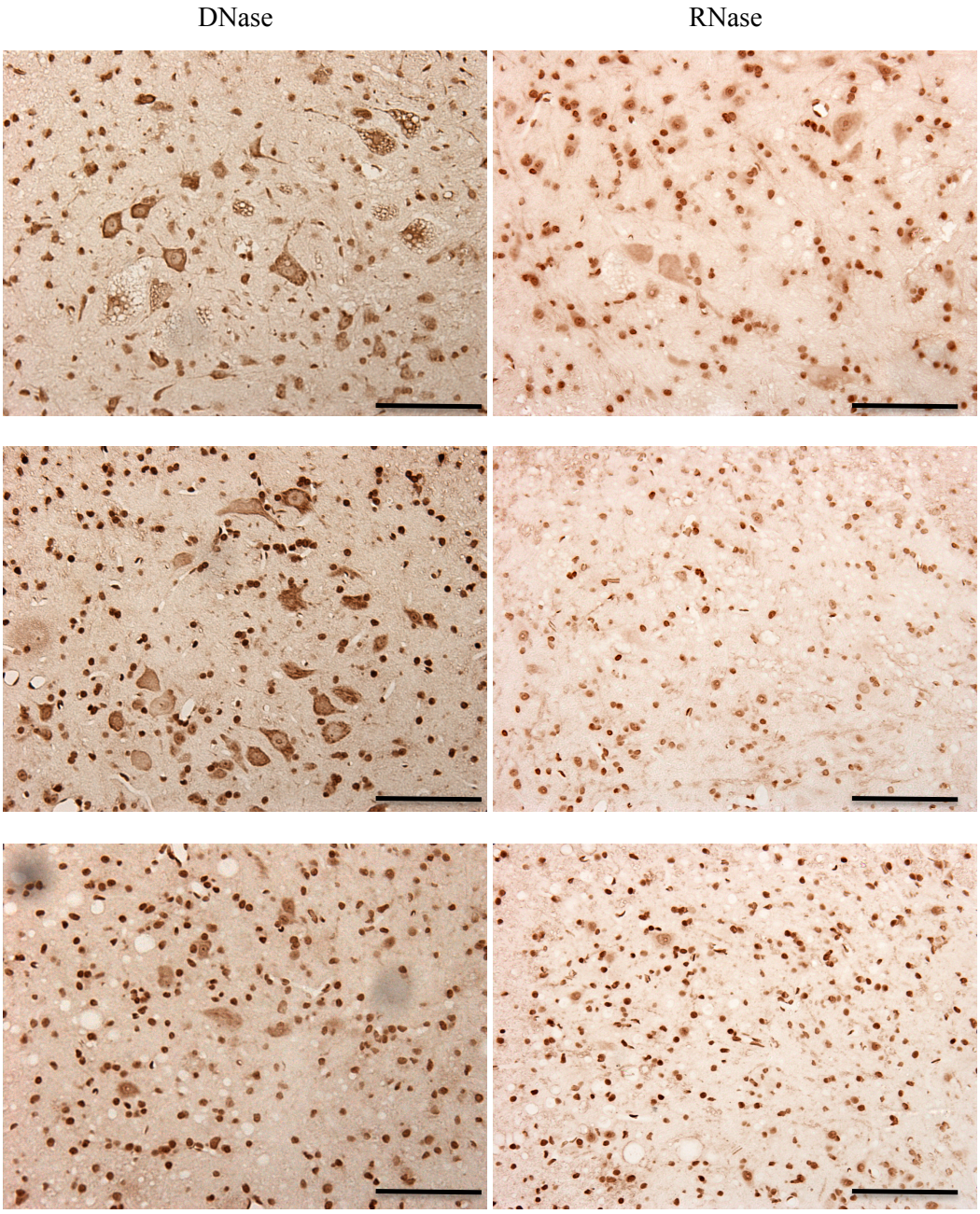


Figure 4.5: 8-OHG reactivity in the anterior spinal cord of human G93A mutant *SOD1* transgenic mice following DNase/Rnase pre-treatment.

Following DNase pretreatment cytoplasmic staining is still prominent in the MNs (arrow) in comparison to RNase pretreatment where it appers diminished (arrowheads). These are representative images (n=3). Scale bar represents 100µm.

4.2.1.5 Oxidative DNA damage repair response

The DNA damage response is often used to identify oxidative stress, as repair enzymes are activated in response to DNA modifications. Activation of this response within the spinal cord of transgenic mice expressing familial ALS-linked G93A mutant *SOD1* was investigated by immunohistochemical examination of Ogg1, DNA-PK, and γ-H2AX. At presymptomatic stage disease intense cytoplasmic and nuclear reactivity of the DNA glycosylase Ogg1 is observed within MNs (figure 4.7, B, arrow), in comparison to the controls which display a predominantly neuronal cytoplasmic pattern of staining that appeared reduced in intensity (figure 4.7, A, arrow). Ogg1 reactivity was clumpy within the cytoplasm with the presence of perinucleolar staining, which is comparable to the distribution of 8-OHG reactivity. At the symptomatic stage, there is condensed cytoplasmic reactivity for Ogg1 that is clumpy throughout the cell body, and the perinucleolar staining appeared less prominent. Towards end stage disease, cytoplasmic Ogg1 reactivity persisted with a similar staining pattern to that seen at symptomatic stage, but once again MNs appear shrunken with some showing a loss of morphology (figure 4.7, F, arrow).

At presymptomatic and symptomatic stage of disease small cells showed relatively little reactivity for Ogg1, however as disease progresses towards end-stage the number of Ogg1-positive small cells increased. Few small cells showed reactivity in the age-matched controls at all time points investigated. The detectable reactivity was subtle nuclear dot-like staining, which, unlike the MNs, doesn't parallel 8-OHG reactivity.

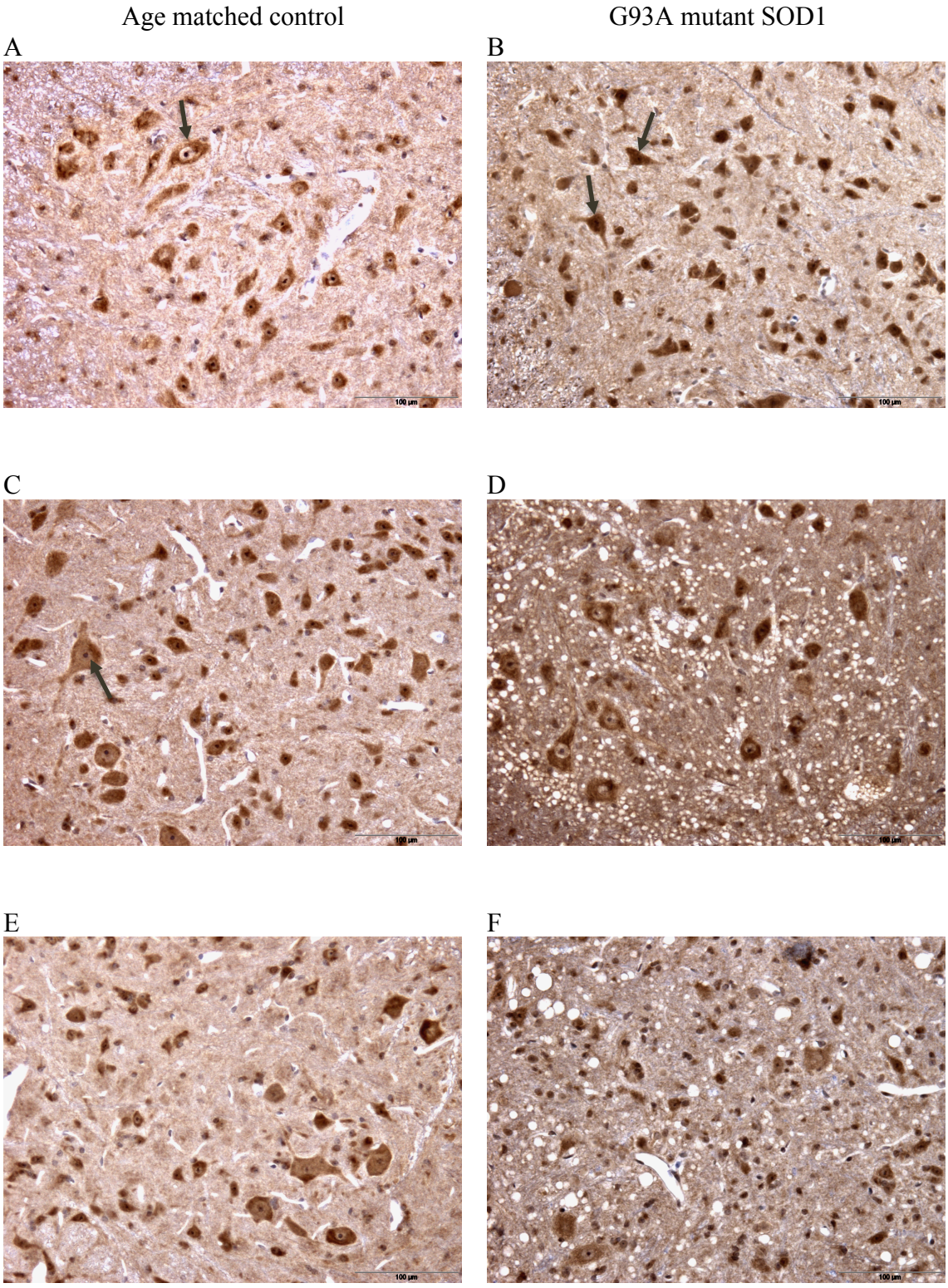


Figure 4.6: Ogg1 reactivity in the anterior horn of human G93A mutant *SOD1* transgenic mice

Presymptomatic, symptomatic, and end-stage disease (B, D, F respectively) in comparison to age-matched controls. Prominent Ogg1 reactivity is demonstrated in the

nucleus and cytoplasm of MNs at presymptomatic stage disease (B, arrows) in comparison to age-matched controls (A, arrows). These are a representation of Ogg1 reactivity in the G93A *SOD1* transgenic mouse model of ALS. Three separate cords for each age were analysed by IHC. Scale bar represents 100μm.

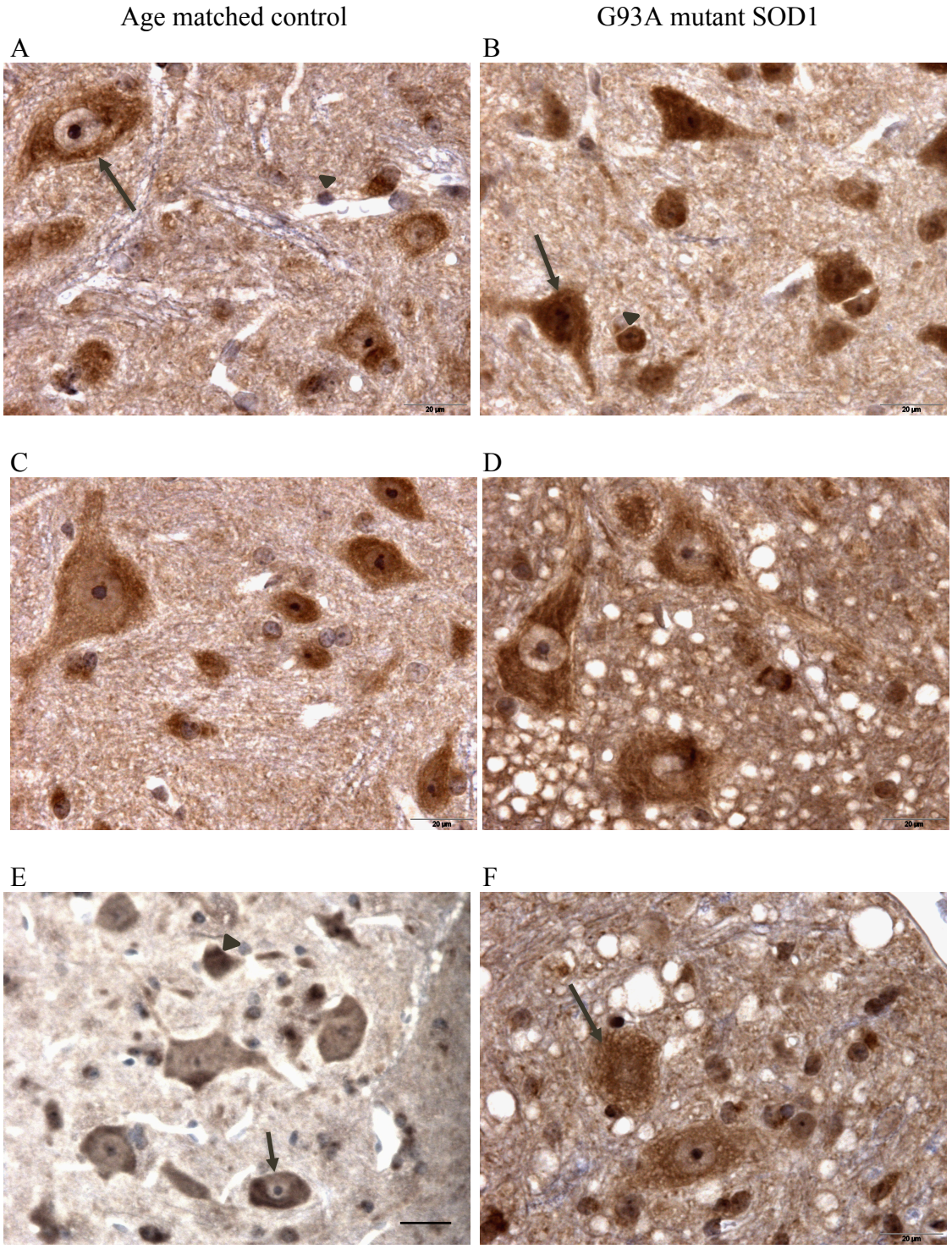


Figure 4.7: Ogg1 reactivity in the anterior horn of human G93A mutant *SOD1* transgenic mice

Presymptomatic, symptomatic, and end-stage disease (B, D, F respectively) in comparison to age-matched controls. (B) presymptomatic stage of disease, intense nuclear cytoplasmic staining in MNs (arrow) and glial cells (arrow head) in comparison to the MN in the age-matched control (A, highlighted by arrow). (D) Symptomatic stage

disease and age-matched control (C). (F) end-stage disease, some MNs show loss of morphology and Ogg1 reactivity (arrow), in comparison to age-matched controls which have negligible nuclear staining (E, arrow). These are a representation of Ogg1 reactivity in the G93A *SOD1* transgenic mouse model of ALS. Three separate cords for each age were analysed by IHC. Scale bar represents 100μm.

4.2.1.6 DNA-damage response markers vary in reactivity with disease progression

At presymptomatic stages of disease, an up-regulation of reactivity in nuclear staining of DNA-PK within MNs and small cells of the anterior horn was seen with variability of staining across cell types (figure 4.8). Some MNs displayed an intensely stained nucleus whereas others have little to no staining. Staining within the nucleus of MNs appeared granular, with no nucleolar pattern of staining. No obvious differences were observed between the intensity and distribution of DNA-PK reactivity within disease cases and controls. In relation to 8-OHG staining in the age-matched controls, oxidative damage and DNA repair response may be maintained at a level that has no detrimental effect on the cells. Levels of endogenous cellular oxidative damage are high, so the observation of damage is expected. At the symptomatic stage, DNA-PK reactivity appeared reduced in comparison to the age-matched controls, however this is only observational and was not quantifiable from the immunohistochemical results. This also correlates with the intense clumpy nucleolus staining observed for 8-OHG in the transgenic sections. DNA-PK showed markedly reduced reactivity in the nucleus of MNs towards end-stage, in comparison to the controls that displayed prominent nuclear staining in the large MNs within the anterior horn. This is consistent with the pattern of staining and morphology of MNs seen with other markers.

DNA-PK reactivity at the presymptomatic stage showed a variable intensity of staining between small cells. It is difficult to ascertain differences in the intensity of staining across these cells due to their size, however is clear that DNA repair mechanisms are activated within these cells. At end-stage disease there appeared to be more positive small cell nuclei within the field, however although this may be due to an up-regulation in DDR it may also be due to atrophy of anterior horns during disease, with synaptic loss producing an apparent increase in cell numbers.

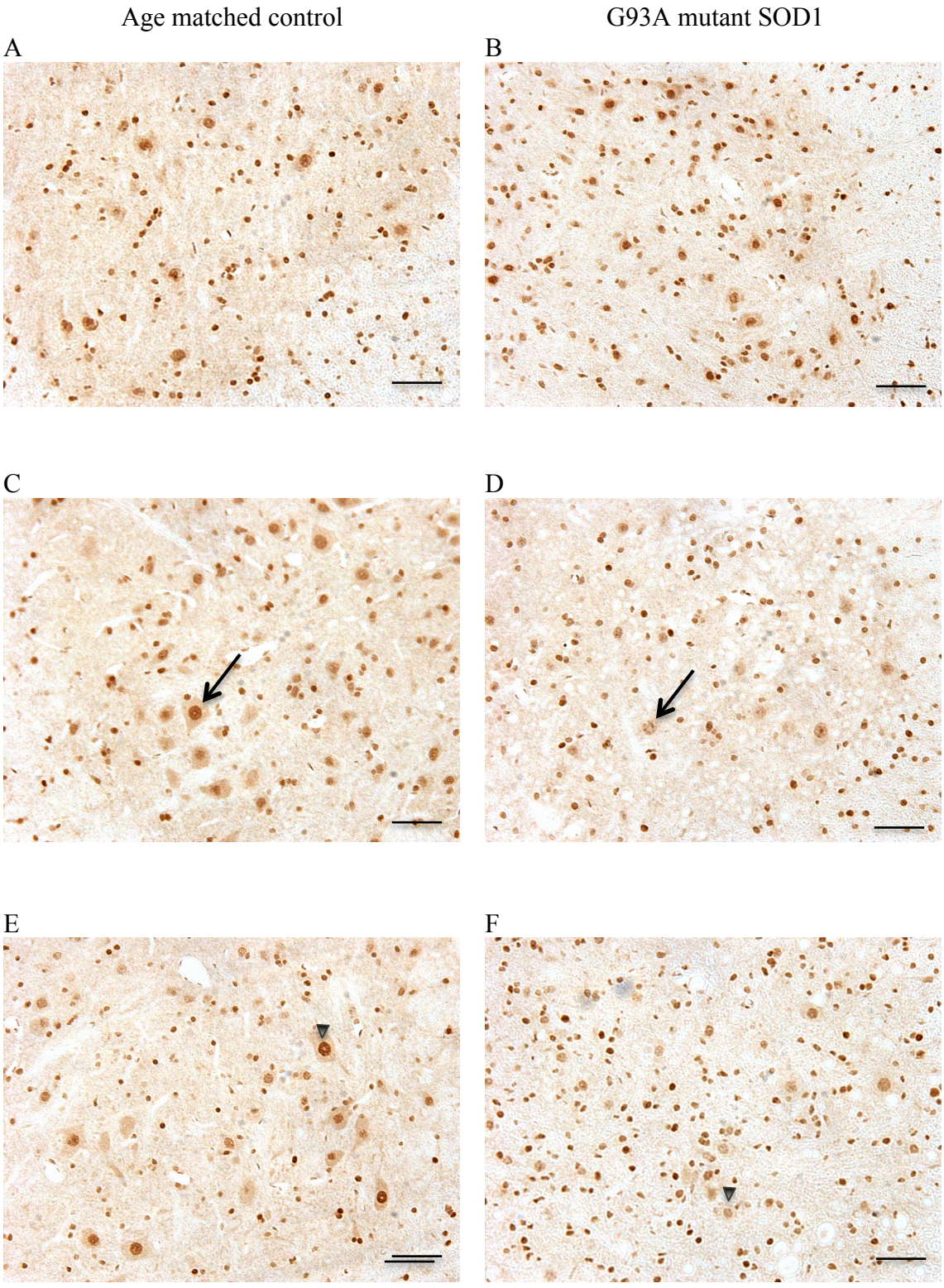


Figure 4.8: DNA-PK reactivity in the anterior horn of human G93A mutant *SOD1* transgenic mice

Presymptomatic, symptomatic, and end-stage disease (B, D, F respectively) in comparison to age-matched controls. At presymptomatic stage disease no differences in

DNA-PK reactivity are observed between disease (B) and age-matched controls (A). At symptomatic (D, arrow) and end-stage disease (F, arrowhead) DNA-PK reactivity with the MNs appears reduced in comparion to the controls (C, arrow). These are a representation of DNA-PK reactivity in the G93A *SOD1* transgenic mouse model of ALS. Three separate cords for each age were analysed by IHC. Scale bar represents 100μm.

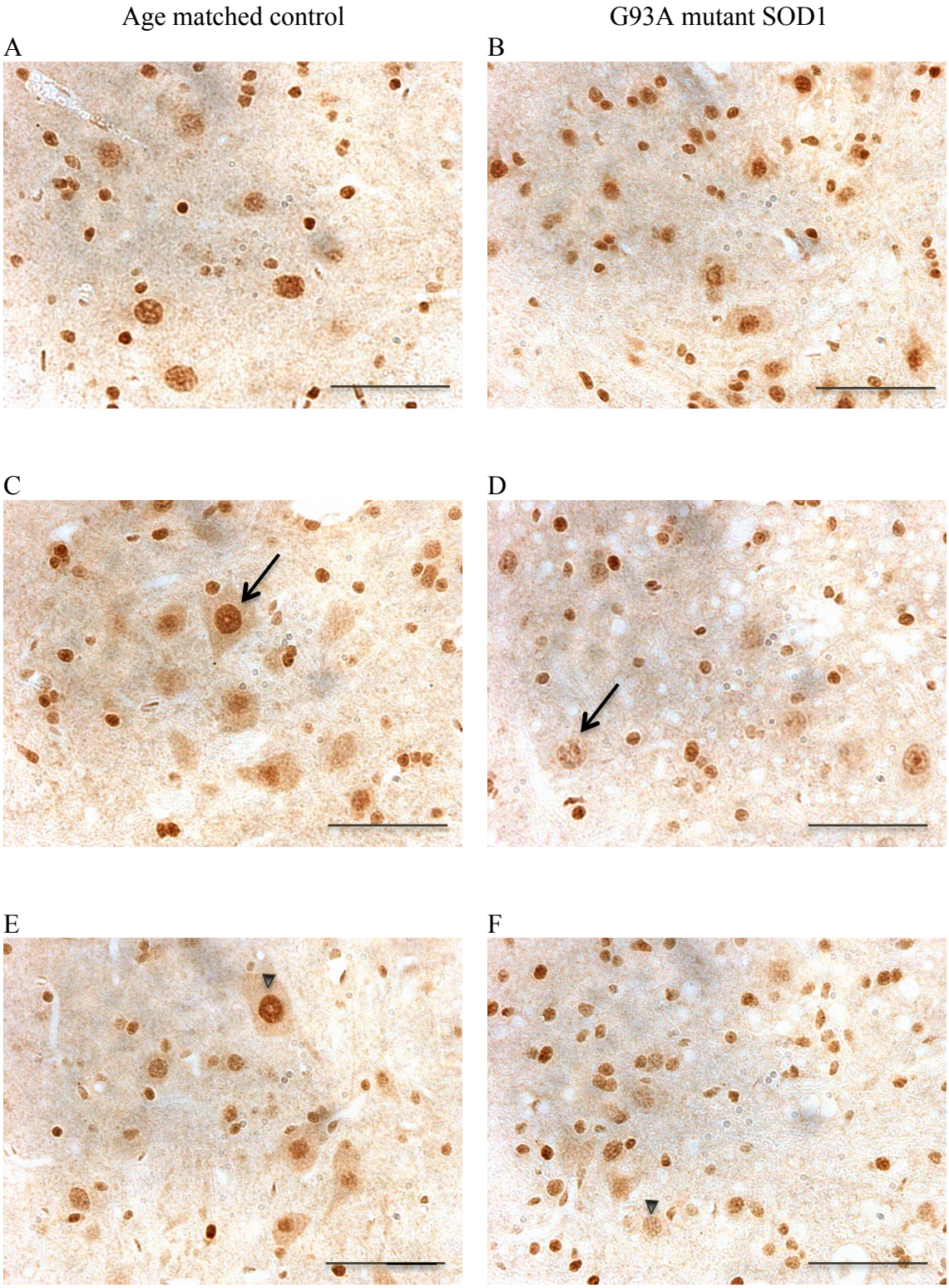


Figure 4.9: DNA-PK reactivity in the anterior horn of human G93A mutant *SOD1* transgenic mice

Presymptomatic, symptomatic, and end-stage disease (B, D, F respectively) in comparison to age-matched controls. At symptomatic (D, arrow) and end-stage disease

(F, arrowhead) DNA-PK reactivity with the MNs appears reduced in comparion to the controls (C, arrow). These are a representation of DNA-PK reactivity in the G93A *SOD1* transgenic mouse model of ALS. Three separate cords for each age were analysed by IHC. Scale bar represents 100μm.

4.2.1.7 DNA-damage markers are still activated at end-stage disease

To further investigate DNA damage response markers to see if a similar pattern was observed during disease progression, the reactivity of γ-H2AX was studied. In the control spinal cord at presymptomatic stage intense nuclear reactivity of γ-H2AX within MNs was observed, specifically the nucleolus was defined and was more prominent in the transgenic cord (figure 4.10). MNs displayed intense staining at the presymptomatic stage in disease cases, however at symptomatic stage larger MNs displayed a reduction in reactivity, which persisted at end-stage disease. γ-H2AX reactivity is prominent in the MNs of the control cord at 90 and 140 days, displaying a similar staining pattern to that seen at presymptomatic stage in the transgenic cord. The intensity of staining within small cells differs across the section, similar to the observations with other markers. At end-stage disease nuclear staining appeared less prominent within the small cells compared to the control cases.

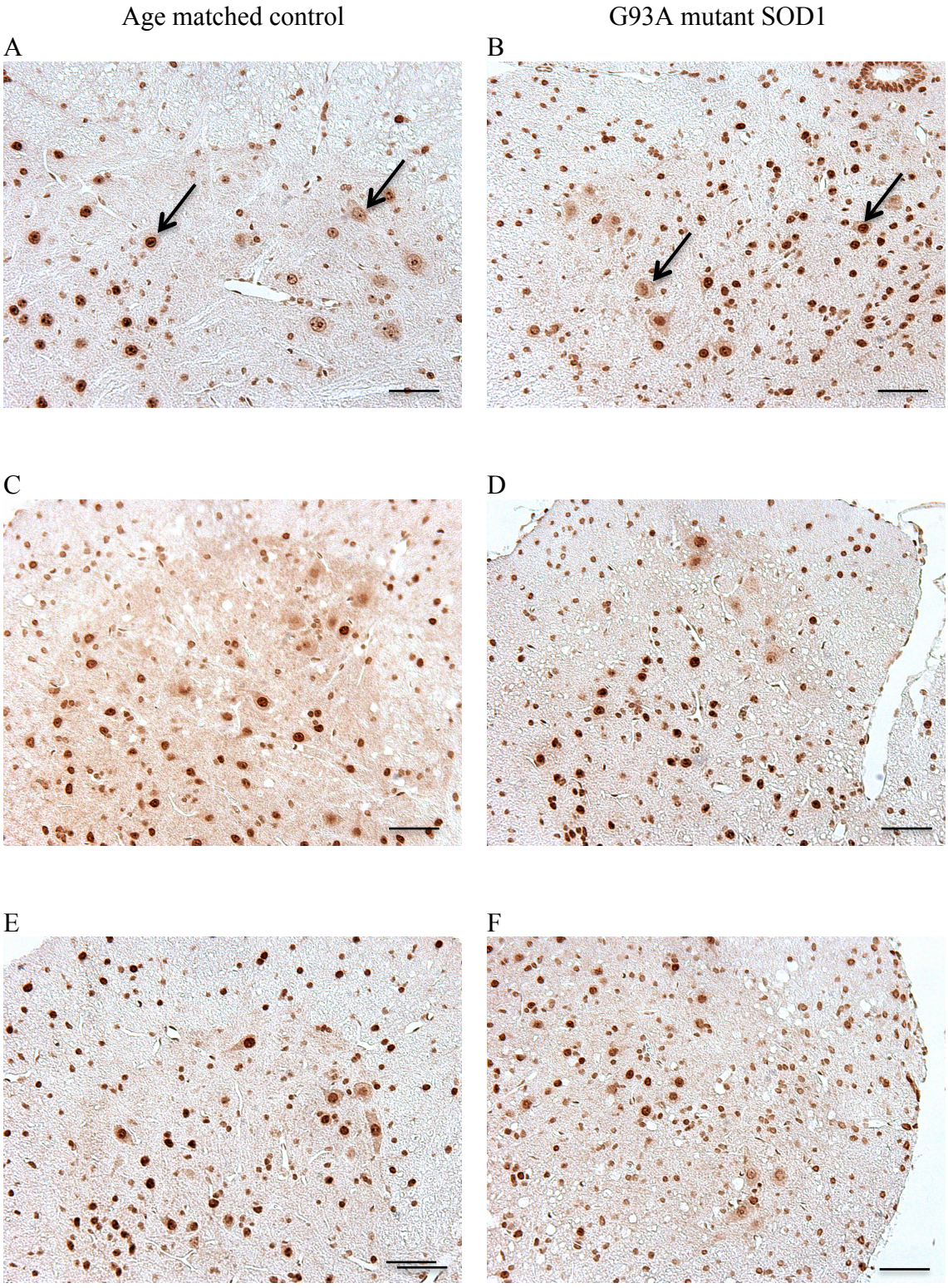


Figure 4.10: γ-H2AX reactivity in the anterior horn of human G93A mutant *SOD1* transgenic mice

Presymptomatic, symptomatic, and end-stage disease (B, D, F respectively) in comparison to age-matched controls. Some MNs display intense γ-H2AX reactivity in

MNs whereas others display little to no reactivity in both the G93A mutant *SOD1* mouse spinal cord and age-matched controls (A&B, arrows). This persists from presymptomatic to end-stage disease. These are a representation of γ-H2AX reactivity in the G93A *SOD1* transgenic mouse model of ALS. Three separate cords for each age were analysed by IHC. Scale bar represents 100μm.

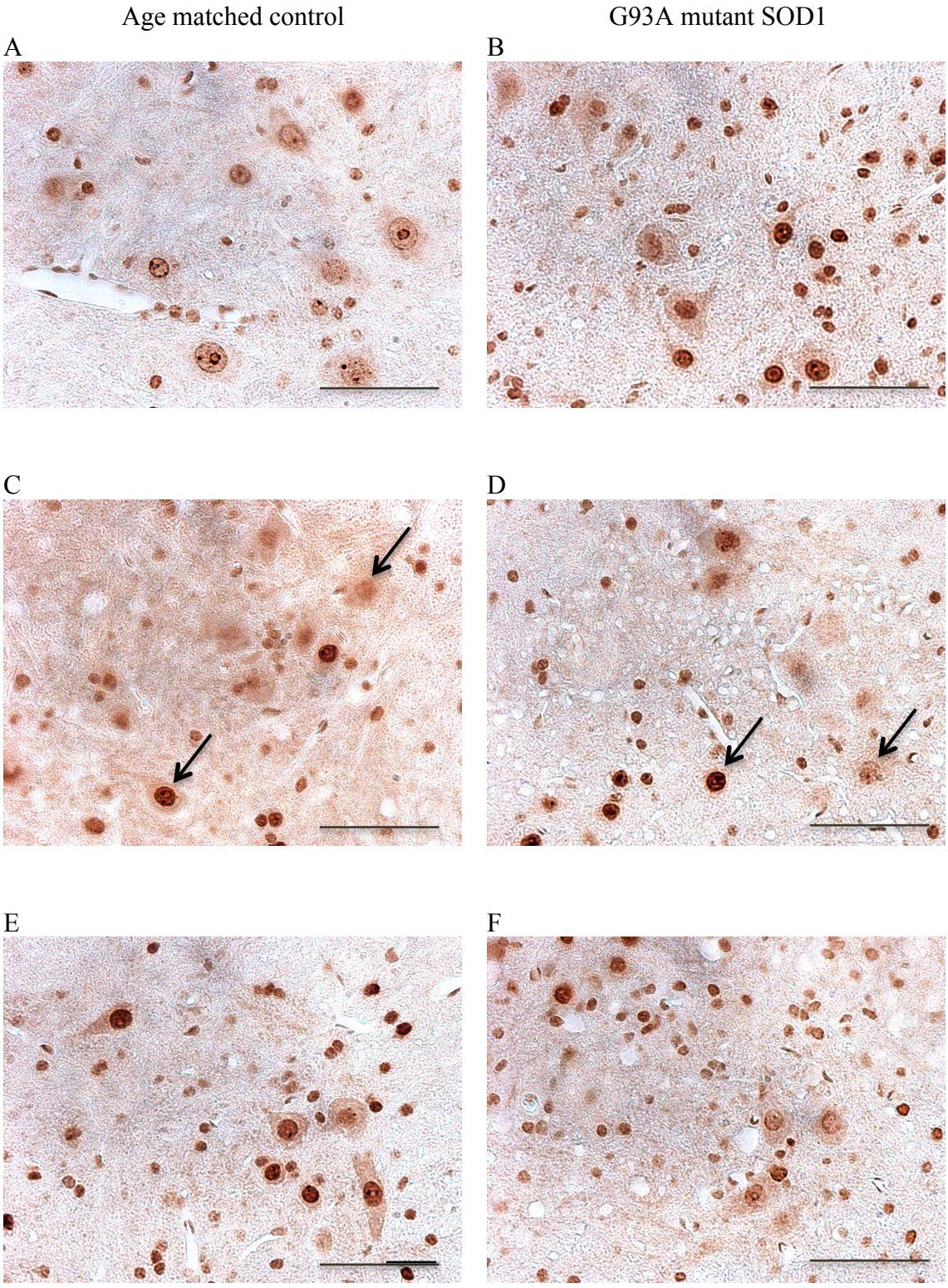


Figure 4.11: γ-H2AX reactivity in the anterior horn of human G93A mutant *SOD1* transgenic mice (higher power)

Presymptomatic, symptomatic, and end-stage disease (B, D, F respectively) in comparison to age-matched controls. Some MNs display intense γ-H2AX reactivity in

MNs whereas others display little to no reactivity in both the G93A mutant *SOD1* mouse spinal cord and age-matched controls (C&D, arrows). These are a representation of γ-H2AX reactivity in the G93A *SOD1* transgenic mouse model of ALS. Three separate cords for each age were analysed by IHC. Scale bar represents 100μm.

4.2.2 RNA Oxidation in an in vitro model of mutant SOD1 familial ALS

4.2.2.1 Human mutant *SOD1* transfection levels in NSC34 cells

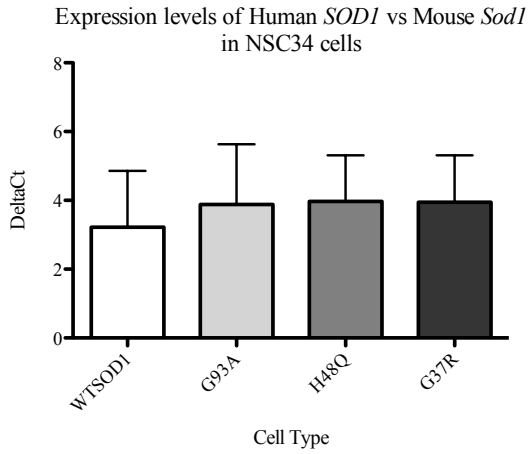
NSC34 cells stably expressing the cDNA of human G93A mutant SOD1, human G37R mutant *SOD1*, human H48Q mutant *SOD1*, human wild-type *SOD1*, or the pIRES mammalian expression vector only, were used to investigate oxidative stress associated dysfunction related to ALS pathology. Initially RT-qPCR and western blotting techniques were used to determine whether the level of transfection of each of the human *SOD1* mutations were comparable in the NSC34 cell lines. The human *SOD1* levels in transfected NSC34 cells were compared to expression levels of endogenous mouse *Sod1* and the housekeeping gene *GAPDH* (figure 4.12). There were no significant differences in the expression level of human wild-type *SOD1* (WTSOD1) and the human *SOD1* mutations in comparison to mouse *Sod1* and *GAPDH*, demonstrating equivalent transfection levels for each of the mutations. Despite the variation across experiments, the levels of transfection within an experiment were comparable, as identified by the similar Ct values which are proportional to the amount of cDNA in the sample (table 4.2).

Table 4.2: *Investigating expression levels of human SOD1 in transfected NSC34 cells*

#1 and #2 represent independent results

Cell line	*Ct(SOD1) #1*	*Ct(SOD1) #2*	*Ct(SOD1)-Ct(Sod1)*		*Ct(SOD1)-Ct(GAPDH)*	
			#1	*#2*	*#1*	*#2*
WTSOD1	18.725	18.695	4.645	4.615	2.265	6.125
G93A	19.18	19.445	6.485	3.3	3.1	4.785
H48Q	19.58	16.705	5.875	2.73	3.89	6.595
G37R	18.76	19.89	5.12	4.215	3.18	6.575

A.



B.

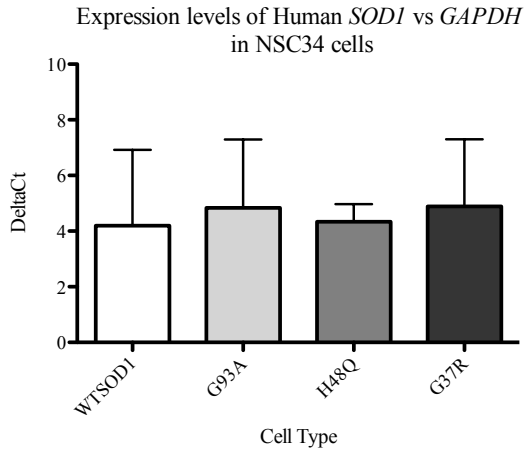


Figure 4.12: Comparing the expression of mouse and human *SOD1* in NSC34 cells

Analysis of the levels of mouse and human *SOD1* expression in the NSC34 cells transfected with different *SOD1* mutations revealed no significant differences between WTSOD1 and mutant *SOD1* expressing NSC34s. (A) The expression of human *SOD1* was related to the expression of endogenous mouse *Sod1*. (B) The expression of human *SOD1* was related to the expression of housekeeping gene *GAPDH*. Data presented as mean with SD (n=3), statistical analysis by one-way ANOVA with Bonferroni post-test.

Investigating the levels of human and mouse SOD1 proteins in protein extracts from the non-transgenic control, pIRES vector control, WTSOD1, and mutant *SOD1* NSC34 cells was used to demonstrate the expression of *SOD1* at the transcriptional level corresponds to equivalent protein levels between the different cell lines. Densitometric analysis

revealed equivalent protein expression of mouse SOD1 in each of the cell lines (figure 4.13, C). However, there was a significant reduction in human SOD1 protein expression for the G93A and H48Q mutant SOD1 transfected NSC34 cells, in comparison to the WTSOD1, and G37R mutant SOD1 transfected cells (figure 4.13, D). The pIRES vector only and the non-transfected cells as expected had no presence of human SOD1 but showed equivalent expression levels as the transfected cells for mouse Sod.

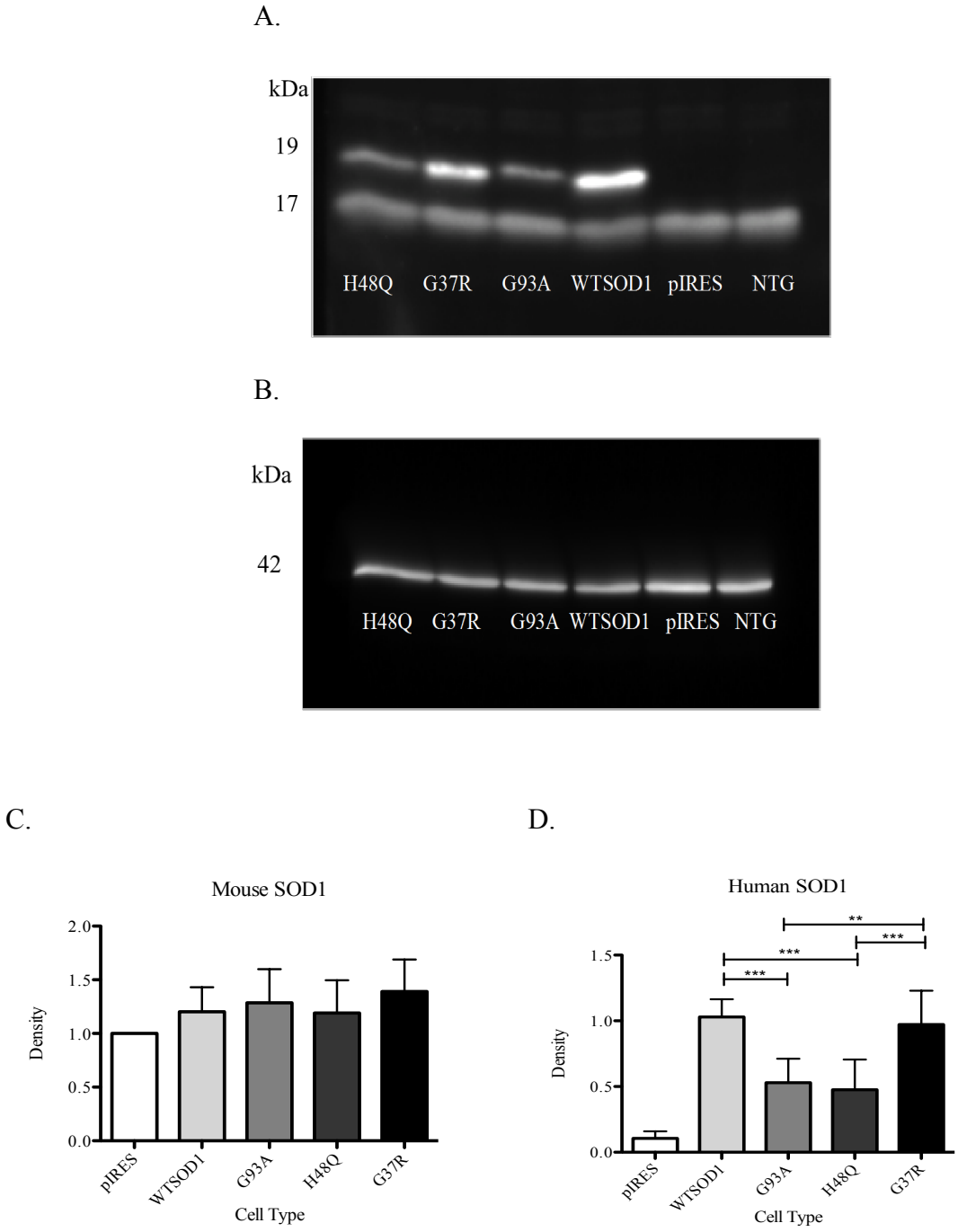


Figure 4.13: Comparing protein expression of mouse and human SOD1 in NSC34 cells

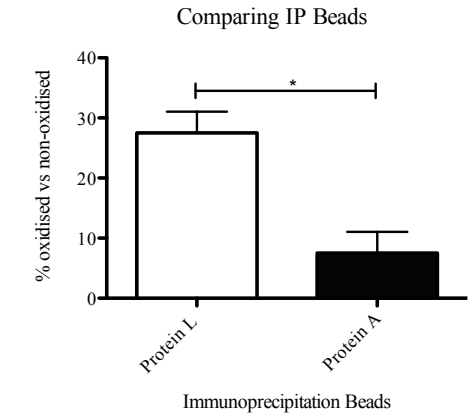
(A) Western blot of human and mouse SOD1. (B) Human and mouse SOD1 were normalised to Actin. (C) Densitometric analysis of the levels of mouse Sod1 protein present in the NSC34 cells transfected with different SOD1 mutations. (D) Densitometric analysis of the levels of human SOD1 protein present in the NSC34 cells transfected with different *SOD1* mutations. Data presented as mean with SD (n=7), statistical analyses by one-way ANOVA with Bonferroni post-test ***=P≤0.001, **=P≤0.01.

4.2.2.2 Quantifying differences in RNA oxidation in NSC34 cells

Investigating differences in RNA oxidation between cells carrying a human *SOD1* mutation in comparison to controls was initially examined by Northwestern blotting. This involved extracting total RNA and electrophoresing on a denaturing formaldehyde agarose gel at a low voltage. The RNA was transferred on to positively charged nylon membrane, and probed with an antibody against 8-OHG to determine differential levels of oxidative damage to RNA. Despite previous papers publishing data displaying differences between in RNA oxidation between models of neurodegenerative diseases and controls using similar methods, we failed to produce data using this method.

As an alternative we attempted to investigate differences in levels of RNA oxidation between cells carrying a *SOD1* mutation in comparison to controls by separating and subsequently quantifying the oxidised and non-oxidised fractions of RNA by RT-qPCR. Separation of the fractions was achieved by immunoprecipitation (IP) using an antibody against 8-OHG and protein L agarose beads (section 2.2.1.1.4), as previously described (Chang, et al. 2008). The optimum concentration of RNA used for IP was investigated by trialling a titration of RNA concentrations. RNA for optimisation was extracted from pIRES-vector only NSC34 cells, as these were to be used as controls in the subsequent experiments. The oxidised and non-oxidised fractions were transcribed to cDNA and RT-qPCR compared levels of 18S rRNA and GAPDH. 18S rRNA was selected as this would provide a measure of all RNA species rather than individual genes. A standard curve was set up for each experiment using 18S rRNA primers and serial dilutions of total RNA extracted from pIRES-vector only NSC34 cells (figure 2.3). This allowed the relative concentration in each sample to be titrated back to a value on the standard curve. Further to this, the optimal type of bead used to immunoprecipitate the oxidised RNA was investigated. Protein L agarose beads were initially chosen due to their affinity for mouse antibodies with kappa light chains. Protein A Dynabeads (Invitrogen, UK), which are magnetic beads and have high affinity for mouse IgG2, were tested in comparison to the protein L agarose beads. RT-qPCR to compare the IP efficiency of the two types of bead revealed the protein L agarose beads yield a greater percentage of oxidised RNA in comparison to non-oxidised RNA, when the starting concentration is equal (figure 4.14).

A.



B.

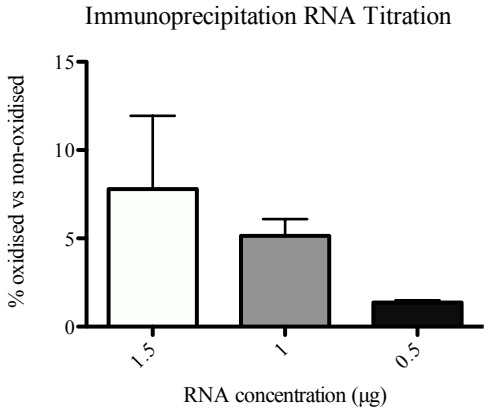


Figure 4.14: Optimising immunoprecipitation efficiency

To optimise the IP of oxidised RNA different protein beads and starting concentration of total RNA were trialled. The proportion of oxidised RNA immunoprecipitated was significantly greater using the protein L agarose beads so these were used for subsequent experiments (A). Other beads trialed were unsuccessful in capturing a proportion of RNA that could be quantified. The percentage of oxidised RNA immunoprecipitated from total RNA increased as the starting concentration of total RNA was increased (B). There were no significant differences between the percentages of oxidised RNA IP between samples. 1µg of RNA was chosen as the optimum concentration for IP as this shows sufficient IP with little variation. A greater concentration of starting material was not used as this method was being optimised for the immunoprecipitation of RNA from the anterior horn of mouse spinal cord for the microarray experiment (chapter 3). Data presented as mean with SD (n=3), statistical analyses (A) two-tailed unpaired t-test (B) one-way ANOVA with Bonferroni post-test *=P≤0.05.

Using the standard curve the percentage of oxidised to non-oxidised RNA from each sample was calculated. A starting concentration of 1µg RNA was used for subsequent IP experiments as this was considered the most consistent in terms of the percentage of oxidised RNA that is immunoprecipitated. Our aim was to investigate whether differential levels of RNA oxidation exist between NSC34 cells carrying a *SOD1* mutation compared to controls. A standard curve for 18S rRNA was used to determine the relative levels of 18S rRNA in the oxidised and non-oxidised fraction from the Ct threshold of the respective fractions. The percentage of oxidised RNA relative to non-oxidised was calculated, and the results show greater levels of oxidative damage to RNA in the cells carrying the G93A and H48Q *SOD1* mutation in comparison to pIRES and human wild-type *SOD1* NSC34 cells (figure 4.15). The G37R mutant *SOD1* NSC34s were also investigated, however the IP was unsuccessful as amplification did not occur until after cycle 26 of the PCR. This generated a low Ct value, which is indicative of the amount of template present at the start of amplification, so this sample was excluded from the results.

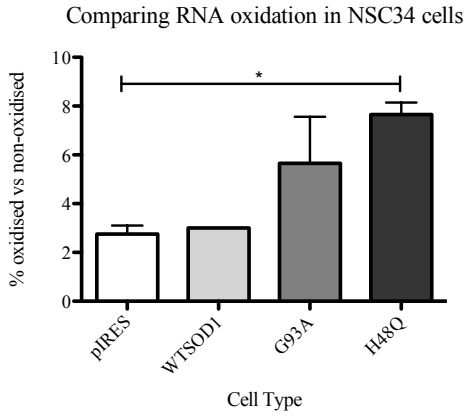


Figure 4.15: Investigating RNA oxidation in NSC34 cells

Levels of RNA oxidation between cells carrying human *SOD1* mutations were investigated in comparison to controls. A significant increase in oxidation is seen for the H48Q mutation in comparison to the pIRES vector control cells. An increase in RNA oxidation within the G93A mutant *SOD1* cells was also seen, however this was not statistically significant. Data presented as mean with SD (n=3), statistical analyses by one-way ANOVA with Bonferroni post-test, *=P≤0.05.

The finding that G93A and H48Q mutant *SOD1* NSC34s have a greater level of oxidative damage to RNA in comparison to controls correlates with the reduction in protein levels seen for these mutations. Transfection levels appear to be comparable across all cells types at the transcriptional level as determined by RT-qPCR. The increase in oxidative damage to these transcripts may consequently lead to a reduction in the level of functional protein due to the inability for the transcript to be accurately translated, which supports previous studies that have investigated the consequences of oxidative modification to nucleic acids (Ding, et al. 2005, Tanaka, et al. 2007).

4.2.3 Investigating susceptibility of NSC34's to oxidative stress

4.2.3.1 *Cell viability of control and mutant NSC34 cells after exposure to oxidative stress*

The effect of oxidative stress on cell viability in the control and mutant NSC34 cell lines was investigated by treating the cells with various concentrations of hydrogen peroxide (H_2O_2); their survival was determined by trypan blue exclusion. We were investigating the hypothesis that cells carrying a human mutant *SOD1* transgene are more susceptible to oxidative stress mediated cell death. The treatment of control and mutant cells with moderate stress (50μM to 100μM) produced a slight reduction in cell viability over time, with the G93A and H48Q mutations beginning to show susceptibility (figure 4.16, figure 4.18). After two hours of a 50μM and 100μM treatment, WTSOD displayed a significantly greater viability in comparison to the G93A mutant cells, however this significance does not persist with six and ten hour treatments at these concentrations (figure 4.17). The G37R mutant cells begin to show greater resistance to the exogenous stress at 100μM, with a significant increase in viability seen in comparison to the G93A mutant cells at six hours (figure 4.18). Vulnerability to oxidative stress for the G93A mutation persists with a 250μM treatment with significant differences in cell viability compared to the other mutations and controls seen at two, four and six hours ($p \leq 0.01$). The G37R mutant cells continue to show a greater viability in comparison to the other mutations. A ten-hour treatment results in comparable levels of viability across all cell types, with the G93A showing slightly reduced survival (figure 4.16).

An exposure of 500μM to 1mM H_2O_2 produced an exponential reduction in viability with time, with certain mutations displaying rapid inductions in cell death (figure 4.16, figure

4.18). The G93A mutation displayed a significant reduction in cell viability with a 500μM treatment in comparison to both controls and the other mutations ($p \leq 0.05$) and the H48Q mutation showed a significant reduction in viability in comparison to controls at six and ten hours ($p \leq 0.01$). The other mutations and controls may be activating a more successful stress response at two and four hours, as viability between them is comparable, maintaining approximately 80% viability up to six hours with a 500μM dose. The G37R mutation showed a steady decline over time with both a 500μM and 1mM dose, but displayed the greatest survival at ten hours, with viability significantly greater than the other mutations ($p \leq 0.001$) (figure 4.18). These differences may be indicative of the effect the different mutations have on the cells. The patterns in viability between the different cells is similar with both 500μM and 1mM treatments, with the G93A showing the greatest susceptibility and the G37R showing the greatest resistance to stress. In comparison, the H48Q mutation demonstrates rapid inductions of cell death at six hours with 500μM and 1mM doses.

Prolonged treatment with H_2O_2 (>six hours) would be expected to significantly reduce viability across all cell types. The G93A mutant *SOD1* NSC34 cells were the most severely affected with the H48Q mutant showing similar effects as time and H_2O_2 concentration increases, in comparison to the G37R mutation that displayed a greater percentage viability across all concentrations at all the investigated time points ($p \leq 0.05$). The pIRES vector and WTSOD1 controls show reductions in viability but these did not become pronounced until persistent exposure to high concentrations of H_2O_2 (figure 4.16, figure 4.18).

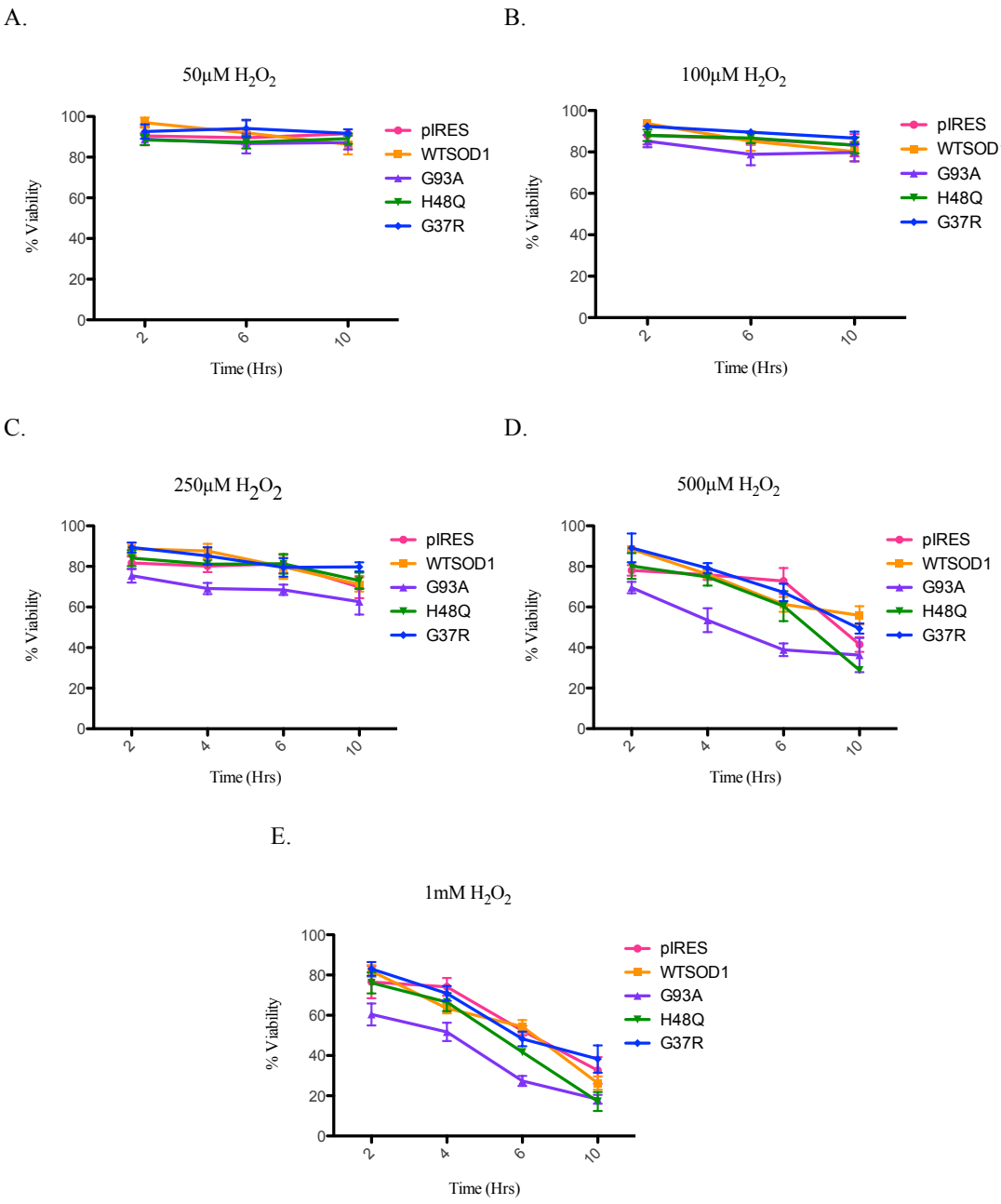


Figure 4.16: Cell viability after H_2O_2 treatment of cells transfected with vector only, normal human *SOD1*, or the G93A, H48Q, or G37R mutant form of human *SOD1*

(A) 50µM H_2O_2 treatment (B) 100µM H_2O_2 treatment (C) At 250µM H_2O_2 treatment, the G93A mutation shows increased susceptibility to stress. A ten hour exposure results in substantial cell death across all cell lines. (D) At 500µM H_2O_2 treatment, a notable reduction in cell viability over time across all cell types investigated is seen. (E) A 1mM H_2O_2 treatment produced an exponential decline in cell viability over time with the most rapid induction taking place between two and six hours for all cell types. Data presented as mean with SD (n=5). Statistical analyses shown on the following graphs.

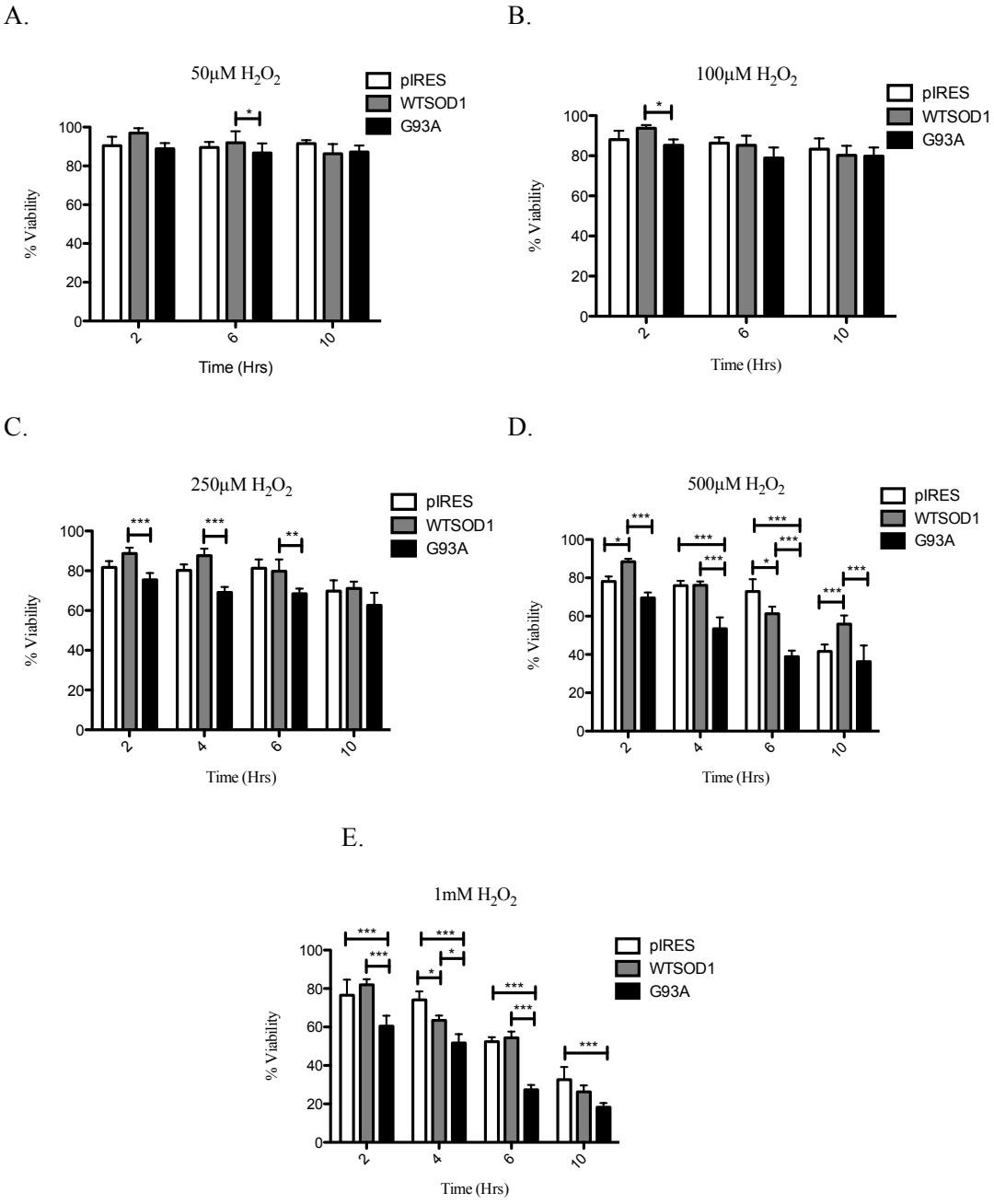


Figure 4.17: Cell viability after H_2O_2 treatment of cells transfected with vector only, normal human *SOD1*, or human G93A mutant *SOD1*

Human G93A mutant *SOD1* transfected NSC34 cells begin to show susceptibility to stress with 50μM and 100μM H_2O_2 treatment (A&B). At 250μM H_2O_2 treatment, the G93A mutation shows increased susceptibility to stress in comparison to the controls investigated (C). At 500μM H_2O_2 treatment, a notable reduction in cell viability for the G93A mutation is seen in comparison to controls (D). 1mM H_2O_2 treatment produced an exponential decline in cell viability over time. Data presented as mean with SD (n=5). Statistical analysis by two-way ANOVA with Bonferroni post test, *P=<0.01, **P=<0.001, ***P=<0.0001

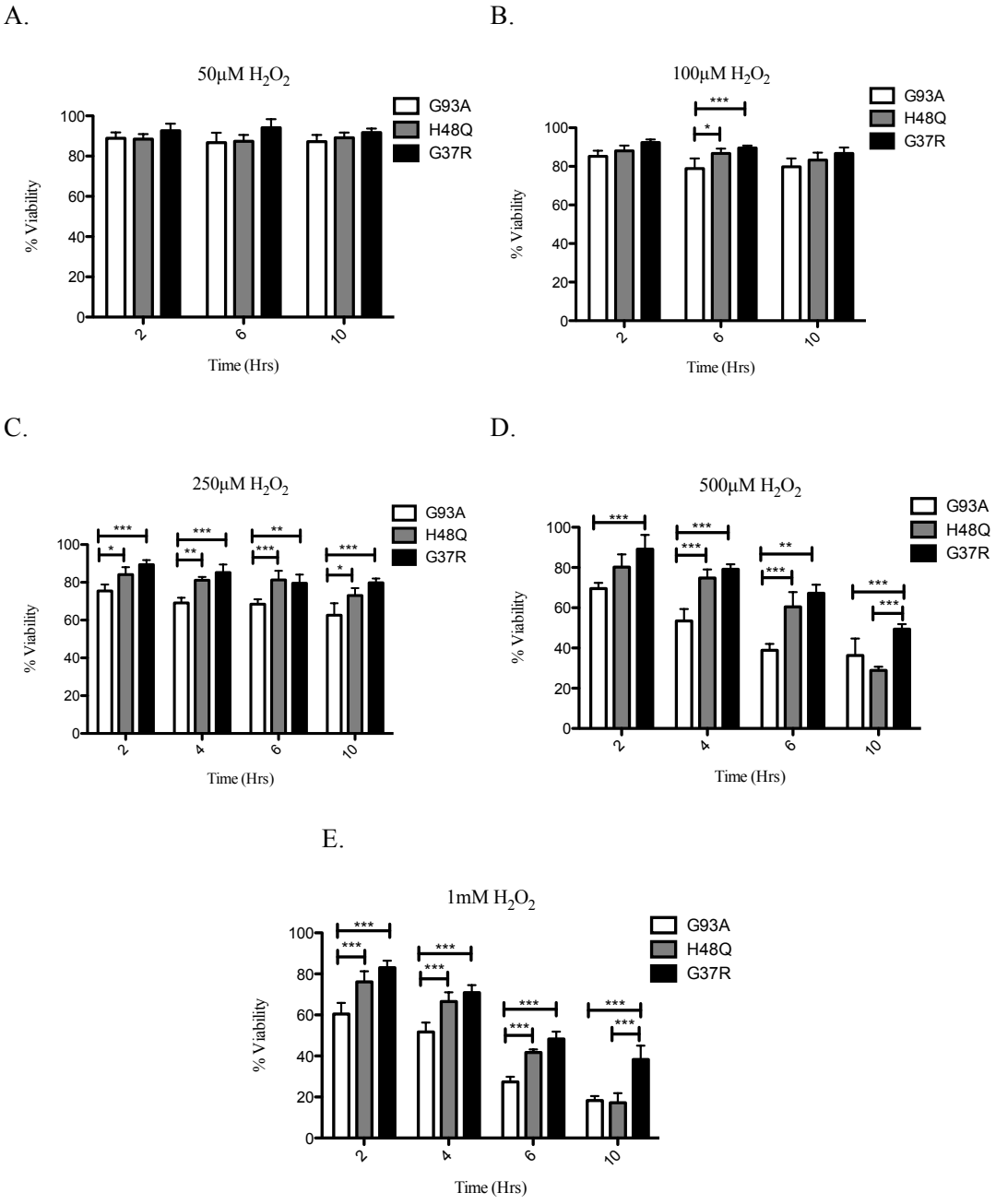
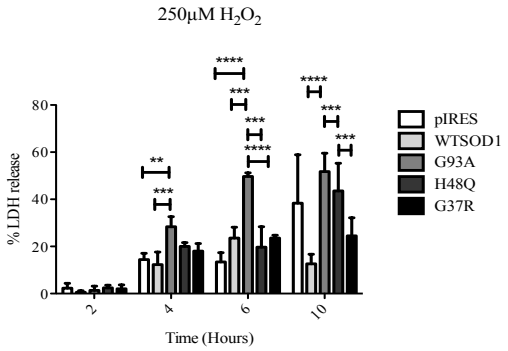


Figure 4.18: Cell viability after H_2O_2 treatment of cells transfected with human G93A, G37R, or H48Q mutant *SOD1*

At 50μM and 100μM stress, the G37R begins to show resistance to stress-induced cellular death in comparison to the other two mutations (A&B). The G93A mutation continues to show susceptibility with a 250μM treatment, with cell viability significantly lower than the H48Q and G37R mutation at all time points (C). This persists with a 500μM treatment (D). Following prolonged exposure to increased stress, all mutations have a substantial reduction in viability, with the G93A and H48Q mutations showing the greatest susceptibility (D&E). Data presented as mean with SD (n=5). Statistical analysis by two-way ANOVA with Bonferroni post test, *P=<0.01, **P=<0.001, ***P=<0.0001

To further investigate the effect of oxidative stress on cellular injury, Dr Scott Allen carried out lactate dehydrogenase (LDH) assays. The release of LDH from the cell was measured to quantitatively measure cell lysis in the presence of H_2O_2 (figure 4.19). No difference in LDH release was observed between mutants and controls following a two-hour treatment with a 250µM, 500µM, or 1mM concentration of H_2O_2. A significant increase in LDH release was observed in the G93A mutant compared to the control and H48Q/G37R mutant cell lines ($p \leq 0.01$) following exposure of cells to 250µM, 500µM and 1mM H_2O_2 for four and six hours. The G37R mutant cells displayed similar LDH release to the controls at the time points and H_2O_2 tested, which is consistent with the trypan blue cell viability data (figure 4.16). The H48Q mutant cells in general showed greater LDH release than controls at 250µM H_2O_2.

A.



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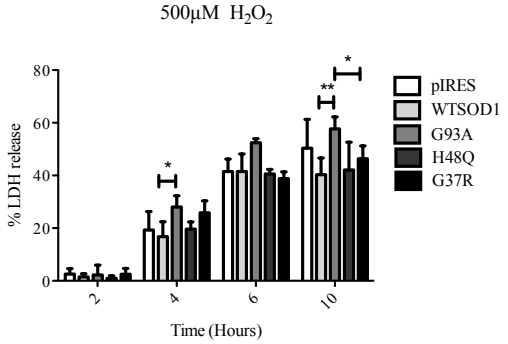


C.

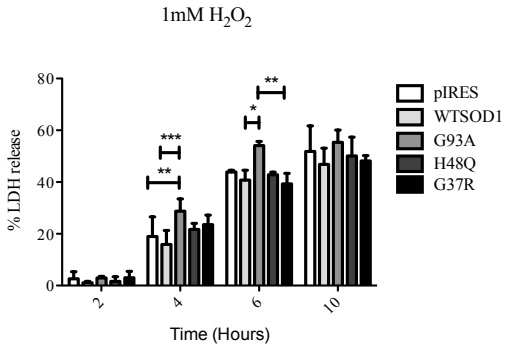


Figure 4.19: LDH release in control and mutant NSC34 cells following exposure to H_2O_2

Data presented as mean with SD (n=3). Statistical analyses by two-way ANOVA with Bonferroni post-test, *=P≤0.05, **=P≤0.01, ***=P≤0.001, ****=P≤0.0001

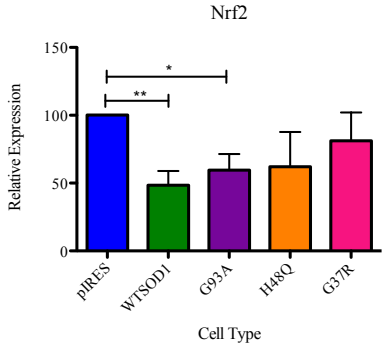
4.2.4 Antioxidant response of control and mutant NSC34 cells

4.2.4.1 Activation of antioxidant response under basal conditions

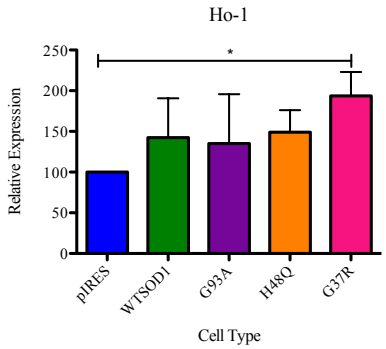
To investigate the effect oxidative stress has on the expression of stress response genes in mutant NSC34s, a selection of oxidative stress response genes were investigated by RT-qPCR under basal and stressed conditions. We hypothesised the increased susceptibility to cell death of mutant *SOD1* NSC34s following exposure to oxidative stress was in part due to their inability to up-regulate a defensive response through altering their transcriptome. This has been shown previously in gene expression profiling studies in both the *in vitro* and *in vivo* models of mutant SOD1-associated fALS (Kirby*, et al.* 2005, Vargas*, et al.* 2005). Genes whose expressions have previously been reported to change in response to oxidative stress conditions were chosen. The 100μM and 250μM H_2O_2 treatment conditions were chosen, since 100μM H_2O_2 represents a dose at which the cells are exposed to an oxidative environment but display no overt cell death, suggesting they are mounting some form of response and 250μM H_2O_2 which leads to cellular susceptibility to death with increasing lengths of treatment, suggesting the protective mechanisms are lost or cannot compensate for the level of stress the cells are presented with. The genes investigated were mouse *SOD1, Ogg1, Nrf2, Ho-1*, and *Nqo1*. The expression level of stress response genes was initially compared to the relative expression levels of *GAPDH*. However, studies have shown *GAPDH* expression to be altered in response to stress (Ito*, et al.* 1996, Schmittgen and Zakrajsek 2000), and the samples were re-analysed in comparison to Actin expression levels.

Under basal conditions, the G93A has significantly lower expression of *Nrf2* in comparison to the pIRES control. This was also seen in the WTSOD1 transfected NSC34 cells (figure 4.20 A). Expression levels of Ho-1 were similar for the WTSOD1 and human *SOD1* mutations, however G37R showed a significant increase in expression in comparison to the pIRES control (figure 4.20, B). Nqo1 displayed a reduction in expression in the G93A mutant *SOD1* NSC34s, however this did not reach significance, which could be due to the variability of the data (figure 4.20, C). The H48Q and G37R mutation also displayed slightly reduced expression levels of *Nqo1*, but this data was also variable.

A.



B.



C.

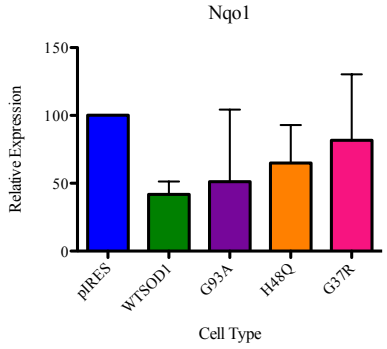


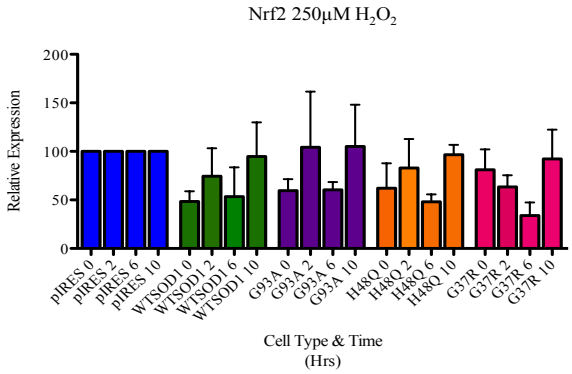
Figure 4.20: Expression of oxidative stress response genes in control and mutant NSC34 cells under basal conditions

The expression levels of *Nrf2*, *Ho-1*, and *Nqo1* were examined in the pIRES control, WTSOD1 and human mutant *SOD1* carrying NSC34 cells under basal conditions. *Nrf2* was found to be significantly reduced in the WTSOD1 and human G93A mutant *SOD1* NSC34s in comparison to the pIRES control (A). The G37R mutant *SOD1* NSC34s showed a significant increase in *Ho-1* expression in comparison to the pIRES control (B). WTSOD1 showed reduced expression of *Nqo1* in comparison to the pIRES control, along with G93A however this was highly variable (C). Data presented as mean with SD (n=4). Statistical analyses by one-way ANOVA with Bonferroni post-test*=P≤0.05, **=P≤0.01

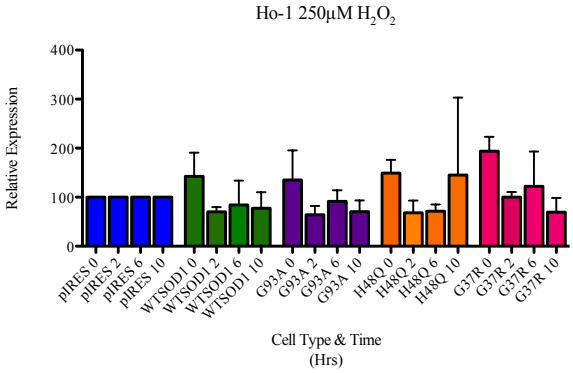
4.2.4.2 Activation of oxidative stress response following exposure to H_2O_2

The induction of antioxidant response genes was also investigated after exposure to oxidative stress; RNA was extracted following H_2O_2 treatment for the cell viability assays. The genes investigated under basal conditions were studied (*Nrf2, Ho-1*, and *Nqo1*). Levels of relative expression of these oxidative stress response genes were investigated after exposure to 250μM H_2O_2 for two, six, and ten hours. A slight reduction in expression is seen across all cell types in comparison to pIRES control cells for *Nrf2*, following exposure to 250μM H_2O_2 for six hours (figure 4.21, A). Following exposure to oxidative stress the induction of *Ho-1* is reduced in G93A and G37R mutant *SOD1* transfected NSC34s in comparison to pIRES control cells and their basal expression levels, however this was not significant (figure 4.21, B). This may represent a reduction in the ability to up-regulate an antioxidant response following exposure to stress. The expression of *Nqo1* is increased in G93A mutant *SOD1* NSC34s in comparison to their basal expression levels, however this does not differ significantly from the controls (figure 4.21, C).

A.



B.



C.

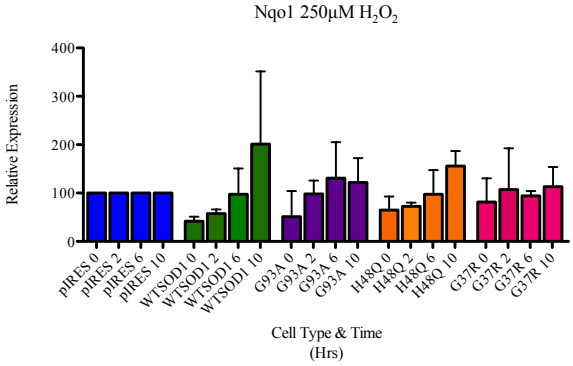


Figure 4.21: Expression of oxidative stress response genes in control and mutant NSC34 cells following exposure to H_2O_2

Expression levels of *Nrf2*, *Ho-1*, and *Nqo1* were investigated in control and mutant NSC34 cells following exposure to 250µM H_2O_2 for two, six, or ten hours. No significant differences were seen for the induction of stress response genes following exposure to oxidative stress for each of the cell lines investigated. Levels of expression were highly variable across and between control and mutant cells. Data presented as mean with SD (n=3), and statistical analysis by two-way ANOVA with Bonferroni post-test

4.2.5 Gene expression levels of DNA damage response in NSC34 cells

4.2.5.1 DNA damage response under basal conditions

We also investigated expression levels of *Ogg1*, to determine whether there is an increase in the activation of DNA damage response within cells carrying human *SOD1* mutations. The G93A mutant *SOD1* NSC34s showed a non-significant ($p>0.05$) increase in expression in comparison to the pIRES and WTSOD control cells, and a significant increase ($p\leq0.05$) in comparison to the H48Q mutant *SOD1* NSC34 cells (figure 4.22).

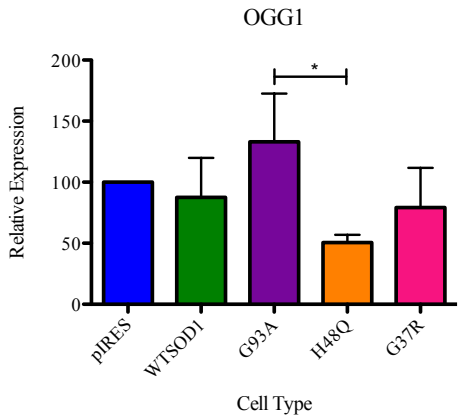


Figure 4.22: Expression of DNA damage response gene *Ogg1* in control and mutant NSC34 cells under basal conditions

The expression levels of *Ogg1* were investigated in the pIRES, WTSOD1, and human mutant NSC34 cells under basal conditions. The G93A mutation had the greatest level of expression in comparison to all cell types, and its expression was significantly increased in comparison to the H48Q mutation. Data presented as mean with SD (n=3). Statistical analysis by one-way ANOVA with Bonferroni post-test *=$P\leq0.05$

4.2.5.2 DNA damage response following exposure to oxidative stress

The expression levels of *Ogg1* in control and transfected NSC34 cells were investigated following a 250µM exposure to H_2O_2 for two, six, and ten hours (figure 4.23). After two hours exposure, a non-significant reduction in *Ogg1* expression is seen in all the G93A *SOD1* mutant cells in comparison to basal expression levels and the controls. At six hours the H48Q mutation shows great variability in expression, whereas the G93A and G37R

mutations, despite showing a reduction in expression in comparison to WTSOD1, expression is slightly greater than what is seen in the pIRES control NSC34s. Following a ten hour exposure to oxidative stress, the H48Q mutant *SOD1* NSC34s demonstrate the greatest expression level of *Ogg1*. Again, there are no significant differences between expression levels across the cell lines.

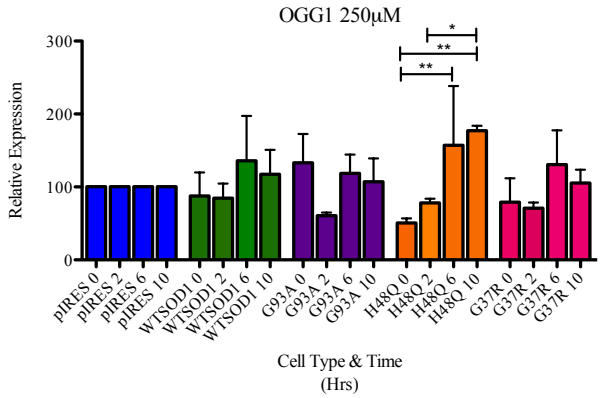


Figure 4.23: Expression of DNA damage response gene *Ogg1* in control and mutant NSC34 cells following cellular exposure to oxidative stress

No significant differences in expression were seen in *Ogg1* expression between the mutations at each time point. Increased expression was seen across all cell lines following a six and ten hour exposure to oxidative stress, with only H48Q mutant *SOD1* showing a significance increase in expression over time. Data presented as mean with SD (n=3). Statistical analysis by two-way ANOVA with Bonferroni post-test *=P≤0.05, **=P≤0.01

4.3 Discussion

4.3.1 Nucleic acid oxidation and DNA repair in the spinal cord of an in vivo model of ALS

The oxidative modification of lipids, proteins and nucleic acids and/or the activation of damage response pathways are generally used as markers of oxidative stress. While oxidative modifications have been shown to increase, a reduction in DNA damage response (DDR) has been reported in the neuropathology of ageing and neurodegenerative disease (Borgesius*, et al.* 2011, Jackson and Bartek 2009, Weissman*, et al.* 2007b), and linked to increased cellular stress. Evidence suggests the accumulation of damaged macromolecules to be a causative factor of the progress of ageing and specific diseases (Esiri 2007). Here we demonstrate the presence and distribution of oxidative modification to nucleic acids within the large MNs and small cells in the spinal cord during disease progression in the G93A mutant *SOD1* transgenic mouse model. Over 20 different types of oxidatively altered purine and pyrimidine bases have been identified in nucleic acids (Ishibashi*, et al.* 2005). Guanine has the lowest oxidation potential compared to cytosine, thymine/uracil, and adenine; it is the most readily oxidised and consequently 8-hydroxydeoxyguanosine/8-hydroxyguanosine are commonly used as biomarkers of oxidative damage to DNA and RNA respectively (section 1.6).

Here we demonstrate the presence of 8-OHG reactivity at the presymptomatic stage of ALS progression, providing evidence of oxidative damage to nucleic acid prior to MN death and confirming this is not merely a consequence of dying cells. Nucleic acid oxidation was previously observed to be most prominent at the early presymptomatic stage and then to have subsided at later stages of disease progression in a transgenic murine model expressing human G93A mutant *SOD1* (Chang*, et al.* 2008), which is similar to our observations. The antibody against 8-OHG identifies oxidatively modified DNA and RNA, however cytoplasmic predominance of oxidative damage, and the reduction in staining intensity following RNase treatment, suggests that during normal ageing and disease states ROS generated by the mitochondria inflict damage on surrounding RNA species. This is consistent with previous findings of RNA susceptibility to oxidative modification during ageing and neurodegenerative disease (Chang*, et al.* 2008, Nunomura*, et al.* 1999). The predominance of cytoplasmic staining may also be due to the lack of repair mechanisms for oxidatively damaged RNA. In both AD and PD 8-OHdG/8-OHG immunoreactivity was predominantly localised in the

cytoplasm with a significant increase identified in patients compared to age-matched controls (Nunomura*, et al.* 2001, Zhang*, et al.* 1999).

Changes in MN morphology were identified during disease progression in the G93A mutant *SOD1* transgenic mouse model. At the presymptomatic stage, the shrunken morphology may represent their imminent atrophy and the loss of structure at end-stage suggests MN degeneration. The reduction in 8-OHG reactivity within MNs at end-stage may represent the loss of ribonucleic acid as the cells degenerate. A previous study demonstrated that levels of RNA oxidation were significantly higher in control mice compared to the transgenic mice at 120 days of age, which may represent degeneration of those cells previously demonstrated to be vulnerable to oxidative damage (Chang*, et al.* 2008). This evidence substantiates the view that an increase in oxidative damage to nucleic acid accompanies neuronal degeneration during ALS pathogenesis, which is then reduced as the neurons die, and also suggests a reduction in the capacity to repair oxidatively damaged DNA (Aguirre*, et al.* 2005). The extension of 8-OHG reactivity to neurites at presymptomatic stage of disease and during normal ageing suggests the oxidation of RNA molecules targeted for transportation, or damage to those confined within ribonucleoprotein complexes. The perinucleolar staining is also diminished with RNase treatment suggesting that the RNAs within this compartment are oxidatively damaged. Perinuclear dots of intense staining can also be seen, which may represent the aggregation of oxidised RNA or areas of increased susceptibility to damage. The granular staining pattern seen at presymptomatic and symptomatic stages suggest oxidative damage to ribosomal RNAs within the cytoplasm, and also may represent the oxidation of specific mRNA complexes.

Following RNase pre-treatment, some cytoplasmic staining is still observed in the cytoplasm of MNs. This may be a result of inefficient removal of all RNA due to the concentration of RNase used, or may represent the oxidative modification to mtDNA, which has been demonstrated in previous studies (Yakes and Van Houten 1997). DNase pre-treatment also revealed a slight reduction in staining, which provides increasing evidence for mtDNA damage however pre-treatment with both DNase and RNase failed to diminish all staining suggesting the concentration of the nucleases need to be optimised. Isolation of the nuclear and cytoplasmic compartments, and further isolation of the mitochondria would allow investigation into how much reactivity is attributable to nuclear DNA, mitochondrial DNA, and RNA oxidative damage. Small cell reactivity is similar following pre-treatment with either DNase or RNase. Increased reactivity is observed in the small cells following DNase treatment at symptomatic and end-stage

disease, suggesting increased RNA oxidation in comparison to the MNs, however this is speculative as it is difficult to distinguish the cytosol from the nucleus in these cells.

The intensity of small cell reactivity differs across the sections at all ages in both disease and control mice. Towards end-stage disease an increase in small cells infiltrate the field; this may not be due to an increase in cellular proliferation, but may be a consequence of dendritic regression and reduced synaptic densities (Resnick*, et al.* 2003, Terry 2000), allowing more cells to be distinguished within a given area. The prominent staining observed in small cells at end-stage demonstrates the selective vulnerability of MNs to disease, and suggests these cells are more resistant to oxidative stress induced damage.

Cresyl violet staining is typically used to identify the neuronal structure in brain and spinal cord tissue. Here, the Nissl substance (rough endoplasmic reticulum) appears intensely stained due to the presence of ribosomal RNA, and is particularly prominent at presymptomatic stage of disease. The staining here matches the morphology of the MNs and the pattern of 8-OHG staining at each stage of disease, highlighting the loss of nucleic acid and cellular structure as the MNs degenerate. The staining in the transgenic sections at presymptomatic stages could appear intensified due to the reduced volume of the MNs, or could be due to the cells mounting compensatory mechanisms to the upstream pathogenic mechanisms. An increase in protein synthesis in addition to increased oxidative damage to nucleic acids may occur presymptomatically, and as the disease progresses and homeostasis cannot be maintained the cell shifts towards decline and degeneration. This is shown by a loss of morphological features of MNs towards end-stage disease, with a general reduction in Nissl staining.

Although nucleic acid oxidation is a feature of normal ageing, the survival and morphological maintenance of MNs suggests damage does not independently cause cellular decline and degeneration in neurological disorders. Since the persistence of 8-OHG in RNA would cause a reduction in the fidelity of gene expression and processing of transcripts, organisms must have a mechanism for scavenging oxidised molecules or coping with the downstream effects of abnormal transcripts. In neurodegeneration, a reduction in cellular repair processes accompanied by increasing disruption to homeostatic mechanisms may cause cells to succumb to the disease. One hypothesis suggests proteins specifically bind to oxidatively damaged RNA to target them for removal (Hayakawa*, et al.* 2001, Hayakawa*, et al.* 2002). The selective elimination of mRNA containing 8-OHG may be essential to prevent the formation of erroneous proteins, and may maintain cellular viability during normal ageing. The Ogg1 DNA

glycosylase acts to eliminate oxidised guanine bases from DNA, so we aimed to determine the presence and distribution of Ogg1 in the G93A mutant *SOD1* transgenic mice during disease progression. The small cells in comparison to the MNs showed relatively little staining across the section at presymptomatic and symptomatic stage of disease, however as disease progresses towards end-stage the number of Ogg1-positive small cells increased. Other studies have shown the presence of Ogg1 reactivity in astrocytes (Araneda*, et al.* 2001), and increased expression of Ogg1 was identified in pre-clinical AD patients (Lovell*, et al.* 2011). This supports what is seen here with the increased reactivity in MNs at presymptomatic stage of disease suggesting cells are trying to compensate for the increase in oxidative damage at early stages of disease.

The intense reactivity of Ogg1 within the nucleus and cytoplasm of MNs at presymptomatic stage suggests the cells are up-regulating mechanisms to counteract the inflicting damage, and may explain the reduced intensity of 8-OHG within the nucleus of the MNs. For further study, it would be interesting to investigate Ogg1 reactivity at an early time point in the G93A mutant *SOD1* transgenic mouse model and age-matched controls, to determine whether the up-regulation of Ogg1 is an early feature of MND. Investigating differences in Ogg1 protein levels in the G93A mutant *SOD1* transgenic mouse model in comparison to controls by western blot would also provide quantification of the differences identified here by immunohistochemistry. A single RT-qPCR experiment was carried out on the anterior horn from G93A mutant *SOD1* transgenic mice at one to five months of age, to investigate *Ogg1* expression levels in this model. A slight increase in *Ogg1* expression was identified up to late-stage of disease (four months), however this was substantially reduced at end-stage disease (five months) (data not shown). Further work would expand the sample size to identify any significant differences.

The distribution of Ogg1 reactivity at other stages of disease and in controls parallels what is seen for 8-OHG. Other DNA damage markers are also present at the early stages but seem to be less active as disease progresses, suggesting a decline in the functionality of these mechanisms. Although the majority of 8-OHG cytoplasmic staining was diminished with RNase treatment, some MNs still show some reactivity to 8-OHG suggesting damage to mtDNA. The cytoplasmic reactivity of Ogg1 suggests repair mechanisms are functioning for the removal of oxidative lesions from mtDNA, potentially maintaining damage at benign levels within MNs. In eukaryotes, two major isoforms of Ogg1 exist, termed α-Ogg1 and β-Ogg1, generated by alternative splicing of the transcript (Aburatani*, et al.* 1997, Takao*, et al.* 1998). The N-terminus of this gene

contains a mitochondrial localisation signal that is present in both isoforms, however a nuclear localisation signal is only present in α-Ogg1, suggesting the specific targeting of Ogg1 to the mitochondria in mice for repair of oxidative lesions in mtDNA (Jensen*, et al.* 2003, Nishioka*, et al.* 1999, Rachek*, et al.* 2002), and an explanation for the distribution of cytoplasmic reactivity seen here.

Investigation in an *in vitro* model of fALS revealed an increase in expression of Ogg1 in G93A mutant *SOD1* transfected NSC34 cells compared to pIRES and human WTSOD1 controls, and the G37R and H48Q mutations under basal conditions. The G37R and H48Q mutant cells show a slight reduction in Ogg1 expression in comparison to the controls, which may indicate an inability to up-regulate repair mechanisms within these cells. Following oxidative stress by H_2O_2 treatment, all mutant NSC34 cells demonstrated an up-regulation in Ogg1 expression following six and ten hour treatments with 250μM H_2O_2. Expression levels following exposure to exogenous stress may be more representative of what is happening *in vivo*, and the contribution of toxic species from cells surrounding the MNs. The lack of a significant increase in Ogg1 expression in mutant cells under basal conditions may be a consequence of cellular adaptation to culture conditions and therefore an elevated stress response is only seen following induction of stress.

Neurons have complex mechanisms to defend their genome to ensure functionality and longevity. Diverse mechanisms incorporating many aspects of cellular metabolism, repair pathways and cell death are interlinked and act in combination in response to DNA damage (Bakkenist and Kastan 2004, Callegari and Kelly 2007, Shiloh 2003). Cumulative damage to DNA during the ageing process of CNS neurons and in various neurodegenerative diseases has been documented (Coppede and Migliore 2010, Fishel*, et al.* 2007, Weissman*, et al.* 2007b). Considering this, we also investigated the activation of the DNA repair enzymes DNA-PK and γ-H2AX. DNA repair pathways are activated within MNs during normal ageing and disease progression, but the activation of these pathways may be diminished during neurodegenerative disease. Studies of DNA damage response in post-mortem tissue from AD patients revealed reduced DSB repair during NHEJ, which coincided with reduced DNA-PK activity (Shackelford 2006). Reduced uracil DNA glycosylase (UDG), Ogg1 and polymerase-β activities in both affected and unaffected brain regions of AD patients led to the suggestion that an impairment of BER is a general feature of AD brain (Weissman*, et al.* 2007b). Overall, our study revealed no qualitative difference in reactivity for DNA-PK and γ-H2AX in disease cases compared to controls at presymptomatic and symptomatic stages of disease. Some MNs and small

cells displayed prominent intense nuclear reactivity for these enzymes and others demonstrated a lack of activation. In the age matched controls, reduced reactivity of the DNA damage markers may represent a low level of DNA DSBs, and the ability of the cells to activate repair mechanisms and manage the inflicting damage. The reduction of reactivity within the transgenic cord may represent a lack of activation/DNA damage response as disease progresses. In ALS patients it has been shown that the level of the apurinic/apyrimidinic endonuclease (APE) protein, which is involved in DNA BER, was reduced in the motor cortex, and PARP1 activity, which is activated in response to DNA breaks was reduced in MNs of the spinal cord, but was elevated in the motor cortex, parietal cortex and cerebellum (Kim*, et al.* 2004). This may represent regional differences in the activities of these enzymes, or the types and levels of damage different neurons are encountering. The elevation of Ogg1 and the reduction in APE1 and DNA polymerase-γ expression in the spinal cord MNs in *SOD1* transgenic mice further supports *in vivo* data establishing a reduction in DNA repair processes in neurodegenerative disease (Manabe*, et al.* 2001, Murakami*, et al.* 2007).

The reactivity for DNA-PK observed at end-stage disease appears reduced in MNs and small cells, which may represent a loss of DNA damage response mechanism as disease progresses, or indicate a degradation of mitochondrial and nuclear DNA available for repair. MNs also display changes to their morphology, as was previously seen with the other markers. However, the activation of H2AX at end-stage disease suggests other factors regulating its activation, or a redundancy among the DNA repair enzymes. This redundancy among the DNA glycosylases for example, is supported by the absence of a significant phenotype in a single glycosylase knockout model, which reflects the important cellular roles for these enzymes (Hazra*, et al.* 2002, Parsons and Elder 2003). It may be that DNA repair proteins can be recruited to sites of DNA DSBs but they are unable to propagate the signal downstream to initiate the recruitment of further repair factors. Even though activation of DNA damage response proteins occurs at end-stages of disease, the consequences of increased oxidative stress and the impact of other disease associated factors may have more of a detrimental effect causing cellular decline and degeneration. If the ageing series were extended you would expect to see a similar pattern in staining as is seen in the transgenic cords, with reactivity declining with age due to a reduction or inability to activate the damage response.

During the symptomatic stages of ALS, nucleic acid oxidation may persist but with a greater impact on the production of functional proteins, and the damage may already be irreversible. The majority of remaining MNs towards end-stage disease no longer exhibit

a clearly defined soma and in some cases there appears to be loss of the nucleus, which is supported by cresyl violet and haematoxylin staining. In some of the disease cases glia can be seen in close proximity or at the edge of the MN at end-stage. This could be an indication of neurophagia, the phagocytic destruction of nerve cells, where inflammatory cells cluster around degenerating or dead neurons and actively remove them.

Different cell types in the CNS might have an increased resistance to stress, different stress capacities, and different active repair mechanisms, which may explain some of the differences in reactivity for the markers investigated between MNs and small cells. Different types of MNs may also differ in their stress/damage response. The prominent reactivity in small cells suggests the damage may impact their functionality, which in turn would affect their ability to maintain a homeostatic environment and support for the MNs. Our results display a lack of Ogg1 reactivity within glial cells, suggesting other repair enzymes/pathways may function in these cells, the removal of free oxidised bases may be more efficient, or these cells are less susceptible to DNA oxidation.

4.3.2 Oxidative stress related cellular decline in an in vitro model of ALS

The NSC34 cell line is a well-established model of MNs (Cashman*, et al.* 1992). Introducing human mutations into these cells makes them a suitable *in vitro* model for investigating mechanisms of disease. Here we initially investigated the level of transfection for the human *SOD1* transgenes in the NSC34 cells at both the RNA and protein level. The expression of human mutant *SOD1* within the cells is consistent at the mRNA level, demonstrating equivalent levels of transfection for the different *SOD1* transgenes. This was determined by comparing the Ct value generated for the human *SOD1* to the Ct value for the endogenous mouse *Sod1* (Pan*, et al.* 2012). Despite equivalent transgene expression, significant differences between the mutations are observed at the protein level. In particular, the G93A and H48Q mutations display significantly reduced protein expression in comparison to WTSOD1 and G37R mutant SOD1. This may represent a reduction in the ability of the cell to produce the full-length protein, which consequently may lead to the production of abnormal or short polypeptides, which have a greater tendency to aggregate. These aggregations may lead to increased cellular toxicity, and have been demonstrated in models of familial-linked ALS (Bruijn*, et al.* 1997, Bruijn*, et al.* 1998). Previous studies have highlighted an inconsistency between protein and mRNA levels in wild-type and mutant human *SOD1*

transgenic murine models, which may be due to differences in protein stability (Jonsson, *et al.* 2006, Rumfeldt, *et al.* 2006, Stathopulos, *et al.* 2003). siRNA silencing of mutant *SOD1* in a familial-linked ALS model, led to reduced protein levels which coincided with increased motor performance and delayed symptom onset (Ralph, *et al.* 2005). The reduction in protein levels seen here in the G93A and H48Q mutant *SOD1* NSC34 cells could lead to a reduced disease phenotype in these cells, which may account for some of the differences seen in oxidative stress response in comparison to other studies using this model (Kirby, *et al.* 2005). Oxidative modification of the *SOD1* transcript may affect its ability to be translated and processed. The increased levels of oxidised RNA identified in the G93A and H48Q mutant *SOD1* NSC34 cells in comparison to controls may result in the reduced human SOD1 protein levels seen in these cells in comparison to controls. Oxidative modification to the RNA transcript has been demonstrated to reduce the fidelity of translation, leading to premature termination or the production of abnormal proteins and short polypeptides (Ding, *et al.* 2005, Tanaka, *et al.* 2007).

Previous studies revealed a reticular cytoplasmic distribution of mutant SOD1 in NSC34 cells by immunocytochemistry and a reduction in G93A SOD1 protein levels in comparison to wild-type SOD1, as shown here (Sau, *et al.* 2007). Reduced protein levels and specific activity for human G93A mutant SOD1 in comparison to human WTSOD1 has also been demonstrated in fALS patients; however, the proteins displayed equivalent turnover rates, suggesting differences in stability between the mutant and wild-type proteins accounts for the differences in cellular concentration of the respective proteins (Bowling, *et al.* 1995). Although reduced protein expression was seen for the G93A and H48Q mutant SOD1, these cells were still the most susceptible to cell death following exposure to oxidative stress (figure 4.13). Disrupted folding of mutant proteins has been shown to enhance cellular sensitivity to stress (Zhang and Zhu 2006). The degradation of misfolded or shortened mutant SOD1 proteins would lead to a reduction in the total levels of protein, increase the chance of toxic aggregate formation, and in turn increase cellular vulnerability to oxidative stress.

Previous studies have revealed an accumulation of SOD1 immunoreactive bands in the insoluble protein fraction, when the soluble and insoluble fractions from WTSOD1 and mutant SOD1 transfected NSC34s are investigated (Cozzolino, *et al.* 2008). These bands corresponded to a proportional reduction in the amount of SOD1 migrating in the monomeric form; however, under denaturing conditions the high molecular weight bands were not present. This demonstrated the stability of these oligomers is attributable to intermolecular disulfide bonds. It would be interesting to investigate this in our fALS

model, to determine SOD1 oligomer formation in transfected NSC34 cells and investigate whether exposure to oxidative stress alters SOD1 oligomer formation. An explanation for the reduction in protein seen for the human G93A and H4Q8 mutant SOD1 in the NSC34s could be due to an increase in the proportion of insoluble SOD1, which may increase their tendency to aggregate (Basso*, et al.* 2006, Basso*, et al.* 2009, Karch*, et al.* 2009), as has been demonstrated in models of ALS and familial SOD1 associated ALS patients (Hart 2006, Prudencio*, et al.* 2009, Shibata*, et al.* 1996, Watanabe*, et al.* 2001).

The G93A and H48Q mutations, in addition to showing increased levels of RNA oxidation, and reduced mutant SOD1 protein expression, were also increasingly susceptible to cell death under oxidative stress conditions, as demonstrated by trypan blue exclusion and LDH assay. Treatment of cells with various H_2O_2 concentrations was used to investigate differences in vulnerability to stress between the cell lines. Concentrations of 50μM to 1mM H_2O_2 were chosen for cell treatments as these have generally been cited as used for similar work in the literature; accompanied by a study demonstating no effect on the cellular viability of mouse cells treated with up to 200 μM H_2O_2. The H_2O_2 gradient that exists across cell membranes means for the same extracellular concentration of H_2O_2 there is a lower H_2O_2 concentration intracellularly. Our study demonstrated an exposure to a dose of 50μM H_2O_2 had a minor impact on cell viability across cell types, with mutant *SOD1* G93A and mutant *SOD1* H48Q beginning to show susceptibility to the stress ($p \leq 0.05$) after two hours. A 250μM H_2O_2 treatment had an effect on cell survival over time, but this was moderate in comparison to the changes seen with higher doses. The G93A and H48Q mutant cells showed increasing cell death following periods of prolonged exposure to stress. The increased RNA oxidation observed in these cell lines in comparison to controls may contribute towards their susceptibility to cell death, by reducing the fidelity of translation and thus affecting protein levels and subsequently cellular metabolism. The G37R mutant cells, in contrast, showed an exponential reduction in cell viability over time with increasing doses of hydrogen peroxide, but maintained cell viability at a level equal or in some cases above that of the controls.

There is evidence from both cellular and animal models of ALS suggesting that SOD1 mutation leads to varying levels of cellular toxicity depending on the mutation in question. ALS-associated SOD1 mutations show the propensity to aggregate both with self and other proteins, which may be a result of disruption to the native protein folding (Prudencio*, et al.* 2009). A study investigating the correlation between the propensity for aggregation and conformational stability of SOD1 showed G93A to have the highest conformational instability, and was therefore more prone to aggregation, in comparison to

other SOD1 mutations, including G37R (Stathopulos, *et al.* 2003). Another study also highlighted the differences between the mutations in transgenic mouse models of ALS. The G93A mice exhibited the fastest disease onset and had the shortest lifespan in comparison to the G37R and H46R/H48Q mutations (Karch, *et al.* 2009). The G37R mutation was also shown to be less prone to forming insoluble aggregates. This may be part of the reason why the G93A mutation appeared more toxic in our study.

These experimental findings may reflect differences observed clinically between *SOD1* mutation-types in ALS patients. In human ALS patients the G37R mutation displays an earlier onset of disease but has a longer disease progression in comparison to the G93A mutation (Cudkowicz, *et al.* 1997). Here we observed that the G37R mutant cells are not greatly affected in terms of viability in response to oxidative stress, suggesting the mutant transgene confers greater resistance to this insult in comparison to the other mutations. The H48Q mutation displays a later disease onset in ALS patients but the clinical course is rapidly progressive with a much shorter duration of disease compared to the other two mutations investigated here (Orrell, *et al.* 1997, Orrell, *et al.* 1999). In terms of cell viability the H48Q mutant NSC34 cells were gradually more susceptible to oxidative stress until a certain level of insult when a rapid induction in cell death was observed. Progression of disease in patients harboring the G93A mutation is relatively rapid with typical survival of two to five years (Radunovic and Leigh 1996). Variations between the individual mutations likely underlie the differences seen in susceptibility to oxidative stress in these experiments. This phenotypic heterogeneity is not unusual between patients with different *SOD1* mutations (Battistini, *et al.* 2005), adding further complexity to studying the pathology of the disease. The rapid induction of cell death following exposure to 500μM and 1mM concentrations of H_2O_2 may indicate a sudden loss of, as yet undefined, compensatory mechanisms and an inability of the cells to recover from the insult. The cells may be able to compensate at lower concentrations, and hence only show minor reduction in viability. The redox system is part of a complex signaling network. Previous studies have shown that low concentrations of ROS can influence the regulation of intracellular signaling such as the phosphoinositide 3-kinase (PI3K) pathway and the mitogen activated protein kinase (MAPK) cascade (Seo, *et al.* 2005, Son, *et al.* 2011) and which potentially explain the maintained viability observed with moderate H_2O_2 treatment. From these data we can identify where the major changes in viability occur, allowing a focus on mechanisms operating prior to cell death, potentially in compensated and decompensated phases.

This work supports previous studies that have shown oxidative stress by serum withdrawal causes an increase in cell death of NSCS4 cells transfected with human G93A mutant *SOD1* in comparison to cells transfected with human wild-type *SOD1*. The mutation also rendered cells more sensitive to toxicity induced by exogenous nitric oxide (Cookson*, et al.* 2002). H_2O_2 diffuses readily across cell membranes and cellular compartments and consequently is commonly used for studies of oxidative stress and redox-regulated processes (Chance*, et al.* 1979). However, considerable variation exists between cells in the concentration of exogenous hydrogen peroxide required to initiate a particular biological response. Exogenous hydrogen peroxide has been shown to be less effective at eliciting a signaling response than endogenously produced hydrogen peroxide (Chen*, et al.* 2005, Sablina*, et al.* 2005). This must be taken into account when investigating antioxidant responses in *in vitro* models and relating them to human disease and may account for the absence in major differences in activation of antioxidant responses in the cell lines investigated here. Although H_2O_2 elicited a cell death response after increased exposure, it would have been interesting to treat cells with a low dose of H_2O_2 and measure their response in time intervals following removal of the stress. Co-culture systems would also give an enhanced insight as *in vivo* mutant MNs are influenced by their surrounding cells. Mutant NSC34s have also demonstrated a dose and time dependent increase in apoptotic cell death following treatment with cobalt chloride (Xu*, et al.* 2011), indicating the use of multiple stressors to determine effect on cellular function. Perhaps a more relevant form of cellular stress to impose would be proteasome inhibition, as the accumulation of abnormal and misfolded proteins is described as a pathological hallmark of many neurodegenerative diseases (Karch*, et al.* 2009, Watanabe*, et al.* 2001). Other studies, including this one, show that mutant SOD1 generates oxidative toxicity within MNs and this might intensify other perturbed mechanisms produced due to the mutation. However exactly how this leads to pathogenesis remains unknown. What is emerging is the increasing complexity and differences between mutations, which may confer resistance and susceptibility in the human form of disease.

Measurement of indirect markers such as an increase in oxidative stress related gene expression or antioxidant defense is often used to investigate oxidative stress (Floyd and Hensley 2002). Differences in antioxidant response gene expression in cells carrying human *SOD1* mutations were investigated under basal conditions and following exposure to oxidative stress. Here we investigated whether the presence of these mutations affect the ability of cells to protect themselves from stress, and whether this may account for some of the differences seen in terms of cellular vulnerability to oxidative stress in NSC34s carrying different human *SOD1* mutations. *GAPDH* is a well-established

housekeeping gene used for calculating the relative expression levels of genes of interest by RT-qPCR. *GAPDH* mRNA levels have been shown to increase during periods of increased oxidative stress (Ito, *et al.* 1996). This may be due to the role of GAPDH in cell death signalling following oxidative stress (Hara, *et al.* 2005). Due to this, our samples were re-analysed in comparison to Actin expression levels. Further research has shown both *Actin* and *GAPDH* expression levels differ depending on experimental conditions, and ribosomal proteins are often considered the most reliable sample to compare the expression of the gene of interest against (Dheda, *et al.* 2004, Schmittgen and Zakrajsek 2000). In light of this, future work would involve validating the internal references under the desired experimental conditions to avoid misinterpretation of results. Support for this comes from conflicting results demonstrating no changes in *GAPDH* expression levels following oxidative stress (Zainuddin, *et al.* 2010).

Antioxidant response element (ARE) containing genes encode detoxifying enzymes and cytoprotective antioxidant proteins, and are regulated by the transcription factor Nrf-2. In response to oxidative stress Nrf2 is released from Kelch-like ECH associated protein 1 (Keap1), an actin binding protein localised to the cytoplasm, and translocated to the nucleus where it activates the expression of genes with antioxidant activity, including haem oxygenase 1 (Ho-1) and NAD(P)H:quinone oxidoreductase 1 (Nqo1) (Zhang and Gordon 2004). Reduced Nrf2 expression in an *in vitro* model of familial linked ALS and MNs from ALS patients was associated with a reduction in expression of phase II detoxifying enzymes and antioxidants (Kirby, *et al.* 2005, Petri, *et al.* 2012). Several of the genes that are regulated by Nrf2 have been implicated in protection from neurodegenerative disease, and targeting Nrf2 activity is becoming regarded as a candidate for neurodegenerative therapy (Muller, *et al.* 2007). Ho-1 catalyses the oxidative degradation of haem to biliverdin in the brain and other tissues protecting cells against programmed cell death (Ewing and Maines 1991, Gozzelino, *et al.* 2010), and Nqo1 is a quinone reductase involved in detoxification pathways. Both genes are inducible in response to oxidative stress, which may act to protect cells from degeneration (Dore, *et al.* 1999). The Ho-1 promoter exerts its protective effects through an antioxidant response element located in its promoter, which has a consensus sequence similar to that of other antioxidant enzymes (Balogun, *et al.* 2003, Poon, *et al.* 2004), and gene knockout studies have revealed its biological significance as an antioxidant (Poss and Tonegawa 1997). Histological analysis has shown Ho-1 activity to be increased in the grey and white matter of both sALS and fALS patients, with prominent reactivity in large MNs (Ferrante, *et al.* 1997a).

Gene expression of *Nrf2* and its downstream targets *Ho-1* and *Nqo1* were investigated in all cell lines, initially under basal conditions. WTSOD1 and human mutant *SOD1* transfected cells all show a reduction in *Nrf2* under normal culture conditions. Expression is increased following two hours H_2O_2 treatment but is reduced after six hours exposure across all cell types. *Ho-1* expression however is different, with basal levels increased above pIRES across all cell types and this being reduced following two hours H_2O_2 exposure. This may represent the inability of *Nrf2* to activate its downstream targets in response to exogenous stress, or indicate it is acting through different pathways to maintain cellular viability. A two-hour 250μM exposure only produced a significant reduction in cell viability for G93A mutant *SOD1* cells, with the other cell lines showing slightly greater viability in comparison to the pIRES control cells. *Nqo1* expression is reduced under basal condition for G93A mutant *SOD1* NSC34 cells only. There is about a 50% increase in expression for this cell line following two hours H_2O_2 exposure, however this is not significant and expression is highly variable across all cell lines. A reduction in the expression of antioxidant response genes following exposure to stress could represent an inability of the cell to respond to this due to a mutation, which may be an explanation for the differences seen across the *SOD1* mutations in cell viability after exposure to oxidative stress. It may also be the result of other compensatory mechanisms activated to maintain viability. Investigation of a broader range of antioxidant response genes, combined with apoptotic and cell signalling pathway markers would overall give a reasonable insight into what is happening at the transcriptome level in response to stress. A microarray study of MNs isolated from the spinal cord of the G93A mutant *SOD1* transgenic mouse model also failed to identify differential expression of Nrf-2, or Nrf-2 regulated genes in comparison to controls (Ferraiuolo*, et al.* 2007). Antioxidant potential in astrocytes has been shown to be greater in astrocytes in comparison to neurons (Shih*, et al.* 2003), which may represent a reliance on glial cells for activation of oxidative stress response *in vivo* (Pehar*, et al.* 2005). A single RT-qPCR experiment for *Nrf2* was also carried out on the anterior horn from G93A mutant *SOD1* transgenic mice at one to five months of age. This identified an increase in *Nrf2* expression at presymptomatic stage of disease, which then was reduced during disease progression (data not shown). As with *Ogg1* further work investigating these differences is needed to identify any significant changes.

Model systems are often used to understand how oxidative stress can produce neurotoxic effects leading to cellular degeneration. However, *in vitro* models can often be quite difficult to interpret in terms of what is happening in response to exogenous compounds,

especially stressors, as cells in culture are likely to already be in a state of oxidative stress, or have adapted to their culture conditions. This may result in only small changes seen in the redox state of the cell following exposure to stress. NSC34s are an immortalised cell line, are robust and grow easily in culture. H_2O_2 is widely used to exert oxidative stress on cell culture systems, has been shown to mediate growth-promoting or metabolic effects of growth factors and cytokines at various concentrations, and ROS are now known for their importance in redox sensitive signalling *in vivo*. Here we see increased variability of *Nrf2*, *Ho-1*, and *Nqo1* expression under basal conditions and following exposure to H_2O_2, both across cell lines and between samples from the same cell line. This could be due to a number of factors related to the culture conditions. Although confluency is maintained between 70-80% for harvesting, differences in cell number must be taken into account. All the experiments were carried out at a passage number of between 15-20.

Previous studies investigating oxidative stress and its impact on gene expression changes have documented the importance of these antioxidant genes in stress response. For example, gene expression profiling demonstrated transcriptional repression in NSC34 cells stably expressing human mutant *SOD1* G93A (Kirby*, et al.* 2005). Looking at relative expression levels by RT-qPCR is not as robust as gene expression profiling, so taking this approach and comparing expression levels following exposure to stress may give a comprehensive insight into how the cells respond and adapt to oxidative stress. Using an *in vivo* model would also be a more robust approach, from which the analysis of pathway changes could be further investigated *in vitro*.

4.4 Conclusion

During ageing, an increase in oxidative stress and a reduction in the activity of repair/defense mechanisms may lead to an accumulation of oxidative damage, which is maintained under threshold levels for initiating cell death mechanisms, as cell loss is not a major feature of non-pathological ageing. Breaching this ‘threshold’ for damage in subsets of disease vulnerable post-mitotic neurons during ageing may lead to neurological disorders.

Oxidised nucleotides within cells may not only serve as substrates for nucleic acid biosynthesis but also participate in energy metabolism and signal transduction. Some particular biological effects, for example the activation of apoptotic pathways, are caused

by the accumulation of oxidised nucleotides within cells (Nakabeppu, *et al.* 2010). The chemically modified nucleotides may serve functionally as a sensing mechanism for ROS and RNS to induce cellular adaptive responses to oxidative stress, or as a way of sequestering the toxic species, which can subsequently be removed by surveillance mechanisms (Ihara, *et al.* 2011). The increase in RNA oxidation in comparison to DNA oxidation may represent a protective mechanism for cells to sequester toxic species to prevent modifications to genomic DNA. Sub-lethal insults to RNA are likely to be less damaging than modifications to DNA, which may produce alterations in its coding properties and normal function in transcription or replication. An increase in RNA oxidation alternatively may be the result of inefficient repair and removal mechanisms for these modifications in comparison to DNA. The true quantification of nucleic acid damage is difficult as here we only refer to one modified base, when guanine, adenine, thymine/uracil, and cytosine can all be oxidatively modified with numerous oxidation products subsequently generated (Henderson, *et al.* 2005, Niles, *et al.* 2004). Mass spectrophotometry is one method being used to get a more accurate interpretation of oxidative damage to nucleic acids. Hydroxyl radicals provide high-resolution probes which enable the structural and conformational changes of DNA and RNA to be detected and quantified (Brenowitz, *et al.* 2002, Erb, *et al.* 2012, Taghizadeh, *et al.* 2008).

In neurodegenerative disorders components of the DDR machinery have also been reported to be defective within cells (Barzilai, *et al.* 2008) and studies have revealed reduced activity of DNA repair enzymes within the brains of AD, PD, and ALS patients. Quantification of DNA damage response proteins within a murine model of ALS would enable us to identify differences in the activation of these pathways during disease progression. Here we show the presence of DDR in the spinal cord of transgenic mice expressing familial ALS-linked G93A mutant *SOD1* and in littermate controls immunohistochemically, however limitations of this technique mean it only provides qualitative information. Double labelling immunohistochemistry would also enable us to distinguish which small cells are showing 8-OHG reactivity.

We aimed to compare the differences in RNA oxidation in an *in vitro* model of familial linked ALS, to determine whether the presence of different *SOD1* mutations affects the quantity of RNA that is oxidised. A limitation of the IP technique is the antibody binding capacity may be saturated, or the optimum volume of beads to use is unknown. The majority of IP kits are for protein so we had to optimise the method to obtain sufficient RNA for downstream analysis. In comparison to previous studies, we were using a low concentration of starting RNA. Increasing the starting concentration of RNA would mean

increasing the concentration of antibody to maximise the IP and may increase chances of non-specific binding. We had difficulty in quantifying the proportion of RNA in the oxidised fraction, as the concentration was too low to be accurately quantified using the Nanodrop Spectrophotometer. Using RT-qPCR the expression level of 18S rRNA in the oxidised fraction was related back to a standard curve of known RNA concentrations and compared to the amount of non-oxidised RNA. It is also important to consider is a proportion of the non-oxidised fraction may be lost during the IP, or only a percentage of the oxidised fraction is captured, and a more specific and direct quantification would be beneficial.

Further work using the NSC34 cell model of familial linked ALS could be used to investigate levels of DNA oxidation between the cells carrying different human mutant *SOD1* transgenes. Alternative RT-qPCR methods to the one used here have also been described as a method of measuring RNA oxidation (Rhee*, et al.* 1995). A more robust model would be to use primary cells from transgenic murine models of ALS to investigate differences in levels of nucleic acid oxidation and oxidative stress responses. This would also provide a model in which differences in nucleic acid damage and repair could be quantified, and comparisons could be made between different cell types in the CNS. This is difficult as only a limited number of cells are available from each animal used. The use and combination of genomic and proteomic research tools enable identification of alterations at the mRNA and protein levels and determine how these differ in disease. Microarray analysis has identified differentially expressed genes in this NSC34 cell model of familial linked ALS, and proteomic work provides further evidence for alteration of corresponding proteins (Allen*, et al.* 2003, Kirby*, et al.* 2005). To determine how RNA and protein expression levels differ in response to oxidative stress and in a co-culture environment (which may be more physiologically relevant) would be interesting approaches to take.

Chapter 5
Investigating mitochondrial bioenergetics in an *in vitro* model of ALS

5.1 Introduction

5.1.1 Mitochondrial energy metabolism

The integrity and functionality of mitochondria are key determinants of neuronal function and survival. Mitochondria form highly dynamic networks and are the primary site of ATP synthesis through two main metabolic pathways, oxidative phosphorylation and glycolysis (Otera and Mihara 2011, Shi*, et al.* 2010). During cellular metabolism, pyruvate, fatty acids, and amino acids are generated, which can be further broken down by mitochondria to nicotinamide adenine dinucleotide dehydrogenase (NADH) and/or flavin adenine dinucleotide ($FADH_2$). These reduced equivalents are used by oxidative phosphorylation for energy production by the electron transport chain (ETC). The ETC located in the mitochondrial inner membrane is composed of four multi-subunit enzyme complexes (complex I-IV), which are involved in a series of oxidation-reduction reactions between redox pairs, and two electron carriers (coenzyme Q and cytochrome c).

NADH and the hydroquinone form of $FADH_2$ are the electron donors and generate the redox potential gradient driving electron transport and ATP synthesis. The oxidation of NADH and/or $FADH_2$ facilitates the transfer of electrons through the complexes. Complexes I, III, and IV, of the electron transport chain comprise the energy-conserving core, the transfer of electrons facilitates the pumping of protons across the inner mitochondrial membrane. This generates a reduction in redox potential as the electrons pass through the complexes, establishing an electrochemical gradient (proton-motive force) (figure 5.1). This force is used to drive proton re-entry, and during ATP synthesis this predominantly occurs through ATP synthase, catalysing the phosphorylation of ADP to ATP. Ion channels and transporters in the inner membrane regulate proton and ion flux. The proton circuit set up across the inner membrane is central to mitochondrial bioenergetics. In summary, oxidisable substrates are used by mitochondria to set up a membrane potential in the form of a proton gradient, which operates across the inner mitochondrial membrane to facilitate energy production.

In addition to the production of ATP, mitochondria are also central to the intrinsic apoptotic cascade, calcium signalling, and calcium homeostasis. The dysfunction of mitochondrial function can therefore be linked to multiple phenotypic changes of cells.

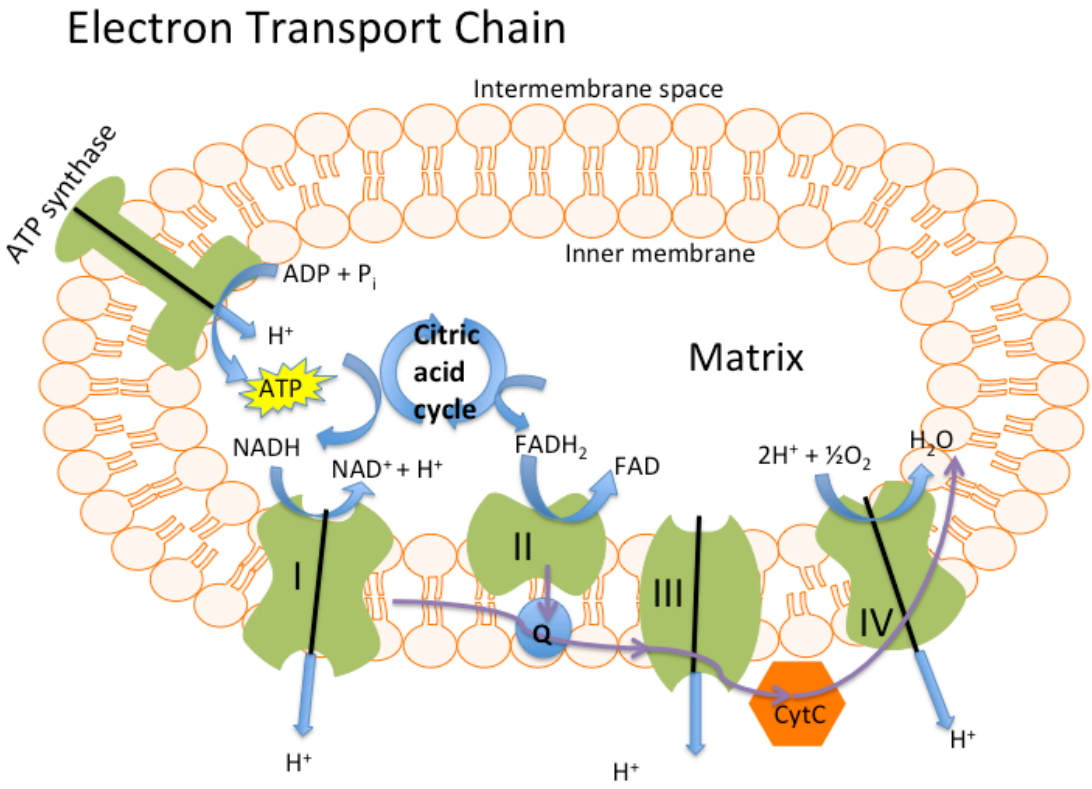


Figure 5.1: Electron Transport in Mitochondria

NADH is oxidised by complex I, which donates electrons to coenzyme Q. Complex III oxidises reduced coenzyme Q, which in turn reduces the mobile electron carrier protein cytochrome C. Electrons are transferred from cytochrome C to complex IV, for the reduction of molecular oxygen. Complex II (succinate-Q oxidoreductase) forms a separate entry point in to the ETC. Succinate is oxidised to fumarate, and a hydride is transferred to FAD to form $FADH_2$. $FADH_2$ transfers its electrons reducing coenzyme Q. ATP synthase catalyses the synthesis of ATP, which is coupled to transmembrane proton transfer. The proton motive force (pmf) generated during electron transport drives the exchange of ADP and P_i for ATP.

5.1.2 Mitochondrial dynamics

Mitochondria are dynamic organelles, continuously remodelling to meet the changing cellular energy demand. Mitochondrial dynamics of fusion and fission allow the organelle to form branched interconnecting networks. In mammals, the mitofusins MFN1 and MFN2, and OPA1 are involved in mitochondrial outer membrane and inner membrane fusion respectively (H. Chen*, et al.* 2003, Santel and Fuller 2001). Proteins involved in fission include FIS1 and DRP1 (Cipolat*, et al.* 2004, Mozdy*, et al.* 2000). Mitochondrial division, turnover, and network formation create an efficient system for mitochondria to deliver ATP to subcellular compartments, and maintaining their critical functions through communication with the cytosol and quality control (Koopman*, et al.* 2005, Rube and van der Bliek 2004). The quality of a mitochondrial population is maintained by mitophagy, a selective removal process for damaged mitochondria by autophagosomes and their subsequent catabolism by lysosomes (reviewed in Ashrafi and Schwarz 2013). Although reactive oxygen species (ROS) are generally known for their detrimental effects on cells, they act as signalling molecules during the early induction events of autophagy (Mammucari and Rizzuto 2010). However, if the pro-survival attempt is unsuccessful, ROS can induce cell death. The cellular context and the modulators of ROS activity determine whether cell death is initiated through the autophagic or apoptotic pathway.

Another aspect of mitochondrial dynamics is motility, the transport of mitochondria to distinct subcellular locations. Mitochondria have been shown to accumulate in regions with high energy demands, such as synapses and areas of increased protein synthesis (Chang*, et al.* 2006, Morris and Hollenbeck 1993). Mitochondrial transport depends on the actin cytoskeleton and microtubules composed of tubulin (Hollenbeck and Saxton 2005, Ligon and Steward 2000, Morris and Hollenbeck 1995). The molecular motor proteins kinesin and cytoplasmic dynein also mediate axonal transport of mitochondria, to facilitate their distribution.

5.1.3 Mitochondrial dysfunction and neurodegenerative disease

Mitochondrial dysfunction is documented in many age-associated diseases including ALS. Because of the essential functions carried out by mitochondria, a disturbance to their properties may confer an intrinsic susceptibility to stress and MN decline in neurological disorders. Studies from both ALS patients and models of disease have demonstrated changes in mitochondrial function including a reduction in the activity of

respiratory chain complexes and decline of mitochondrial bioenergetic capacity, suggesting dysregulated energy metabolism is one mechanism contributing to MN degeneration in ALS (Arciello*, et al.* 2010, Duffy*, et al.* 2011, Jung*, et al.* 2002).

Studies of mitochondrial morphology and function in spinal cord MNs from ALS patients have revealed swollen and vacuolated mitochondria. These morphological changes were accompanied with the presence of mitochondrial aggregates, defective respiratory chain function, and oxidative damage to mitochondrial proteins and lipids (Sasaki and Iwata 1996, Sasaki and Iwata 2007, Siklos*, et al.* 1996, Wiedemann*, et al.* 2002). *In vitro* studies have demonstrated human mutant *SOD1* variants cause a shift in redox potential, have increased mitochondrial superoxide dismutase levels, show increased toxicity, and a demonstrate reduction in respiratory chain complex activity (Cozzolino*, et al.* 2009, Estevez*, et al.* 1999, Ferri*, et al.* 2006). *In vivo* murine studies have demonstrated impairment in mitochondrial axonal transport within mutant *SOD1* MNs, and observed membrane vacuoles derived from degenerating mitochondria (Dal Canto and Gurney 1995, De Vos*, et al.* 2007, Wong*, et al.* 1995). At the time of disease onset, the G93A mutant *SOD1* transgenic mice also exhibit reduced mitochondrial respiration and energy production (Jung*, et al.* 2002, Kirkinezos*, et al.* 2005, Mattiazzi*, et al.* 2002).

A functional compromise to energy production results in a loss of mitochondrial membrane potential, in addition to impaired electron chain transport activity and a reduction in ATP production, with an accompanying increase in ROS production (Arciello*, et al.* 2010, Browne*, et al.* 2006, Menzies*, et al.* 2002). The vulnerability of mitochondria to damage may be increased in ALS, accompanied with reduced ability of the MNs to compensate for the damage/energy deficit. Mitochondrial defects including impairment in the axonal transport of these organelles will impair mitochondrial localisation at critical sites with high energy demands, thus contributing to the denervation process (De Vos*, et al.* 2008). Although mitochondrial impairment and increased oxidative stress have been extensively documented in models of ALS, the causal relationship between impaired bioenergetics, physiological malfunction, and oxidative damage needs to be further established in relation to the underlying pathogenesis of the disease.

Nucleic acid oxidation during ageing and ALS may link to mitochondrial dysfunction through direct modification of mtDNA or indirectly as a consequence of nuclear DNA and RNA oxidation. The oxidative modification to DNA may affect the fidelity of transcription, reducing transcription rates and potentially altering the transcriptome. The

ETC relies on the concerted function of both the mitochondrial and nuclear genomes to express functional components of the respiratory chain complexes. The integration of transcriptional regulatory pathways controlling the expression of nuclear and mitochondrial genes determines respiratory complex formation. The reliance of mtDNA upon nuclear encoded proteins for its maintenance and transcription will impact energy production during periods of oxidative stress, which in turn will lead to mitochondrial dysfunction and increased ROS production; the vicious cycle (figure 5.2). mtDNA is also vulnerable to ROS attack which may also impact upon their function. Further disruption to mitochondrial maintenance and protein quality control during and ageing and neurodegenerative disease may result in the accumulation of dysfunctional mitochondria, which are unable to maintain sufficient energy generation (Karbowski and Neutzner 2012).

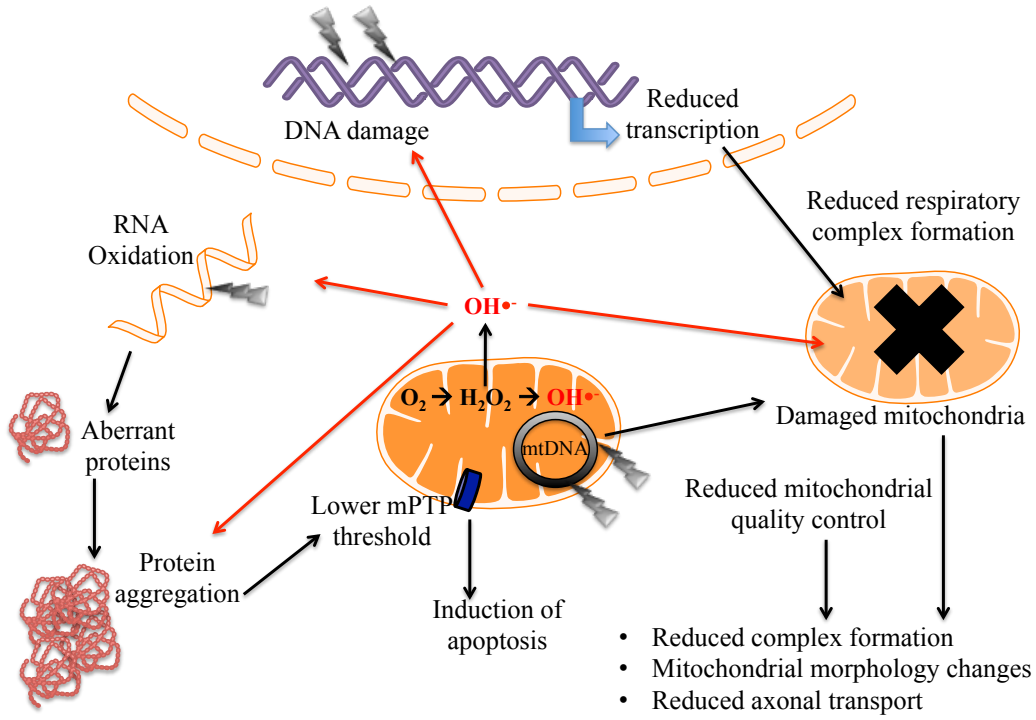


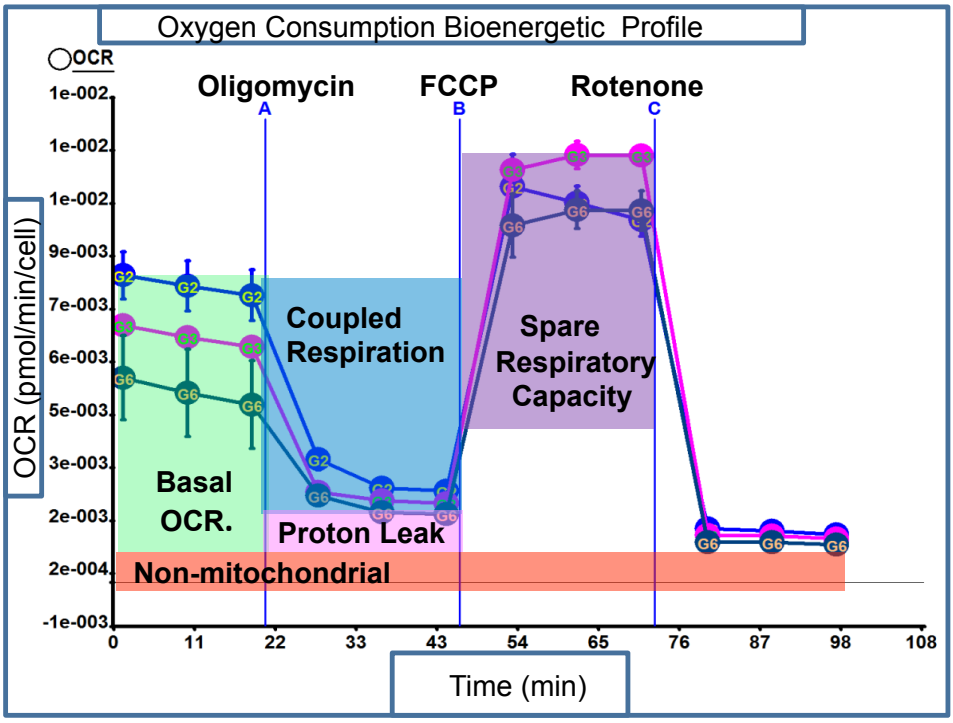
Figure 5.2: Nucleic acid oxidation and disruption to mitochondrial function during ageing and neurodegenerative disease (Adapted from Yankner, *et al.* 2008)

5.1.4 Investigating mitochondrial morphology and bioenergetics in an in vitro model of familial-ALS

The physiological state of cells and differences in metabolic state between cell types can be assessed through measuring the rate of oxygen consumed by the cells. The Seahorse extracellular flux analyser simultaneously measures the two major energy-yielding pathways in cells, aerobic respiration and glycolysis, by measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in intact cells with an undisturbed cellular environment (figure 5.3). OCR provides an indication of mitochondrial respiration, and ECAR measures glycolysis, the breakdown of glucose to lactate, which is the primary source of protons. When investigating cultured cells the measurement of the acidification of the extracellular media (change in pH, caused via the release of protons) provides an indication of the ECAR. OCR and ECAR for a cell is related to the flux through catabolic pathways used to generate ATP. During steady state, the ATP synthesis rate is counterbalanced against ATP consumption and thus, the OCR and ECAR are mainly related to ATP turnover. Although quantitative estimates of ATP turnover are technically challenging, it can be shown that the changes in extracellular fluxes show a concordance with changes in ATP turnover rates.

Mitochondrial metabolism can be investigated by shifting the bioenergetic profile of cells through addition of mitochondrial inhibitors such as oligomycin, carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone (figure 5.3). Oligomycin is an ATP synthase inhibitor and prevents ATP synthesis (inhibits oxidative phosphorylation) in the coupling experiment by blocking the proton conductance (Fo subunit) necessary for oxidative phosphorylation. It is used to prevent state 3 (phosphorylating) respiration but does not affect electron flow among the complexes. FCCP is a protonophoric uncoupler of oxidative phosphorylation (uncoupling of ATP synthesis from electron transport) by transporting hydrogen ions across the mitochondrial membrane instead of the proton channel of ATP synthase and thus causing loss of the mitochondrial membrane potential. Rotenone is an electron transport inhibitor, and inhibits mitochondrial complex I activity by interfering with electron transport chain activity. It inhibits the transfer of electrons from iron-sulfur centres in complex I to ubiquinone, and therefore blocks NADH dehydrogenase during the creation of ATP. Altering the normal physiological state of cells allows the investigation of differences in cellular metabolism within different cell lines, and may highlight mitochondrial dysfunctions for example in the presence of various mutations.

A.



B.

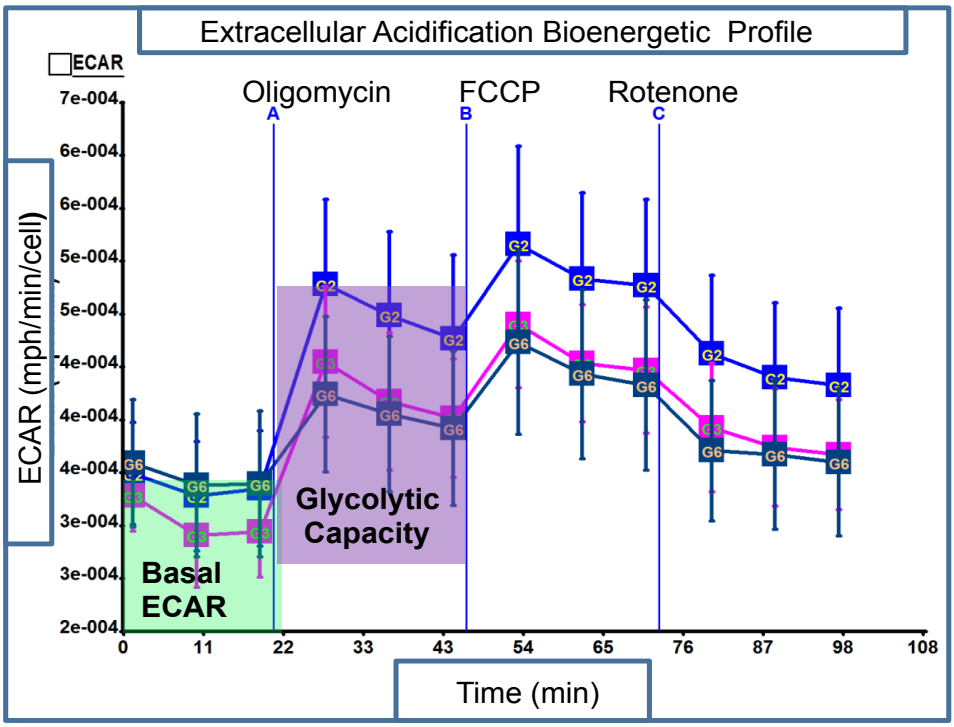


Figure 5.3: Representative OCR (A) and ECAR (B) bioenergetic profile of NSC34 cells.

Blue represents pIRES, pink represents WTSOD1, green represents G93A *SOD1* NSC34 cells. In (A) A, B, and C correspond to the addition of oligomycin, FCCP, and rotenone respectively. Both OCR and ECR are measured three times for each condition.

In this study we utilised the murine neuroblastoma spinal cord (NSC34) hybrid cell line (Cashman*, et al.* 1992), stably transfected with various human mutant *SOD1* transgenes. *SOD1* mutations in these cell lines have previously been shown to increase oxidative stress and mitochondrial dysfunction, with key genes down regulated in their metabolic pathways (Kirby*, et al.* 2005, Menzies*, et al.* 2002). We expressed wild-type human *SOD1* (WTSOD1), G93A mutant human *SOD1*, H48Q mutant human *SOD1*, G37R mutant human *SOD1*, and pIRES vector control in the NSC34 cell line and determined mitochondrial and metabolic function under basal and oxidative stress conditions. We show that significant differences in mitochondrial bioenergetics are identified in the G93A mutant *SOD1* cells compared with the controls and other mutations investigated, both under basal conditions and after exposure to oxidative stress. Morphological examination of the mitochondria by live cell imaging was used to determine whether the functional deficit correlated with differences in mitochondrial morphology between the cell lines investigated.

5.2 Results

Having established differential susceptibility to oxidative stress related cellular decline in NSC34 cells transfected with different *SOD1* mutations we sought to identify whether this was associated with mitochondrial dysfunction. Mitochondrial bioenergetics were measured for intact transfected NSC34 cells prior to and preceding a sublethal oxidative insult. We show significant differences in mitochondrial bioenergetics in the G93A mutant *SOD1* cells compared with the controls and other mutations investigated, both under basal conditions and after exposure to oxidative stress. This functional deficit correlated with a morphological change of mitochondria within these cells, but was not seen in the cells carrying the G37R and H48Q mutations. The G37R mutation, which demonstrated increased resistance to oxidative insult in the previous chapter, showed comparative oxygen consumption and glycolytic flux prior to and post oxidative insult, which indicates the increased susceptibility to stress seen in the other mutations may be correlated with reduced mitochondrial function.

5.2.1 Mitochondrial bioenergetics in an in vitro model of familial-ALS

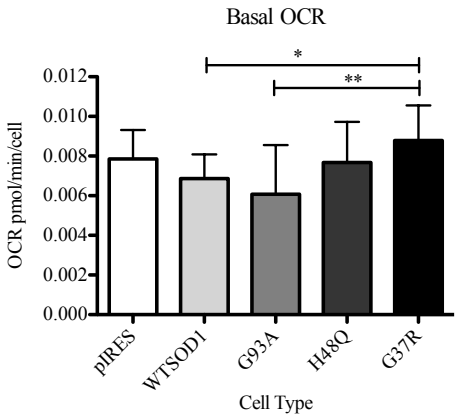
5.2.1.1 Mitochondrial bioenergetics under basal conditions

To create a bioenergetic profile of the neuronal cell model control and transfected NSC34 cells were analysed using an XF24 Seahorse Bioanalyser. Basal conditions were compared with addition of the ATP synthase inhibitor oligomycin, the mitochondrial membrane uncoupler FCCP, and the mitochondrial complex I inhibitor Rotenone to assess mitochondrial function.

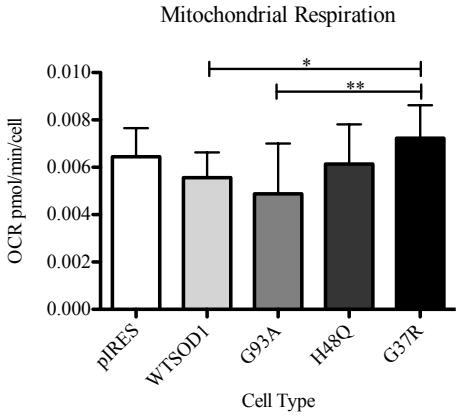
Basal cellular oxygen consumption (OCR) is an indicator of both mitochondrial and non-mitochondrial respiration and is controlled strongly by ATP turnover and partly by substrate oxidation and proton leak (Ainscow and Brand 1999, Brown*, et al.* 1990). Basal OCR was significantly lower in the G93A mutant cells in comparison to the G37R mutant cells ($p \leq 0.01$) (figure 5.4, A), indicating the different effects of *SOD1* mutations on mitochondrial function. A non-significant ($p > 0.05$) reduction in basal OCR was seen between the G93A mutant and control cells. G37R mutant cells showed significantly higher basal OCR than WTSOD1, indicating that the presence of this mutation has a significant difference in coupled respiration under basal conditions. The application of Rotenone was used to determine the fraction of cellular oxygen consumption linked to mitochondria. The G37R mutant cells showed significantly higher ($p \leq 0.01$) mitochondrial respiration compared with WTSOD1 and G93A *SOD1* cells (figure 4.3, B) following the addition of Rotenone.

The application of FCCP dissipates the proton gradient across the mitochondrial inner membrane and allows investigation of maximal mitochondrial respiration. The spare respiratory capacity of cells can be calculated following application of FCCP and the induction of maximal respiration. Spare respiratory capacity represents the amount of additional ATP that can be produced when the cell experiences a sudden increase in energy demand. It is the difference between ATP production by oxidative phosphorylation at basal and maximum respiratory capacity. The mean spare respiratory capacity was variable for all cell types investigated. The G93A and G37R mutant cells displayed reduced spare respiratory capacity compared to controls and the H48Q *SOD1* mutation, however the reduction did not reach significance ($p > 0.05$) (figure 5.4, C).

A.



B.



C.

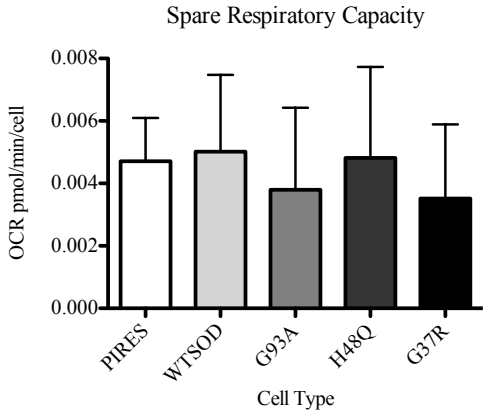


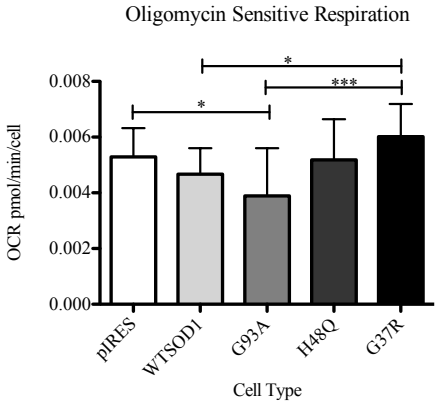
Figure 5.4: The effect of *SOD1* mutations on oxygen consumption

Basal OCR was measured (A) and mitochondrial respiration calculated by subtracting OCR in the presence of Rotenone from basal OCR (B). The spare respiratory capacity calculated by subtracting basal OCR from respiratory capacity after addition of FFCP (C). Data presented as mean with SD (n=4), statistical analyses by one-way ANOVA with Bonferroni post-test *=P≤0.05, **=P≤0.01

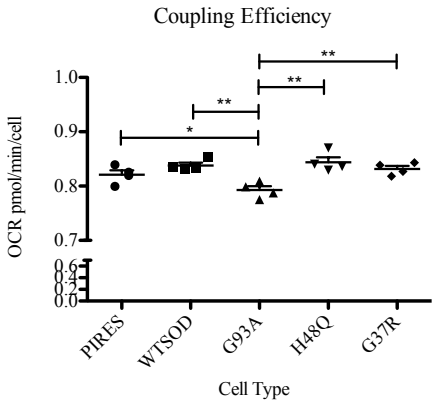
The rate of mitochondrial ATP synthesis was investigated by the application of an ATP synthase inhibitor (oligomycin). The addition of oligomycin shifts the entire cellular ATP synthesis towards glycolysis so that subtraction of the post-treatment OCR from basal OCR indicates oligomycin sensitive respiration. The G93A mutant cells showed significantly lower ATP turnover ($p \leq 0.01$) in comparison to the G37R mutants and the pIRES vector control (figure 5.5, A); however only minor differences in turnover were seen for the mutations in comparison to the controls. Mitochondrial coupling efficiency is the fraction of mitochondrial oxygen consumption used for ATP synthesis. The coupling efficiency can be determined from the change in basal respiration rate following application of oligomycin, and can detect whether the mitochondria are dysfunctional. The G93A mutant *SOD1* cells displayed a significant reduction in coupling efficiency in comparison to WTSOD1 and the G37R and H48Q mutant cells (figure 5.5, B).

During ATP production, a percentage of protons leak across the inner mitochondrial membrane. These protons are able to pass back into the mitochondria, and in the absence of ATP synthesis the proton circuit is largely completed by proton leak. Proton leak can be determined by the subtracting the respiration rate after the application of Rotenone from the oligomycin sensitive respiration rate. The G37R mutant cells showed a significantly greater ($p \leq 0.05$) proton leak in comparison to the WTSOD1 cells (figure 5.5, C). Dysfunctional mitochondria are expected to show an increase in proton leak as much of their energy generation is linked to uncoupled respiration.

A.



B.



C.

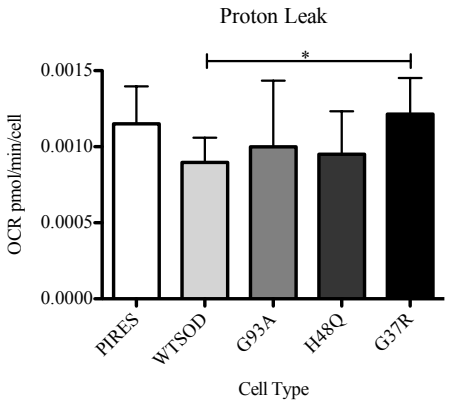
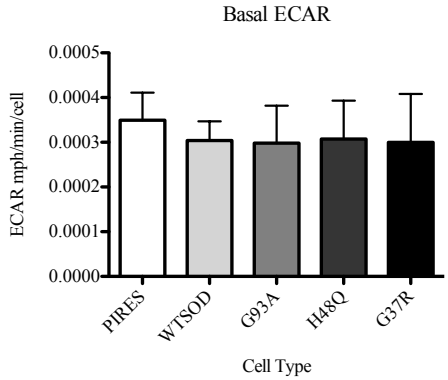


Figure 5.5: Mitochondrial function following complex inhibition

(A) The rate of ATP turnover (coupled respiration) in a basal state can be determined from the decrease in OCR on inhibiting ATP synthase with oligomycin, displayed here as oligomycin sensitive respiration. (B) Coupling efficiency determined from the change in basal OCR after addition of oligomycin. (C) OCR in the presence of rotenone subtracted from OCR in the presence of oligomycin determines proton leak. Data presented as mean with SD (n=4), statistical analyses by one-way ANOVA with Bonferroni post-test ***=P≤0.001, *=P≤0.05

Together with measurements of aerobic respiration, the XF24 Seahorse bioanalyser also enables investigation of the extracellular acidification rate (ECAR), a direct measure of lactate produced by glycolytic flux. Lactate is the primary source of protons, and ECAR is measured indirectly from the change in pH as protons are released in to the surrounding media (Nicholls 2010). No significant differences were observed for basal ECAR between the controls and the mutations (figure 5.6, A). The response of the glycolytic flux to mitochondrial inhibition to restore the energy deficit can be measured. When ATP synthase is inhibited, the cell responds by up-regulating glycolysis to recover the energy deficit, this increase above basal levels is termed the glycolytic capacity. A reduction in OCR after the application of complex I inhibitor Rotenone, would normally be accompanied by a concomitant increase in ECAR to maintain energy production. A collapse in the mitochondrial membrane potential after the addition of uncoupling agent FCCP also results in an increase in ECAR, as cells attempt to maintain their energy balance through glycolysis. The induction of ECAR in the presence of the mitochondrial inhibitors oligomycin, FCCP, and Rotenone was measured, however no significant differences in induction were seen between controls and mutant cells lines ($p>0.05$) (figure 5.6, B).

A.



B.

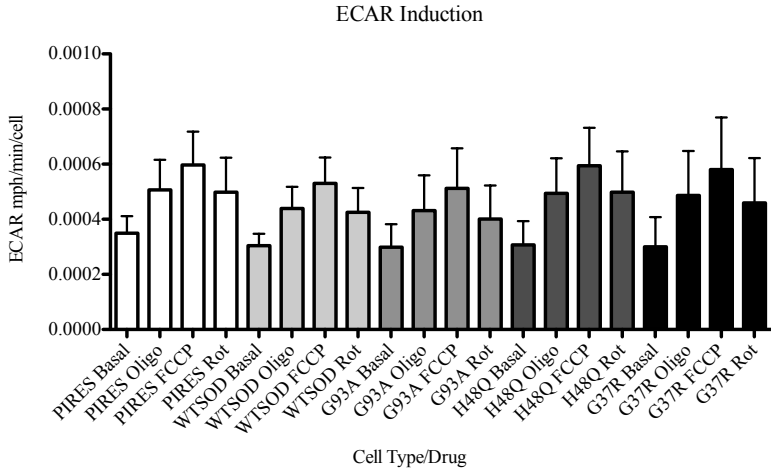


Figure 5.6: Glycolytic flux under basal conditions and following the application of mitochondrial inhibitors

(A) No significant differences were observed for ECAR when measured under basal conditions. (B) ECAR induction did not vary significantly between the controls and mutations investigated following the application of oligomycin (Oligo), FCCP, and rotenone (Rot)

5.2.1.2 Mitochondrial bioenergetics under stress conditions

Following the measurement of mitochondrial function under basal conditions, the cell lines were subjected to three sub-lethal doses of H_2O_2 (up to 200μM for one hour) as determined from cell viability assays (section 4.3.1). OCR and ECAR were measured after exposure to oxidative stress to determine whether these conditions introduced significant metabolic defects.

Basal ECAR was unaffected in the control cells at 50μM and 100μM. However, the G93A mutant cells showed a significant reduction in ECAR at 100μM ($p \leq 0.01$) in comparison to both the pIRES and WTSOD1 controls (figure 5.7, A), which may indicate a susceptibility of this particular mutation to oxidative stress. It was also significantly reduced ($p \leq 0.05$) compared to the H48Q mutation. A reduction in ECAR was seen for both controls and mutants at 200μM. A significant reduction in basal OCR was seen for the G93A mutant cells in comparison to the WTSOD1 ($p \leq 0.01$) and the G37R mutant cells ($p \leq 0.05$) following treatment with 100μM H_2O_2 (figure 5.7, B).

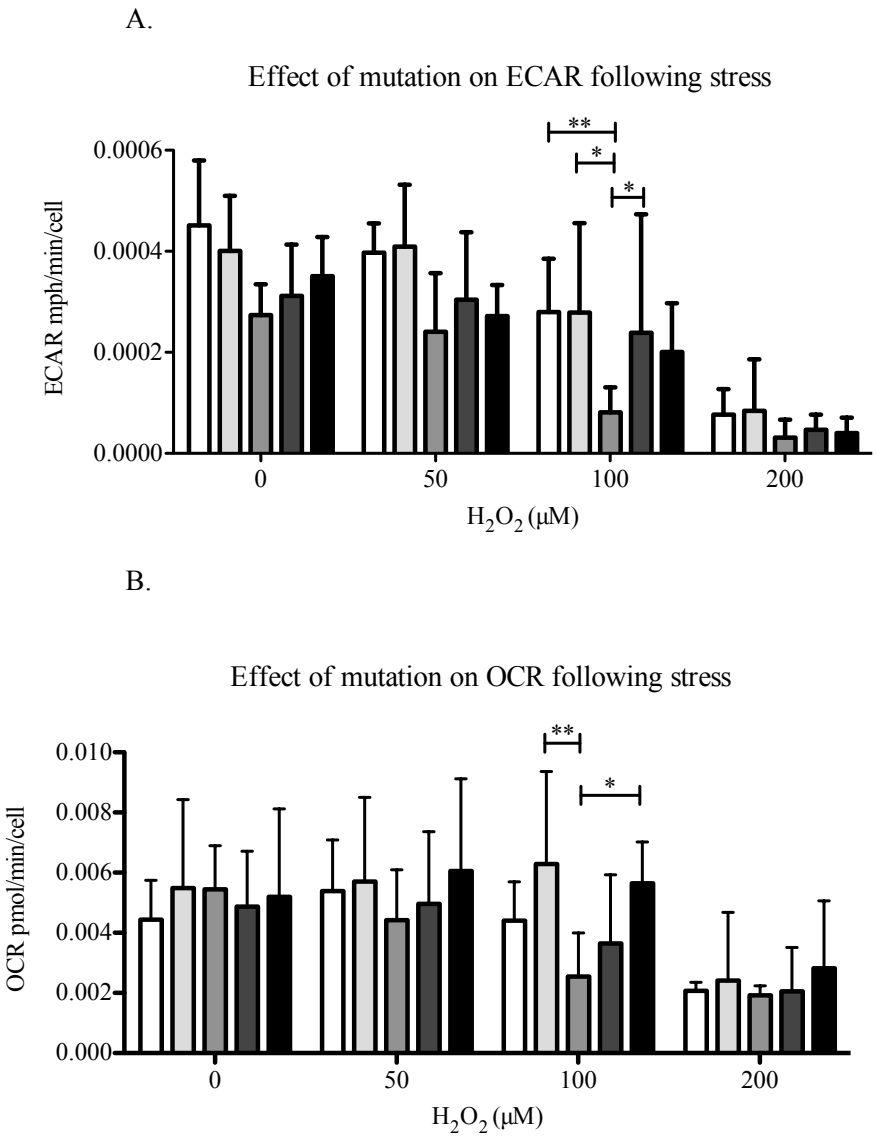


Figure 5.7: The effect of oxidative stress on mitochondrial metabolic function (A) After H_2O_2 treatment the G93A mutation displayed a significant reduction in ECAR at 100μM in comparison to both the vector and human *SOD1* controls and the

H48Q mutation. 200μM H_2O_2 treatment showed reduced ECAR in all cell types. (B) OCR was measured following H_2O_2 stress. The G93A mutation had significantly reduced oxygen consumption in comparison to normal and G37R mutant human *SOD1* after 100μM stress. At 200μM all cells displayed diminished basal OCR. Data presented as mean with SD (n=3), statistical analyses by two-way ANOVA with Bonferroni post-test **=P≤0.01, *=P≤0.05

5.2.2 Mitochondrial morphology in an in vitro model of familial-ALS

Mitochondrial morphology was investigated in the control and mutant NSC34 cells using rhodamine 123 under basal conditions, and following exposure to 100μM H_2O_2 for one hour. The G93A mutation showed significantly reduced basal oxygen consumption rate when exposed to this oxidative insult. The aim was to determine whether the dysfunction of mitochondria seen following exposure to an exogenous stress correlates with a change in their morphology. Qualitative assessment revealed increased network formation in the G93A mutant cells under basal conditions, in comparison to the controls and other mutations investigated. Initial analysis carried out on the three dimensional z-stack projections (Mortiboys*, et al.* 2010) failed to produce reliable measurements. Mitochondria that qualitatively were forming networks or close in proximity were frequently identified by the macro as a single organelle. Subsequent to this analysis was repeated using the same macro (Dagda*, et al.* 2009), however only the middle image from each stack was analysed. . This revealed an increase in the area/perimeter percentage of the G93A mutation under basal conditions, which was significant compared to both the controls, and the G37R, and H48Q mutations (figure 5.8, A). Mitochondrial elongation was greatest in the pIRES cells under basal conditions, but the increase was not significant in comparison to the other cell lines investigated (figure 5.8, B). The area/perimeter is a measure of the interconnectivity of mitochondria within a cell. Following exposure to oxidative stress the G93A mutation displayed a reduction in area/perimeter in comparison to all the other cell lines investigated, and this was significant in comparison to the pIRES control (Figure 5.9, A), and in comparison to itself under basal conditions (figure 5.10). Mitochondrial elongation following exposure to oxidative stress displayed no significant differences between the cell lines investigated (figure 5.9, B).

A. B.

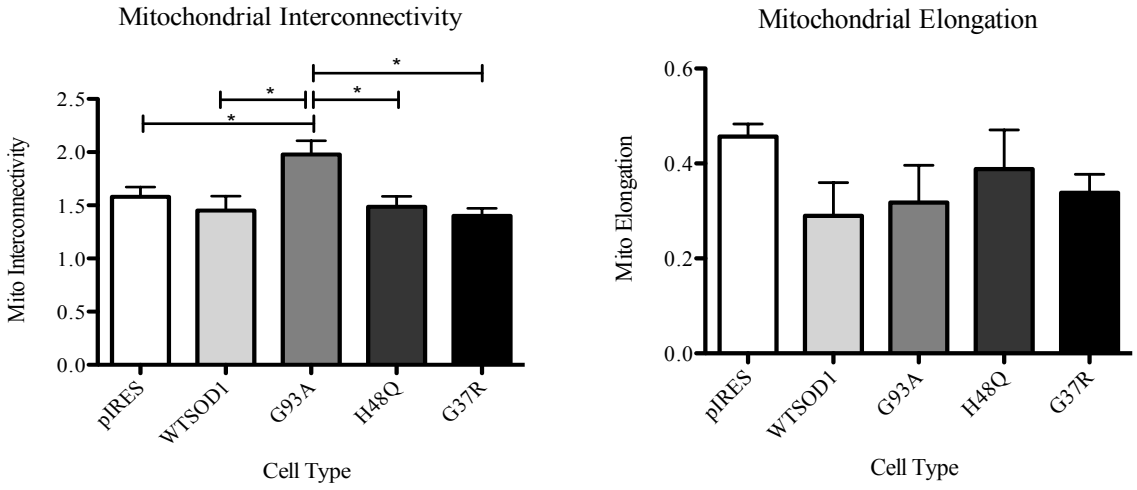


Figure 5.8: Mitochondrial interconnectivity (A) and elongation (B) in control and mutant NSC34 cells under basal conditions.

Data presented as mean with SD (n=3), statistical analyses by one-way ANOVA with Bonferroni post-test *=P≤0.01

A. B.

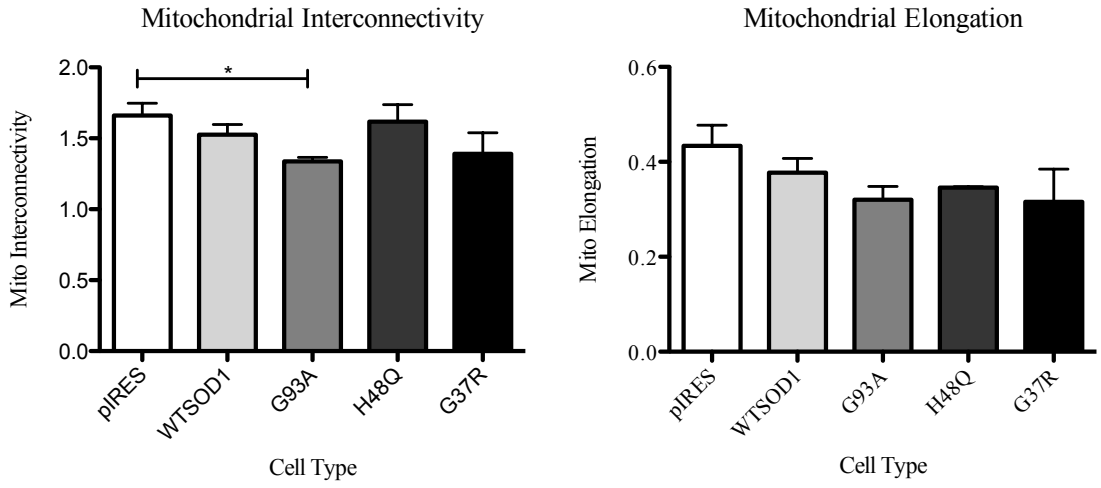
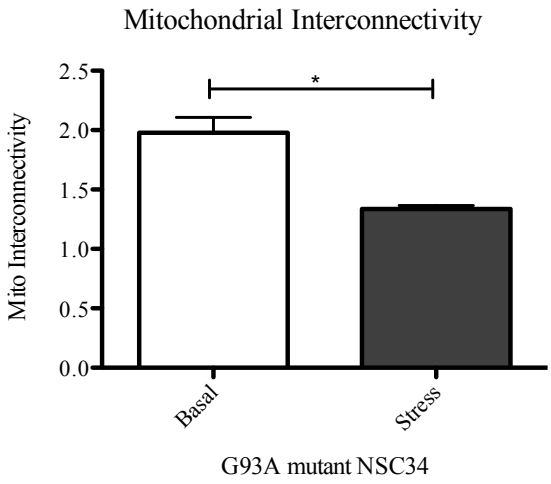


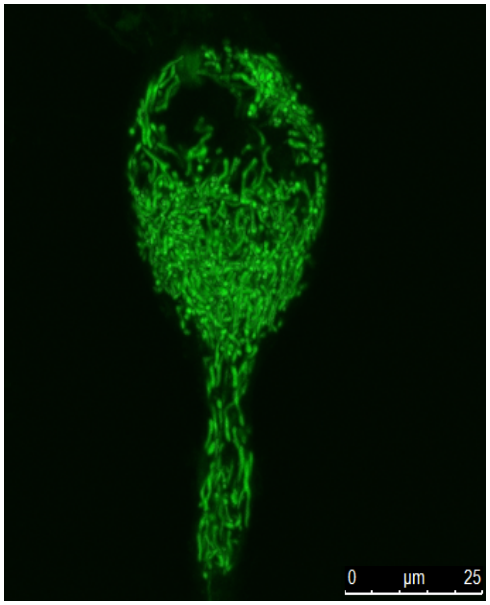
Figure 5.9: Mitochondrial interconnectivity (A) and elongation (B) in control and mutant NSC34 cells following exposure to 100μM H_2O_2 for one hour

Data presented as mean with SD (n=3), statistical analyses by one-way ANOVA with Bonferroni post-test *=P≤0.01

A.



B.



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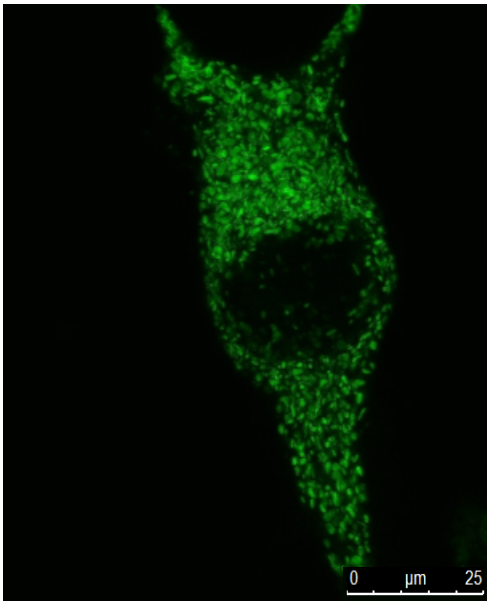

Figure 5.10: Mitochondrial interconnectivity in cells carrying the G93A mutation under basal and stress conditions

(A) Mitochondrial interconnectivity in G93A mutant SOD1 NSC34 cells under basal and stress conditions. Mitochondrial morphology is represented under basal conditions and following exposure to oxidative stress in (B) and (C) respectively. Data presented as mean with SD (n=3), statistical analyses by two-tailed unpaired t-test *=P≤0.01. Images are of the z-stack projection created using Image J.

5.3 Discussion

5.3.1 Mitochondrial bioenergetics in an in vitro model of ALS

To determine whether the presence of different disease-causing mutations have differential effects on mitochondrial function, that could be linked to susceptibility to cell death under oxidative conditions, we investigated the bioenergetic capacity of different human mutant *SOD1* transfected cells. The XF24 Seahorse Bioanalyser simultaneously measures aerobic respiration and glycolysis within intact cells, and has previously been used to investigate mitochondrial bioenergetics in Alzheimer's and Parkinson's disease (Choi*, et al.* 2011, Varghese*, et al.* 2011, Yao*, et al.* 2009). Cellular respiration was assessed under basal conditions (basal respiration) and with the ATP synthase inhibitor oligomycin (to investigate coupled respiration), the mitochondrial membrane uncoupler FCCP (to measure spare respiratory capacity) and the mitochondrial complex I inhibitor rotenone (to assess mitochondrial specific respiration) (figure 5.3). The sequential addition of these compounds shifts the bioenergetic profile of cells allowing differences in mitochondrial function to be compared between cell lines.

Basal oxygen consumption (OCR) is a measurement of mitochondrial respiration that indicates differences in the efficiency of oxidative phosphorylation between cell lines. Oxygen is consumed at complex IV of the respiratory chain, and this is measured as the OCR. The G93A mutation shows a significant reduction in basal OCR in comparison to the G37R mutation, suggesting reduced mitochondrial efficiency, which could explain in part why the G93A mutant cells show an increased susceptibility to oxidative stress in comparison to the G37R mutants. WTSOD1 also demonstrated reduced oxygen consumption in comparison to the G37R mutation, suggesting the presence of WTSOD1 has an effect on the bioenergetics capacity of cells. Individual transfectants did not show significant differences in comparison the pIRES and WTSOD1 in terms of spare respiratory capacity. Although spare respiratory capacity for the G93A and G37R mutations was reduced in comparison to controls, this didn't reach significance, likely due to the variability in the data. The bioenergetic capacity of mitochondria influences the ability of cells to respond to increased energy demands, which may be critical for survival.

Treatment with oligomycin prevents the cells synthesising ATP by oxidative phosphorylation and consequently they revert to glycolysis. This inhibition leads to slight

mitochondrial hyperpolarisation, which may over-estimate the proton leak and under-estimate ATP turnover; however in most cell types the error is relatively small (Affourtit and Brand 2009). Addition of oligomycin allows investigation of the cellular oxygen consumption devoted to ATP synthesis (Nicholls*, et al.* 2010). When proton flux through ATP synthase is inhibited, phosphorylating respiration stops, and proton leak accounts for the residual oxygen consumption. The G93A mutation showed significantly reduced coupled respiration in comparison to the pIRES and G37R mutant cells. Coupled respiration drives oxidative phosphorylation of ADP to ATP mediated by proton pumps across the inner mitochondrial membrane. A reduction in coupled respiration suggests defective ADP/ATP exchange within human G93A mutant *SOD1* transfected NSC34 cells. A decline in the energy generation capacity of cells will lead to changes at the transcriptional level and subsequently protein activities, which could cause normal cellular activities to be compromised. Understanding how mutations disrupt oxidative phosphorylation and the cellular changes this produces, may provide an insight in to how neurons enter a state of degeneration.

Reduced coupled respiration indicates the mitochondria are less efficient, reduced coupling between electron transport and proton extrusion subsequently impacts mitochondrial oxygen utilisation. When oxygen is consumed at complex IV, some of it is incompletely reduced forming the superoxide anion (O_2^-) during normal respiration. A reduction in the efficiency of the ETC and subsequent reduction in coupling efficiency can lead to increased unpaired electrons escaping the respiratory complexes. Redox reactions can lead to increased free radical production, elevating oxidative stress and subsequent molecular damage (Sas*, et al.* 2007). During neurodegenerative disease, impairment in neural energy metabolism accompanied by increased oxidative stress can have several consequences that potentially contribute towards cellular degeneration. Glutamate-mediated disturbances in ion concentrations, excitotoxicity, increased macromolecular damage, and elevated intracellular calcium, would disrupt multiple cellular mechanisms leading to functional decline.

Intracellular and extracellular conditions impact glycolytic flux. A reduction in glycolytic flux as seen here for the G93A mutant cells suggests that enzymatic determinants of glycolytic metabolism or pathway intermediates of glycolysis are affected in cells carrying this mutation, implicating value in investigating changes in specific intermediary metabolites. An increase in glycolytic flux is necessary to meet energy demands and protect cells from oxidative stress induced death during periods of mitochondrial dysfunction. Our previous microarray study revealed that G93A mutant *SOD1* transgenic

mice have an up-regulation in the expression of genes involved in respiratory chain function at presymptomatic stages of disease (Ferraiuolo*, et al.* 2007). This supports the concept that cells carrying the mutation try to compensate for the increase in oxidative stress and reduction in respiratory chain efficiency, by altering their transcriptome. The ability of cells to meet their energy demand by increasing glycolytic flux may be crucial to their survival under compromising conditions, and may explain the resistance of G37R mutant cells to cell death when exposed to oxidative stress. We have also shown in a previous study the detrimental effect on cell viability of inhibiting glycolysis in the G93A mutant NSC34 cells (Menzies*, et al.* 2002). Cells, which are able to meet their energy demands through glycolysis, persist in a state of oxidative stress, which is likely to result in changes in their transcriptome (Bolanos*, et al.* 2010).

The effect of oxidative stress on the bioenergetic profile of the cells was investigated and significant differences in measurements of ECAR and OCR were observed between the mutant-transfected cell lines, again reflecting differences between mutations. These assays were performed under sub-lethal stress conditions (50μM to 200μM H_2O_2 for one hour). Treatment with similar or lower doses for longer periods (although not severe enough to induce cell death) may show different responses of the mutants over time. Overall the data suggest that overexpression of the *SOD1* G93A mutation renders the neuronal cells not only more susceptible than controls to oxidative stress in terms of cell survival (see previous chapter), but in terms of increased susceptibility to perturbations of mitochondrial respiration and glycolytic metabolism, since this was the only mutation to show significant reductions in ECAR and OCR after H_2O_2 treatment.

Previous work using the NSC34 cell model identified significant reduction in the activity of complex II and IV of the mitochondrial respiratory chain in cells transfected with G93A or G37R mutant *SOD1*, in comparison to control vector-only cells (Menzies*, et al.* 2002). No significant differences were seen for complex I and III activity. Defects in the mitochondrial membrane potential in G93A *SOD1* transfected SH-SY5Y cells have also been observed (Carri*, et al.* 1997). However, another study found the activities of mitochondrial ATP synthesis, cytochrome c oxidase, and citrate synthase were unchanged in cells expressing G93A or G85R mutant *SOD1* in comparison to control cells (Magrane*, et al.* 2009), indicating not only the variability of using cell models to study disease but also the complex multi-factorial nature of the disease and how multiple factors are likely to influence functional capacity. The lack of significant reduction in total mitochondrial respiration observed in this study may be due to the fact that we assessed mitochondrial function in real time using intact cells, which gives greater physiological relevance.

Assessing isolated mitochondrial complexes lacks cellular context, our approach gives a more accurate reflection of mitochondrial dysfunction within the cellular environment. Our results agree with both *in vitro* and *in vivo* studies showing that wild-type human SOD1 reduces mitochondrial activity under basal conditions, suggesting over-expression of normal human SOD1 may lead to alterations in mitochondrial function (Jaarsma*, et al.* 2000, Menzies*, et al.* 2002).

Studies have shown an accumulation of oxidative damage to mtDNA during normal ageing and neurodegenerative disease including ALS (Ma*, et al.* 2009). Changes to mitochondrial gene expression cause with transcription and the electron transport chain leading to subsequent increases in oxidative stress and physiological decline (Dranka*, et al.* 2011). Reduced mitochondrial metabolism has been demonstrated in the brain and spinal cord of transgenic mice expressing familial ALS-linked G93A mutant *SOD1* at the onset of disease, supporting the contribution of mitochondrial abnormalities to ALS pathogenesis (Kong and Xu 1998, Mattiazzi*, et al.* 2002). The coordinated expression of mitochondrial and nuclear genomes for respiratory complex biogenesis introduces another point of damage that could potentially affect respiratory efficiency (Lane 2011). Respiratory rate is modulated by transcriptional control, translational regulation, protein stability and phosphorylation, and disruption to any of these processes may affect mitochondrial function due to reduced biogenesis. Although ALS is not primarily a mitochondrial disease, investigating its function in disease models is important, as defects/mutations affecting this organelle are likely to impact other cellular processes underlying pathogenesis (Mammucari and Rizzuto 2010).

NSC34 cells are generated through somatic fusion of murine neuroblastoma and motor neuron-enriched (embryonic day 12–14) spinal cord cells (Cashman*, et al.* 1992). These transformed cells are likely to have different characteristics from the parental cells, including signaling and metabolic differences. Neuroblastoma cells reprogram their metabolism to support rapid metastatic growth, which must be considered when using these lines to study disease mechanisms. Although mutant *SOD1* transfected NSC34 cells are not the perfect model for ALS, they enable study into the potential mechanisms that underlie disease pathogenesis and how neurons carrying these mutations become susceptible to degeneration, insights post-mortem tissue can not provide. These cells are economical compared to rodent models of disease and are a resource for testing potential therapeutics.

For further work investigating mitochondrial bioenergetics in ALS, isolating primary viable cells or mitochondria from transgenic murine models of disease would allow investigation of mitochondrial function during disease progression. Mitochondrial function may be increased transiently at presymptomatic stage of disease as a compensatory mechanism (Ferraiuolo*, et al.* 2007). Serum withdrawal has been shown to cause oxidative stress in NSC34 cells (Cookson*, et al.* 1998). Investigating mitochondrial function after serum withdrawal would determine whether the addition of serum affects mitochondrial function, and whether there are differences between the mutations. In the experiments here, cells are subject to an hour of serum withdrawal after being subject to oxidative stress and before being read by the Seahorse Bioanalyser. This gives a period when cells may start to recover from the stress, potentially explaining why fewer significant differences are seen in OCR and ECAR after oxidative stress exposure. H_2O_2 treatment of cells whilst they are in the bioanalyser would allow the immediate effects of stress on mitochondrial dysfunction to be assessed over a range of time and concentrations simultaneously. Another approach might be to assess mitochondrial function in cells exposed to oxidative stress after a predetermined recovery period. This approach allows for longer treatment time periods that can be associated with additional experimental endpoints such as cell death. The cells would be treated in their normal culture medium and incubated under normal conditions, avoiding potential artifacts due to altered cell culture conditions. Investigating differences in mitochondrial gene expression or protein levels, by RT-qPCR and Western blotting respectively, may give an insight into what is happening at the molecular level and enable further investigation into the mechanisms underlying selective vulnerability to oxidative stress and mitochondrial dysfunction.

5.3.2 Mitochondrial morphology in an in vitro model of ALS

Mitochondria are dynamic organelles, forming interconnected networks to facilitate cellular energy demand. Neuronal survival critically depends on the integrity and functionality of mitochondria. Mitochondrial morphology and function is maintained through fission and fusion, and disrupting the balance of the processes can result in mitochondrial fragmentation, elongation, or aggregation (Chan 2006). Studies in *in vitro* and *in vivo* models of ALS have revealed abnormal mitochondrial clustering in the axons of human mutant *SOD1* transgenic mice, and extensive fragmentation of mitochondria in

cultured NSC34 cells expressing human mutant *SOD1* (Menzies*, et al.* 2002, Raimondi*, et al.* 2006, Sotelo-Silveira*, et al.* 2009).

Mitochondrial morphology was assessed using rhodamine 123, which is a specific probe frequently used for the localisation of mitochondria within live intact cells (Johnson 1980). The Mito-Morphology macro (Dagda 2009) was used for the analysis, and provides measurements of mitochondrial number, area, perimeter, average circularity, and average area/perimeter ratios. In this study, we identified increased interconnectivity under basal conditions within NSC34 cells transfected with human G93A mutant *SOD1* in comparison to the controls and other mutations investigated. Networking of the mitochondria has been shown to potentially represent an adaptive mechanism, allowing them to function more efficiently to deal with cellular stress (Koopman*, et al.* 2005, Mortiboys*, et al.* 2008). The G93A mutation was also previously reported to be the most susceptible to oxidative stress related cellular decline as identified by cell viability and LDH assays (chapter 4). The results from this study suggest the G93A mutation may increase its mitochondrial interconnectivity to compensate for an energy deficit. Although the basal oxygen consumption rate was only significantly reduced in comparison to the G37R mutation and the reduction in spare respiratory capacity did not reach significance, a significant reduction was seen in the coupling efficiency for the G93A mutation cells, suggesting the mitochondria are dysfunctional under basal conditions. The analysis indicates the mutation predominantly affects mitochondrial morphology under basal conditions. The change in morphology may allow the cells to compensate, and therefore the differences seen in terms of mitochondrial bioenergetics were only slightly reduced in comparison to the controls and the other mutations investigated. Previous studies have demonstrated mitochondrial fragmentation facilitates the release of cytochrome c and therefore promotes activation of the apoptotic pathway. When cells are subject to modest levels of stress, the mitochondria fuse to form a closed network, which is referred to as stress-induced mitochondrial hyperfusion (SIMH) (van der Bliek 2009). This potentially allows cells to counter the stress by optimising their ATP production, providing transient protection against apoptosis and mitophagy (Gomes and Scorrano 2011, Rambold*, et al.* 2011, Tondera*, et al.* 2009), which potentially is what is happening in the cells carrying the G93A mutation. However, the mechanisms mediating this response remain poorly understood.

Following exposure to oxidative stress, the G93A mutation showed significantly reduced mitochondrial interconnectivity in comparison to the pIRES control and itself under basal conditions. This correlated with a significant reduction in basal oxygen consumption and

extracellular acidification rate following exposure to the same stress. Cell viability assays revealed this level of stress to be sublethal, further indicating the effect this mutation has on the cells in terms of vulnerability to oxidative stress related dysfunction. A loss of mitochondrial connectivity, accompanied by the formation of punctate mitochondria has been demonstrated under conditions of mitochondrial dysfunction (De Vos*, et al.* 2005, Karbowski and Youle 2003). This study supports previous work demonstrating stress conditions and changes in energy source can induce significant mitochondrial morphological changes (Tondera*, et al.* 2009). The change in morphology following exposure to stress suggests the G93A mutant cells are no longer able to compensate for the energy deficit and therefore induce fragmentation. Changes in mitochondrial cristae and mitochondrial fragmentation, are known to have a vital role in apoptosis (Youle and Karbowski 2005).

Here we have applied a novel technique to investigate mitochondrial function within intact motor neuronal NSC34 cells under basal and oxidative stress conditions. Differences in cellular metabolic and bioenergetics function between the mutations are consistent with the differences observed in viability. The G93A mutation was the most susceptible to oxidative stress in terms of cell survival and showed significantly lower OCR, spare respiratory capacity and mitochondrial respiration in comparison to the G37R mutation, which consequently was the least susceptible to oxidative stress under the conditions investigated. Additionally the G93A mutation was the only cell type to show significant changes in OCR under stress conditions. The H48Q mutation lay between the G93A and G37R mutations in relation to mitochondrial bioenergetic capacity and susceptibility to oxidative stress. The susceptibility of the G93A mutation to oxidative stress induced cell death and mitochondrial dysfunction may be a result of the increased toxicity of this mutation (Section 4.6.2). In addition to this, G93A mutant *SOD1* have been shown to bind mitochondria within the spinal cord of transgenic mouse models of disease, forming high molecular weight aggregates, which are subsequently bound by apoptotic regulator Bcl-2 (Pasinelli*, et al.* 2004). The sequestration of Bcl-2 by mutant SOD1 aggregates will render the protein non-functional, and inhibition of Bcl-2 binding to pro-apoptotic proteins may reduce cellular viability. Bcl-2 is also important for maintaining the mitochondrial membrane potential (Danial and Korsmeyer 2004), therefore its sequestration in to SOD1 aggregates may lead to disruption of the mitochondrial membrane potential. G37R SOD1 were also shown to bind Bcl-2, but to a much lesser degree (Pasinelli*, et al.* 2004).

This work contributes to the growing field of mitochondrial bioenergetic dysfunction in motor neuron disease and further work should focus on the underlying mechanisms by which these changes occur.

Chapter 6
Discussion

ALS involves a progressive neuronopathy, which characteristically results in respiratory failure as the cause of death for most patients. Many of the processes implicated in ALS are mechanisms common to a range of age-associated neurological disorders. However, despite extensive research into these pathways, significant hurdles remain in the discovery of therapeutics, due to the multi-factorial nature of disease aetiology and its clinical heterogeneity. Up to 20% of familial cases of ALS have been linked to mutations in the Cu/Zn superoxide dismutase-1 (*SOD1*) gene, and *in vitro* and *in vivo* models harbouring a human SOD1 mutation have been widely used to study the pathogenic mechanisms. More recently a substantial proportion of fALS cases have been linked to an expansion of the intronic hexanucleotide repeat sequence in *C9ORF72*. The identification of this in sALS patients, potentially allows for greater understanding of common mechanisms underlying pathogenesis in sALS and fALS patients, and providing new models for disease study. An important feature of this mutation is that it seems to reinforce the role of RNA processing in the ALS disease process.

6.1 Gene expression profiling of an oxidised fraction of RNA during ageing

A priority in neurodegenerative research is to understand the interactions of neuronal cell types, and how this interaction and their cellular function changes during normal ageing and disease. Oxidative stress is one common mechanism long associated with ageing and age-associated neurodegenerative disorders. Investigation has focused on the interplay of oxidative stress and other cellular mechanisms proposed to underlie neurodegeneration, including how oxidative modification to proteins, lipids, and nucleic acids may contribute towards the pathology of neurodegenerative disease. A recent study of the human G93A mutant *SOD1* transgenic mouse model of fALS revealed prominent oxidative modification to RNA within the cytoplasm of MNs at presymptomatic stage disease. Further to this select classes of RNA were shown selectively targeted for modification (Chang*, et al.* 2008). Although progressive neuronal loss is a hallmark of neurodegenerative disorders, the pathways and dysfunction identified in disease may reflect an acceleration of the functional impairments identified in ageing. The first aim of our study was to determine whether selective oxidative modification of RNA was a

feature of normal ageing, and for this we analysed the expression profile of an oxidised fraction of RNA from the anterior horn of mice aged six, twelve, and eighteen months. The progression of ageing and neurodegenerative disease can be investigated using model organisms, which makes them extremely valuable for genetic research. Mouse models are commonly used because of their genetic and physiological similarities to humans. Another advantage of using mouse models is the ability to specifically manipulate the mouse genome to create specific genetic changes that are of interest to study.

We identified a change in the number of genes and the enrichment classes of genes differentially expressed in the oxidised RNA fraction during ageing. This may represent the gene expression changes during normal development of the anterior horn, reflecting changes in the requirements of the cell as an organism ages. The increased oxidation of certain transcripts may be due to their availability for modification, or it may be that certain transcript features predispose to selective modification. A significant increase in the length of the transcript and number of exons of genes in the oxidised fraction in comparison to those in the non-oxidised fraction at six and twelve months of age were identified, suggesting these features may influence modification frequency. mRNA abundance has been found to correlate with transcript half-life, however comparison of the half-life for genes identified in the oxidised and non-oxidised fractions in our study revealed no significant differences in terms of mRNA half-life and susceptibility to oxidation. This suggests that while the abundance and presence of a transcript in a cell may affect the opportunity for it to be oxidatively modified, other factors also govern the selective targeting for RNA oxidation.

RNA oxidation has been identified in a wide range of diseases, and evidence suggests the modification and the consequent loss of integrity of RNA is a mechanism of neurodegenerative pathology. In addition, disturbances to RNA processing and metabolism is becoming increasingly evident in ALS, and other neurodegenerative diseases. The expansion of understanding for these mechanisms, along with the identification of numerous regulatory ncRNAs has established a multitude of targets that could lead to cellular decline if their function is dysregulated. We are interested in disturbances to RNA processing and metabolism because of the association with ALS pathogenesis, and interestingly genes identified to be specifically targeted for oxidative modification to their transcript in our study have been associated with mechanisms that have been reported to be dysregulated in ALS. The enrichment of specific groups of genes involved in transcriptional regulation, including transcriptional coactivators and chromatin modifiers, and RNA processing in the oxidised fraction were identified at six,

twelve, and eighteen months. The analysis was subjective in that our focus centred on genes in the oxidised fraction involved in these processes at each age, but other enrichment classes were also identified. Age-related changes in gene expression are associated with insults from endogenous and exogenous factors, and reduced synthesis of transcription factors has been demonstrated (Roy 1997), which may explain the differences in the transcripts targeted for oxidation at each age. Oxidative modification to the RNA transcript may impact on the downstream function of the protein if translational fidelity is affected by the damage. Aberrant alternative splicing or mRNA processing may result from the presence of a modified base. A reduction in transcription factor/co-factor availability would lead to reduced activation/repression of target genes and subsequent downstream changes in expression, potentially causing extensive disruption to normal cellular functioning.

Whether RNA modification and dysfunction is a primary mechanism underlying disease pathogenesis or is a consequence of increased oxidative stress and other disruptions to cellular functioning remains unknown. Prominent neuronal RNA oxidation was identified at presymptomatic stage in an *in vivo* model of ALS, suggesting an early involvement in pathogenesis (Chang*, et al.* 2008). Investigating RNA oxidation in ageing found it to be prevalent during later life, suggesting an accumulation of damage during normal ageing without pathogenic consequences (Nunomura*, et al.* 2012). In this study we identify selective oxidative modification of RNA during normal ageing, with notable transcripts modified at a young age (six months). Although this could be a cellular regulatory mechanism related to a cellular pruning process or a consequence of the availability of the transcript for modification, further work to identify whether targeting of select transcripts for modification may predispose an individual to a neurodegenerative disease. An important area for research is translating whether what happens in pathology is recapitulated in ageing, and the factors determining the transition from normal ageing to neurodegeneration.

Many processes act to regulate RNA stability to coordinate gene expression. RNA oxidation may be another mechanism acting to control mRNA abundance within a cell. Oxidants are known as important regulators of signalling, including the activation/inactivation of various transcription factors. Like ROS, at low levels, oxidised guanine derivatives and the modification of transcipts could have signalling/regulatory properties, but be detrimental after reaching a certain threshold, for example in neurodegenerative disease when oxidative stress is increased and the frequency of modification is likely to increase. This supports the differences seen between our ageing

study, and the study investigating RNA oxidation in ALS (Chang*, et al.* 2008). The targeting of transcripts for modification may be more selective during ageing, with widespread damage occurring during disease.

Although gene expression profiling studies have sought to identify common genes and pathway changes that occur in ageing and specific neurodegenerative disease, the perturbations that lead to pathology remains largely unknown. The changes in gene expression during ageing and disease may be the result of numerous changes including, transcriptional control, RNA processing, protein turnover, making it difficult to identify the causative factor due to the multi-factorial nature of normal ageing and neurodegenerative disease. The changes in nuclear architecture during ageing and disease are likely to impact transcriptional fidelity, which may result from oxidative modification to the RNA transcript as identified here for multiple genes involved in transcriptional regulation. To determine whether this damage does impact transcriptional fidelity, investigation of downstream genes and proteins is needed. It would be interesting to see whether the oxidation of RNA during ageing significantly alters the protein expression for the specific gene, as previously described for *in vitro* models. Interesting questions also remain surrounding the oxidative modification of ncRNAs, and whether selective modification of these alters their function and potentially promotes neurodegeneration.

6.2 The impact of *SOD1* mutations on cellular function

Oxidative stress has been extensively studied with relation to ALS pathogenesis since the identification of a mutation in the antioxidant enzyme *SOD1*. Over 150 mutations in *SOD1* have been identified, which are heterogeneous and specific mutations may have different cellular effects. In our study we wanted to further our investigation to determine whether *SOD1* mutations influence the level of RNA oxidation, and whether differences are seen in terms of oxidative stress related cellular decline. For this we used an *in vitro* model of familial ALS.

Model systems are used to investigate oxidative stress related defects and understand how these neurotoxic effects lead to cellular decline and degeneration. In our study we investigated NSC34 cells stably transfected with the G93A, G37R, and H48Q human *SOD1* mutations. The H48Q and G93A mutations demonstrated increased vulnerability to oxidative stress related mitochondrial dysfunction and cell death. This was pronounced in the G93A mutant cells, which were susceptible to lower doses of stress. These

experimental findings may reflect differences observed clinically between *SOD1* mutation-types in ALS patients. In human ALS patients, the G37R mutation displays an earlier onset of disease but has a longer disease progression in comparison to the G93A mutation (Cudkowicz*, et al.* 1997). Here we observed that the G37R mutant cells are not greatly affected in terms of viability in response to oxidative stress, suggesting the mutant transgene confers greater resistance to this insult in comparison to the other mutations. The H48Q mutation displays a later disease onset in ALS patients but the clinical course is rapidly progressive with a much shorter duration of disease compared to the other two mutations investigated here (Orrell*, et al.* 1997, Orrell*, et al.* 1999). In terms of cell viability the H48Q mutant NSC34 cells were gradually more susceptible to oxidative stress until a certain level of insult when a rapid induction in cell death was observed. Progression of disease in patients harboring the G93A mutation is relatively rapid with typical survival of two to five years (Radunovic and Leigh 1996). Variations between the individual mutations likely underlie the differences seen in susceptibility to oxidative stress in these experiments. This phenotypic heterogeneity is not unusual between patients with different *SOD1* mutations (Battistini*, et al.* 2005), adding further complexity to studying the pathology of the disease.

The investigation of mitochondrial morphology and function within these cells was performed under basal conditions and following exposure to sub-lethal oxidative stress, as determined by cell viability assays. The G93A mutation, although showing no cell death at this dose, had reduced mitochondrial oxygen consumption and displayed significant changes in mitochondrial morphology in comparison to the WTSOD1 control and other mutations investigated. The G93A mutation had increased mitochondrial interconnectivity under basal conditions, suggesting the formation of mitochondrial networks as a compensatory mechanism to counteract an energy deficit within these cells. The loss of this interconnectivity following exposure to stress suggests the cells can no longer compensate, and the mitochondria become more fragmented. The H48Q and G37R mutation displayed no significant changes in mitochondrial morphology and function following exposure to oxidative stress. Interestingly, the G37R mutation showed the greatest resistance to cellular stress in terms of cellular viability and mitochondrial function, demonstrating the different effects these mutations have on cells. This correlates with the previous results; the G93A mutation is more susceptible to oxidative stress induced cellular stress in comparison to the other mutations investigated, which is reflected in its reduced mitochondrial bioenergetics under both basal and stress conditions. This was in comparison to the G37R mutation, which appeared the least susceptible, and demonstrated significantly greater oligomycin sensitive respiration,

coupling efficiency, and mitochondrial respiration. The differences identified between mutations also correlates with what is presented in patients carrying different *SOD1* mutations.

A study investigating the correlation between the propensity for aggregation and conformational stability of SOD1 showed G93A to have the highest conformational instability, and was therefore more prone to aggregation, in comparison to other SOD1 mutations, including G37R (Stathopulos*, et al.* 2003). The G37R mutation was also shown to be less prone to forming insoluble aggregates. This may be part of the reason why the G93A mutation appeared more toxic in our study.

Although the level of expression of the mutant *SOD1* transgenes was equivalent at the transcription level, reduced protein expression for the G93A and H48Q mutation was observed in these cells, suggesting defects at the translational level. These results confirm the cellular vulnerability to oxidative stress observed is not simply a consequence of over-expression of the mutant protein. The G93A and H48Q mutant cells had significantly increased levels of RNA oxidation in comparison to the controls. This suggests the reduction in human SOD1 protein from these cells could be a consequence of increased RNA damage and a subsequent reduction in translation.

To determine whether the presence of a *SOD1* mutation affects the ability of a cell to activate oxidative stress response mechanisms, levels of antioxidant response genes were measured under basal conditions and following exposure to H_2O_2. Expression levels of *Nrf2* were reduced in all mutant cells under basal conditions, and a significant reduction was seen for the G93A mutation. The reduction seen for the cells carrying WTSOD1 suggests the presence of human *SOD1* also impacts cellular functions. Since no significant differences were seen for the levels of the antioxidant response genes investigated here between the *SOD1* mutations, other mechanisms must be contributing towards the selective vulnerability of the G93A mutation to stress-related cell death and mitochondrial function. Expression of the DNA repair enzyme *Ogg1* was significantly increased in the G93A mutant cells in comparison to the H48Q mutant cells. Both these mutations demonstrated increased susceptibility to stress and RNA oxidation, which would suggest an inability of these cells to up-regulate defence mechanisms. However the H48Q mutation demonstrated the greatest increase in *Ogg1* expression following exposure to oxidative stress, which may explain why the G93A mutant cells are susceptible at the lower doses in comparison.

Model systems are used to investigate oxidative stress related defects and understand how these neurotoxic effects lead to cellular decline and degeneration. The differences identified between the mutations in terms of their susceptibility to stress and mitochondrial dysfunction in our study highlights the importance of using multiple models of disease for study. The short lifespan of free radicals and the difficulty in their detection requires indirect markers, such as macromolecular damage and antioxidant response are used for oxidative stress detection (Floyd and Hensley 2002). For future work it would be interesting to determine whether the effects seen here are exacerbated or reduced when the cells are grown in co-culture with normal or human mutant *SOD1* astrocytes, and whether they differ to what is seen in isolated primary MNs from *in vivo* models of disease. Co-culture systems are often used to try and recapitulate the environment of neurons *in vivo.* The lack of significant differences observed in the investigations here could be due to the increased variability between different cultures, or because of a cell culture effect where cells have adapted their survival to the culture conditions. Despite this cell culture studies have enabled significant enhancement of our knowledge regarding the mechanisms underlying neurodegenerative disease. Whilst the factors pre-disposing to a neurodegenerative disease remain unknown, these remain important tools for investigating the events culminating in MN cell death and investigating potential therapeutic mechanisms.

The hypothesis that increased oxidative stress and nucleic acid oxidation contributes towards cellular decline and degeneration in ageing and ALS was investigated, demonstrating specific classes of RNA are targeted for oxidative modification during ageing. The differentially oxidised genes were found enriched in pathways previously implicated in neurodegeneration. Investigating whether the presence of different *SOD1* mutations influences the amount of oxidative damage to RNA identified an increase in RNA oxidation in cells carrying *SOD1* mutations in comparison to controls. Differences in the effects of the *SOD1* mutations on cellular function was identified with the G93A mutation demonstrating increased susceptibility to oxidative stress related mitochondrial dysfunction and cellular viability.

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