The role of ALS associated genes and c-Jun in neurodegeneration

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Declaration

I, Alejandro David Acosta Saltos, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: Date:

Abstract

The molecular pathways mediating motor neuron degeneration in Amyotrophic lateral sclerosis and axonal regeneration following peripheral nerve injury remain elusive.

The transcription factor c-Jun, an important orchestrator of axonal regeneration, has been found upregulated in the spinal cord of ALS cases and the SOD1G93A mouse model of the disease. However the functional role of c-Jun in ALS degeneration has not been defined. The current thesis demonstrates that genetic deletion of c-Jun from neurons and neuroglia prolonged the lifespan of SOD1G93A mice. Associated with this effect on survival, motor neuron loss, axonal loss and neuroinflammation were attenuated in c-Jun deficient SOD1G93A mice. Interestingly, in addition to the neurodegenerative changes, motor neuron pools affected by ALS disease in SOD1G93A mice demonstrated a disease-mediated axotomy response, which was characterised by the expression of regeneration associated proteins and was dependent on the expression of c-Jun.

Since the expression of regeneration-associated proteins changes in ALS, it was next investigated whether the expression of ALS-associated proteins changes during axonal regeneration. This thesis demonstrates that the pattern of expression of ALS associated proteins; TDP-43, FUS and alsin, does indeed change within motor neurons following facial nerve axotomy, in wild type mice. In addition, it describes for the first time that alsin protein may localise to the cellular nucleus in uninjured motor neurons. Interestingly, axotomy resulted in a transient clearing of the normal nuclear immunoreactivity for all three studied proteins. The size of this effect was dependent on the severity of nerve injury for TDP-43 and alsin.

Finally, to begin to investigate the functional role of ALS associated genes in axonal regeneration the A315T TDP-43 model of ALS was assessed following facial nerve transection. Genetic overexpression of ALS associated A315T mutant TDP-43 resulted in delayed motor function recovery following facial nerve axotomy in mice. This effect was associated with increased perineuronal inflammation and increased motor neuron expression of the adhesion molecule

CD44 but reduced perineuronal sprouting. However, overexpression of A315T mutant TDP-43 did not affect anatomical target reinnervation, motor neurons loss or the pattern of expression of other regeneration associate proteins.

Thus, this thesis demonstrates that the molecular changes in ALS-associated motor neurodegeneration and peripheral nerve injury-associated regeneration overlap, and it suggests that insights obtained from studying one of these processes may be applied to further understand the other.

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and

Dr Francia Carolina Acosta Saltos.

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List of Abbreviations

Activating transcription factor 3	ATF-3
Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	AMPA
Amyotrophic lateral sclerosis	ALS
Antioxidant response element	ARE
Autosomal dominant	AD
Basophilic inclusions	BI
Beta2-microglobulin	β2-MG
Brain-derived neurotrophic factor	BDNF
Bunina bodies	BB
Calcitonin gene-related peptide	CGRP
Camp response element-binding	CREB
Catalytic subunit of glutamate cysteine ligase	GCLC
CCAAT/enhancer binding protein	C/EBP
Ciliary neurotrophic factor	CNTF
C-terminal fragments	CTF
Cytoskeleton-associated protein 23	CAP23
Damage-induced neuronal endopeptidase	DINE
Differentially expressed in normal and neoplasia	DENN
Endoplasmic reticulum	ER
End-stage	ES
Excitatory amino-acid transporter	EAAT
Extracellular signal-regulated kinase	ERK
Facial motor nucleus	FMN

Familial Amyotrophic lateral sclerosis	FALS
Fas-Associated protein with Death Domain	FADD
Fibroblast growth factor 4	FGF4
Frontotemporal dementia	FTD
Frontotemporal dementia with ubiquitin-positive inclusions	FTD-U
Frontotemporal dementia tau-positive inclusions	FTD-tau
Fused in Sarcoma	FUS
Galanin, and pituitary adenylate cyclase activating peptide	PACAP
Glial cell line-derived neurotrophic factor	GDNF
Glial fibrillary acid protein	GFAP
Glutamate receptor interacting protein 1	GRIP1
Glutamate receptor subunit 2	GluR2
Granulocyte macrophage colony stimulating factor	GM-CSF
Growth associated protein-43	GAP-43
Guanine-nucleotide-exchange factor	GEF
Heat-shock proteins	HSPs
Heme oxygenase-1	HO-1
Heterogeneous nuclear ribonucleoprotein	hnRNP
Histone deacetylase 6	HDAC6
Immunohistochemistry	IHC
Inhibitor of apoptosis 1	IAP1
Insulin-like growth factor	IGF
Intercellular adhesion molecule 1	ICAM1
Interleukin-1	IL-1

Jun-N-teminal kinase	JNK
Knock down	KD
Knock in	KI
Knock out	KO
Leukemia inhibitory factor	LIF
Lewy body-like hyaline inclusions	LBHI
Macrophage colony stimulating factor	M-CSF
Major histocompatibility complex	MHC
Messenger Ribonucleic acid	mRNA
Metalloproteinases	MMP
Mitochondrial transcription factor A	TFAM
Mitogen-activated protein kinase	MAPK
Mitogen-associated protein kinase	MEK
Monoclonal antibodies	mABs
Morpholino oligo	MO
Myelin-associated glycoprotein	MAG
NAD(P)H: quinone oxidoreductase 1	NQO1
Nephroblastoma overexpressed	NOV
Neuromuscular junction	NMJ
Nicotinamide adenine dinucleotide phosphate-oxidase	NADPH
Nitric oxide	NO
Nuclear erythroid 2-related factor 2	Nrf2
Nuclear export signal	NES
Nuclear factor interleukin 3-regulated	NFIL3

Nuclear factor kappa B	NF-kappa B
Nuclear localization signal	NLS
Oligodendrocyte-myelin glycoprotein	OMGP
Phosphorylated eukaryotic initiation factor 2alpha	Pi-eIF2 alpha
Plasminogen activator	PA
Programmed cell death	PCD
Protein disulfide isomerase	PDI
Reactive oxygen species	ROS
Regulator of chromatin condensation like domain	RLD
Regulator of chromosome condensation 1	RCC1
Rhoa-associated kinase	ROCK
Rounded hyaline inclusion	RHI
Sex-determining region Y box-containing 11	Sox11
Short interfering Ribonucleic acid	siRNA
Signal transducer and activator of transcription 3	STAT3
Skein-like inclusion	SI
Standard deviation	SD
Standard error of the mean	SEM
Stress granules	SG
Superoxide dismutase 1	SOD1
Transactive response DNA binding protein 43 kda	TDP-43
Transforming growth factor-beta 1	TGFb1
Transgenic	Tg
Tumor necrosis factor	TNF

Ubiquitin-proteasomal system	UPS
Unfolded protein response	UPR
Valosin-containing protein	VCP
Vascular endothelial growth factor	VEGF
Wild-type	WT
X-linked dominant	XD

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Chapter 1: Introduction

ALS

Clinical features

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects motor neurons in the spinal cord, brainstem and brain. The combined degeneration of lower and upper motor neurons results in progressive muscle atrophy, weakness, fasciculations and spasticity (Swinnen and Robberecht, 2014). ALS has an incidence of around 2 per 100, 000 per year and a prevalence of around 5 per 100, 000 (Chio et al., 2013). Although highly variable, the onset of ALS clinical symptoms and signs occurs typically after the fifth decade of life. However, juvenile ALS can also occur with an onset age below 25 years, but this is rare (Sabatelli et al., 2008). Patients suffering from ALS generally die around 3 to 4 years from diagnosis. However, the progression of the disease is variable, with some patients living longer than 10 years and others dying within 12 months (Pupillo et al., 2014). Survival can be prolonged by riluzole, the only approved medication for the treatment of ALS. However, its effects are modest, prolonging lifespan by around two months (Miller et al., 2007).

Neuropathology

Post mortem examination demonstrates that ALS is characterised by degenerative changes in all levels of the neuromuscular unit. There is motor neuron cell body atrophy and loss, and the presence of intracellular proteinaceous inclusion bodies. Axonal degeneration can be observed in motor nerves and the corticospinal tract. In addition, neuromuscular junction denervation and associated muscle atrophy are also features of the disease (Swinnen and Robberecht, 2014). All of these degenerative changes are associated with surrounding neuroinflammation (Philips and Robberecht, 2011).

Genetics

Most cases (90-95%) of adult onset ALS occur sporadically without an apparent cause (SALS). Putative risk factors have been identified and include age, smoking, contact with pesticides and insecticides, soldering and welding work, high-fat/low fibre diet, slimness and athleticism, and living in urban areas. However, evidence for any of these risk factors is not conclusive (Longo et al., 2012). Aside from the sporadic form, it is estimated that around 5% of ALS cases are familial (FALS), with a genetic cause driving the disease (Byrne et al., 2011). A large, and growing, number of genes associated with FALS have been identified. The genes responsible for the majority of FALS cases are *C9orf72* (causing around 40% of FALS), *SOD1* (12%), *TARDBP* (4%) and *FUS* (4%) (Renton et al., 2014). Other implicated genes are each associated with only a few cases of FALS; these include *VAPB*, *FIG4*, *CHMP2B*, *OPTN*, *DAO*, *VCP*, *UBQLN2*, *SQSTM1* and *PFN1*. In addition, the rare juvenile form of ALS is linked with mutations in several others genes including, *ALS2*, *SETX*, *Spatacsin* and *SIGMAR1* (Byrne et al., 2011). Table 1 lists the different genes associated ALS, their chromosomal locus, inheritance pattern and putative cellular function.

Animal models of ALS

The elusive nature of the aetiology of ALS has driven the production of genetic animal models which express mutated genes associated with ALS. These encapsulate most of the clinical and neuropathological features of the disease. The use of these has led to the increasing understanding of the molecular and cellular processes driving ALS, and has provided a means of pre-clinical testing of desperately needed new therapies (Islam et al., 2014). Table 2 shows the different animal models of ALS, the genes, species used and type of genetic modifications.

Table 1. Genes implicated in the pathogenesis of ALS. AD, autosomal dominant; AR, autosomal recessive; XD, X-linked dominant; DENN, differentially expressed in normal and neoplasia. Modified from (Renton et al., 2014).

Gene	Location	Inheritance	Putative protein function
TARDBP	1p36	AD	RNA metabolism
SQSTM1	5q35	AD	Ubiquitination; autophagy
C9ORF72	9p21	AD	DENN protein
VCP	9p13	AD	Proteasome; vesicle trafficking
OPTN	10p13	AR and AD	Vesicle trafficking
FUS	16p11	AD and AR	RNA metabolism
PFN1	17p13	AD	Cytoskeletal dynamics
SOD1	21q22	AD and AR	Superoxide metabolism
UBQLN2	Xp11	XD	Proteasome
DCTN1	2p13	AD	Axonal transport
ALS2	2q33	AR	Vesicle trafficking
CHMP2B	3p11	AD	Vesicle trafficking
FIG4	6q21	AD and AR	Vesicle trafficking
HNRNPA2B1	7p15	AD	RNA metabolism
ELP3	8p21	Undefined	RNA metabolism
SETX	9q34	AD	RNA metabolism
HNRNPA1	12q13	AD	RNA metabolism
ATXN2	12q24	Undefined	Endocytosis; RNA translation
ANG	14q11	AD	Angiogenesis
SPG11	15q14	AR	DNA damage repair
VAPB	20q13	AD	Vesicle trafficking
NEFH	22q12	AD	Axonal transport

Table 2. Animal models of ALS. Genetic modification of ALS associated genes: KD, knock down; KI, knock in of ALS associated mutation into mouse gene; KO, knock out; MO, morpholino oligo inhibition of gene expression; Tg, transgenic for human gene with ALS associated mutation. Table modified from (Islam et al., 2014).

ALS Gene	Animal	Genetic modification
SOD1	Mouse	KO, Tg
	Swine	Tg
	Zebrafish	Tg
	Drosophila	Tg, KO
	C. elegans	Tg
TDP-43	Mouse	Tg
	Zebrafish	MO, RI
	Drosophila	Tg, KD
	C. elegans	Tg
FUS	Mouse	Tg
	Zebrafish	RI
	Drosophila	Tg
ALS2	Mouse	KO
	Zebrafish	MO
VAPB(P56S)	Mouse	KI
	Drosophila	Tg
DCTN1/p150 glued	Mouse	KI, Tg
	Drosophila	Tg
DCTN2/p50	Mouse	Tg
VCP	Mouse	KI
FIG4	Mouse	KO

Mechanisms of degeneration

Motor neuron death

Morphological changes of the motor neuron is ALS

Post-mortem histological assessment of ALS cases demonstrates that motor neurons undergo three morphologically distinct stages of degeneration (Martin, 1999). The chromatolytic stage is characterised by cell body swelling, dispersion of the Nissl substance (rough endoplasmic reticulum), displacement of the normally central nucleus to the cellular periphery, and the presence of cytoplasmic inclusion bodies. In the somatodendritic attrition stage, the cell body atrophies and takes a blurred multipolar shape, while the nucleus and cytoplasm take on a condensed homogenous appearance. In the apoptotic stage, the motor neuron takes a fusiform or round shape devoid of any processes, its diameter decreases to a fifth of its normal size and its cytoplasm and nucleus become extremely condensed (Martin, 1999). Interestingly, histopathological examination of human ALS cases analysis fail to demonstrate evidence of cytoplasmic vacuoles or nuclear and chromatin condensation which are normally associated with apoptosis (Martin, 1999).

Similar to changes in human cases, motor neurons in the widely used SOD1 mouse model of ALS demonstrate morphological evidence of chromatolysis (Sasaki et al., 2002) and atrophy. In addition, cytoplasmic vacuoles are found, which correspond to both intracellular organelle dilation (Dalcanto and Gurney, 1995) and the process of autophagy (Chen et al., 2012, Fidzianska et al., 2006). However, unlike ALS human tissue, changes which resemble apoptosis can be observed in the SOD1 mouse model. These changes include cytoplasmic and nuclear condensation, compaction of nuclear chromatin and cellular fragmentation of motor neurons. Nonetheless, morphological features of apoptosis are only observed infrequently (Przedborski, 2004).

Expression and role of death related proteins

Immunohistochemical studies have demonstrated that motor neurons in human ALS cases and mutant SOD1 mice can express a number of apoptosis related markers including the carbohydrate antigen Le^{Y} (Yoshiyama et al., 1994), Par-4 protein (Pedersen et al., 2000) and fractin (Vukosavic et al., 2000). DNA fragmentation, indicative of apoptosis, has also been detected in small

subpopulations of motor neurons of ALS cases and ALS-linked mutant SOD1 mice (Martin, 1999, Hetz et al., 2007). However, this has not been replicated by other groups (Migheli et al., 1994, He and Strong, 2000) and has not been observed in the SOD1 mouse model of ALS (Migheli et al., 1999).

As described above there is evidence that a type of programmed cell death (PCD) may be involved in the loss of motor neurons in ALS (Przedborski, 2004). PCD involves a cascade of molecular signals which may be initiated extrinsically, through death receptors, or intrinsically through the activation of cell death proteins through the mitochondrial apoptotic pathway (McIlwain et al., 2013).

There is evidence that ALS degeneration may be mediated, at least in part, via activation of death receptors on the surface of motor neurons. The TNF, Fas, DR6 and p75 death receptors, as well as their ligands, are all upregulated in ALS-linked mutant SOD1transgenic mice (Veglianese et al., 2006, Raoul et al., 2006, Huang et al., 2013, Kiaei et al., 2007, Bryson et al., 2012, Turner et al., 2003, Copray et al., 2003). In addition, DR6 and p75 receptors, and the cytokine TNF-alpha (TNF receptor ligand) are upregulated in the spinal cords of ALS patients (Huang et al., 2013, Moreau et al., 2005, Seeburger et al., 1993). Functionally, knocking down Fas or p75 receptors using an antisense peptide nucleic acids delayed disease and reduced motor neuron cell loss in ALS SOD1 transgenic mice (Turner et al., 2003, Locatelli et al., 2007). Similarly, pharmacological inhibition of DR6 in mutant SOD1 mice reduced pathology in mutant SOD1 mice (Huang et al., 2013). However, deletion of the TNF-alpha gene did not affect motor neuron disease in ALS SOD1 transgenic mice (Gowing et al., 2006).

Changes in expression and activation of members of Bcl-2 family of cell death proteins have been associated with ALS disease. On the one hand, the pro-apoptotic proteins Puma, Bim, Bid, Bad and Bax have all been found upregulated or activated in mutant SOD1 mice (Kieran et al., 2007, Hetz et al., 2007, Guegan et al., 2002, Vukosavic et al., 1999, Guegan et al., 2001). In addition, Harakiri, Puma, Bak and Bax have are also upregulated or activated in ALS cases (Shinoe et al., 2001, Kieran et al., 2007, Martin, 1999, Mu et al.,

1996). On the other hand, the anti-apoptotic proteins Bcl-2 and Bcl-xL are down-regulated but Bcl-2a1 is upregulated in mutant SOD1 mice (Vukosavic et al., 1999, de Aguilar et al., 2000, Crosio et al., 2006). In post mortem ALS cases, Bcl-2 is also reduced but Bcl-XL expression is unchanged (Mu et al., 1996, Martin, 1999). Interestingly, mutant SOD1 protein has been found to interact directly with Bcl-2 protein and it has been suggested that it interferes with the anti-apoptotic function of Bcl-2 in both ALS cases and mutant SOD1 mice (Pasinelli et al., 2004, Pedrini et al., 2010). Functionally, genetic deletion of pro-apoptotic genes Puma or Bim slows down disease and reduces motor neuron loss in ALS mutant SOD1 mice (Kieran et al., 2007, Hetz et al., 2007). Furthermore, genetic deletion of Bax abolishes motor neuron loss and increases survival in ALS SOD1 mutant mice (Gould et al., 2006). Functionally, overexpression of anti-apoptotic genes Bcl-2 or Bcl-xL mitigates neurodegeneration and prolongs survival of ALS SOD1 mice (Kostic et al., 1997, Vukosavic et al., 2000, Yamashita et al., 2003, Azzouz et al., 2000, Garrity-Moses et al., 2005).

The pattern of expression of mitochondrial proteins involved in apoptosis is also changed in ALS. Cytoplasmic translocation of the pro-apoptotic protein cytochrome C has been found in ALS cases and mutant SOD1 mice (Martin, 1999, Guegan et al., 2001). In contrast, the anti-apoptotic XIAP protein is cleaved and its expression down regulated in mutant SOD1 mice (Guegan et al., 2001, Wootz et al., 2006). Functionally, overexpression of XIAP attenuated disease progression and reduced motor neuron loss in SOD1G93A mice (Inoue et al., 2003, Wootz et al., 2006).

There is evidence that caspases may play a role in motor neuron degeneration in ALS. The inflammatory caspases-1, 11 and 12 (Pasinelli et al., 2000, Kang et al., 2003, Wootz et al., 2004), the initiator caspases-8 and 9 (Guegan et al., 2001, Wootz et al., 2004) and the executioner caspases-3 and 7 have all been found activated in the spinal cord of mutant SOD1 transgenic mice (Pasinelli et al., 2000, Vukosavic et al., 2000, Wootz et al., 2004). In addition, caspase-9 and caspase-3 have also been found activated in the spinal cord of ALS cases (Wootz et al., 2004, Inoue et al., 2003, Martin, 1999). However, the functional roles of most caspases in ALS have not yet been determined (Inoue et al.,

2003, Kang et al., 2003). Nonetheless, irreversible broad-caspase inhibitor attenuates cell death in SOD1 mice (Li et al., 2000a). In addition, using transgenic mice the role for caspases-1, 2 and 11 have been specifically investigated. On the one hand, inhibition of caspase-1 via transgenic expression of dominant negative caspase-1 significantly slowed the progression of ALS disease in mutant SOD1 mice (Friedlander et al., 1997). On the other hand, genetic deletion of caspase-2 or -11 did not affect disease in mutant SOD1 mice (Kang et al., 2003, Bergeron et al., 1998).

The Glial and Inflammatory Response in ALS

In ALS pathology, a glial and inflammatory reaction can be observed around degenerating neuronal cell bodies and axons. This process involves microglia, astrocytes and T-cells (Philips and Robberecht, 2011).

Histopathological changes in ALS

Humans

Microglia, the resident macrophages of the nervous system, are constantly monitoring the extracellular environment, and are the first line of defence against infection and injury (Raivich et al., 1999). In the intact central nervous system microglia have a ramified morphology (resting). However, following neuronal injury, the cell body of microglia enlarges, and the processes shorten and thicken (activated). In the presence of cell death, microglia acquire a round amoeboid shape, characteristic of microglia being involved in the process of phagocytosis (phagocytic), (Del Rio-Hortega, 1932). These morphological changes coincide with the upregulation of immunologically related proteins in the cell surface of microglia.

In post-mortem tissue of patients with ALS, the grey matter and white matter of the precentral gyrus, as well as the lateral funiculus and the anterior horn of the spinal cord contained microglia that have a round/amoeboid morphology. These microglia are strongly immunoreactive for the pan-leukocyte marker CD45, immunoglobulin receptor FcγR1, integrin αMβ2, and MHC class II glycoproteins (Kawamata et al., 1992). Others have found that in the corticospinal tract and the spinal ventral horns of ALS patients, phagocytic microglia highly express IgG in the cell surface and the MHC class II cell surface receptor HLA-DR (Engelhardt and Appel, 1990), In addition, phagocytic cells that express the

glycoprotein CD68, which is found in the cell surface of antigen presenting cells, are evident in the ventral horn, Clarke's column, and mid-dorsal horn (Rexed laminae III–V), as well as the nerve roots of the fifth and seventh nerves. Furthermore, moderate levels of CD68-immunoreactivity are found in the motor nuclei of the trigeminal, facial and hypoglossal nerves (Ince et al., 1996).

Astrocyte activation has also been reported in CNS regions undergoing ALS-associated degeneration. Astrocytes have many functions in the nervous system including, the regulation of extracellular neurotransmitter concentrations and providing trophic support to surrounding neurons (Sofroniew and Vinters, 2010). In the normal brain, fibrillary astrocytes can be found in the white matter. They have long processes that are positive for the cytoskeletal protein glial fibrillary acid protein (GFAP). In the grey matter, astrocytes have numerous short and highly ramified processes that are GFAP-negative. However, the cell body and a few proximal branches can be identified by staining for S100b (Didier et al., 1986).

In the post-mortem brain of humans with ALS, the motor, frontal, temporal, inferior parietal, cingulate, and occipital cortices have intensely stained GFAP-positive astrocyte clusters (Nagy et al., 1994). In addition, in the subcortical white matter of the ALS frontal cortex, GFAP-positive stellate astrocytes with long extended processes can be observed (Kushner et al., 1991). In comparison to the human tissue without the disease, ALS patients demonstrated intense GFAP immunoreativity in the precentral gyrus and spinal cord (Kawamata et al., 1992).

T-cells are recruited to areas of CNS damage (Raivich et al., 1999). T-cell recruitment has been observed in areas affected by ALS. CD4-positive T-cells are observed in close proximity of degenerating corticospinal tracts of ALS patients, while CD4-positive and CD8-positive T-cells are found in the vicinity of motor neurons of the ventral horns (Engelhardt et al., 1993). In addition, there are more CD4- or CD8-positive T-cells in precentral gyrus, the lateral funiculus and the anterior horn of patients with ALS, than those without the disease (Kawamata et al., 1992).

Additional inflammatory processes in ALS include, the increased presence of monocytes/macrophages, dendritic cells and cytokine/chemokine expression in the central nervous system and cerebrospinal fluid of ALS patients (Kuhle et al., 2009, Mitchell et al., 2009, Rentzos et al., 2007, Nagata et al., 2007, Tanaka et al., 2006, Baron et al., 2005, Henkel et al., 2004, Wilms et al., 2003, Lippa et al., 1995).

Mice

, The neuroinflammatory response observed in the mutant SOD1 mouse model of ALS is similar to that found in human cases with ALS (Alexianu et al., 2001). At 80 days of age, when SOD1 transgenic mice do not show any clinical symptoms but only minimal motor neuron cell death, the ventral horn and proximal part of the anterior roots contained numerous αMβ2-positive microglia with round cell bodies and thickened short ramified processes. Microglia activation is predominantly found in the grey matter of the spinal cord, at day 80. However, during the phase of active clinical disease progression, activated microglia are seen in both the grey and white matter of both the ventral and dorsal horn (Alexianu et al., 2001). An increase in microglial density is also found in the spinal cord of SOD1 transgenic mice at 100 days of life compared to non-transgenic controls (Hall et al., 1998b). In addition, large microglial clusters are evident in the spinal cord proximal to large neuronal cell bodies (Alexianu et al., 2001). These activated microglia are highly immunoreactive for IgG throughout the sacral, lumbar, thoracic, and cervical spinal cord. In addition, immunostaining for FcγR1 is significantly increased in mutant SOD1-mice at day 80 and to a greater extend at day 120 (Alexianu et al., 2001). Furthermore, stronger expression of the adhesion molecule ICAM1 is found in the grey matter of the ventral horn of day 80 mutant SOD1-mice, compared to wild type mice. At later time points, ICAM1 staining is widespread, present in microglia of the anterior and posterior horns of the spinal cord (Alexianu et al., 2001). Recently, it has been shown that CD44 is also expressed by some microglia in the spinal cord of mutant SOD1 mice (Matsumoto et al., 2012).

Unlike microglial activation, astrocyte activation is seen at the later time points in ALS transgenic mice. GFAP-immunorectivity is robust in the grey matter of the spinal cord at end stage (Almer et al., 1999). GFAP-positive astrocytes in

the grey matter of the spinal cord have darkly stained cell bodies with elongated processes (Alexianu et al., 2001). However, at early time points, minimal astrocyte activation is seen in the grey and white matter of the spinal cord of mutant SOD1 mice (Almer et al., 1999). At the late stage of the disease, GFAP positive astrocytes strongly express CD44 in the lumbar spinal cord of mutant SOD1 mice (Matsumoto et al., 2012). Finally, at the end stage of the disease T-cells infiltrate the spinal cord. T-cells that are positive for CD3, a pan marker of T-cells, are found to preferentially enter the grey matter of the ventral horn at the late stages the disease (Alexianu et al., 2001).

The role of inflammation in ALS

Motor paralysis associated with ALS is dependent on the progressive degeneration and death of motor neurons. It has become apparent that motor neuron degeneration is not only due to changes within the motor neuron, but also in other cells types such as glia. Mice that selectively express ALS associated mutant SOD1 in neurons do not show any motor neuron loss (Lino et al., 2002, Pramatarova et al., 2001) and developed only mild abnormalities (Jaarsma et al., 2008). In the same way, mice that express mutant SOD1 specifically in microglia (Beers et al., 2006) or astrocytes (Gong et al., 2000) do not show any motor neuron degeneration. However, motor neuron degeneration is evident in mice that ubiquitously express mutant SOD1 (Dalcanto and Gurney, 1994). The notion that motor neuron death is non-cell autonomous is further supported by work on chimeric mice. Chimeric mice have been composed from mixtures of cells carrying wild-type (WT) SOD1 and cells that express human mutant SOD1 (Clement et al., 2003). Using this technique it was demonstrated that motor neurons carrying mutant SOD1 failed to degenerate in an environment of cells carrying WT SOD1 (Clement et al., 2003). This suggests that neighbouring non-neuronal cells carrying mutant SOD1 promote degeneration of motor neurons (Clement et al., 2003).

The specific roles of neuronal and non-neuronal cell types on SOD1 mediated motor neuron damage was shown by cell specific excision of the floxed mutant SOD1 gene using the cre-lox system in mice (Boillee et al., 2006b). In mice, specific neuronal deletion of mutant SOD1 extends survival by delaying onset of disease (Boillee et al., 2006b). Alternatively, the deletion of mutant SOD1

specifically from microglia/macrophages, using the CD11b promoter, extends survival by 99 days by sharply slowing disease progression (Boillee et al., 2006b). The role of microglia/macrophages on survival was further demonstrated in SOD1 mutant mice unable to produce myeloid cells that are known to differentiate into macrophages, dendritic cells, granulocytes, T cells, or B cells (Beers et al., 2006). Myeloid deficient SOD1 mutant mice that underwent transplantation of myeloid cells that express human mutant SOD1G93A at birth, survived up to similar ages as myeloid competent SOD1G93A mutants. However, transplantation of wild type cells of microglial, monocyte, and macrophage lineage into myeloid deficient SOD1 mutant mice slowed down disease progression after onset (Beers et al., 2006). Thus it appears that mutant SOD1 within the motor neurons is a primary determinant of onset of disease, while mutant SOD1 within macrophages/microglial cells determines disease progression.

Microglia may cause neurotoxic effects through the release of reactive oxygen species (ROS) and pro-inflammatory factors. Microglia can induce neuronal injury through the expression of proinflammatory cytokines TNF-α and IL-1β and the ROS superoxide and nitric oxide, and by reducing the expression and release of trophic factors (Michelucci et al., 2009, Ebert et al., 2008, Komohara et al., 2008, Town et al., 2005).

As disease progresses in mutant SOD1 mice, there is an increase in the expression of peroxynitrite and proinflammatory cytokines TNF-α and IL-1β (Beers et al., 2008, Henkel et al., 2006, Hensley et al., 2002, Alexianu et al., 2001, Almer et al., 1999, Hall et al., 1998a). In vivo, the expression of ALS associated mutant SOD1 gene in microglia enhances microglial activation and increases neurotoxicity (Beers et al., 2006, Boillee et al., 2006b). A pathway through which SOD1 may be amplifying microglial mediated neurotoxicity, involves ALS mutant SOD1 binding to Rac1, a member of the Rho familily of GTPases, which subsequently overstimulates NADPH oxidase, which leads to the production of superoxide, which may react with NO to form the toxic compound peroxynitrite (Harraz et al., 2008).

Lymphocytes have been shown to prolong survival of ALS mice (Beers et al., 2008). ALS mutant SOD1 mice that lack functional T- and B- cells have shorter life spans than mutant SOD1 mice with normal T- and B-cells. In addition, mutant SOD1 mice without functional T-and B-cells that receive a bone marrow transplant from normal mutant SOD1 or wild type mice, show longer survival than those which do not (Beers et al., 2008). In the SOD1 mice that lack T and B cells, following bone marrow transplant, CD4-positive T-cells can be found in the spinal cord from the time of disease onset up to end stage, while CD8-posiitve T-cells are only observed at end stage (Beers et al., 2008). There is no consistent evidence for the presence of B cells in the spinal cord at any time point in disease progression, suggesting that T-cells may be driving neuroprotection (Beers et al., 2008). Evidence confirming that T-cells are responsible for prolonging disease duration comes from CD4 deficient mice which lack CD4-positive cells, but have unaltered myeloid-, CD8-postive T-cells and B cells. Mutant SOD1 mice lacking CD4-positve cells have shorter survival times than mutant SOD1 mice that have CD4-positive cells (Beers et al., 2008).

T-cells may prolong survival by altering microglial activation in mutant SOD1 mice (Beers et al., 2008). SOD1 mice without functional T- and B-cells show less microglial activation than those that have T- and B-cells. SOD1 mice lacking CD4-positive T-cells have a reduced expression of microglial markers CD11b and CD68 in the spinal cord, when compared to mutant SOD1 mice that have CD4-positive cells. However, bone marrow transplants from SOD1 mutant or wild type mice to gamma-irradiated SOD1 mice lacking functional T- and B-cells, restores the CD11b, CD68, CD11c, CD40, and MHC II microglial immunoreactivities, as well as CD68 mRNA expression in the spinal cord (Beers et al., 2008). In addition, in the spinal cords of mutant SOD1 mice that lack lymphocytes, the mRNA levels for neurotrophic factors IGF-1 and GDNF, and glutamate transporters that affect neuronal viability are all reduced, as well as those for anti-inflammatory cytokines IL-4 and TGF-β (Beers et al., 2008). However, pro-inflammatory factors, such as TNF-α and peroxynitrite are increased (Beers et al., 2008). This data suggests that T-cells have an important anti-inflammatory and neuroprotective function in mutant SOD1 mice.

Astrocytes also contribute to ALS pathology. In vitro, astrocytes derived from neural progenitor cells of ALS post-mortem spinal cord are toxic to mouse motor neurons in co-culture. By 96 hours, motor neurons atrophy and shorten their neurites, and by 120 hours motor neuron number is reduced to 50% (Haidet-Phillips et al., 2011). This coincides with the production of numerous proinflammatory cytokines and chemokines, known to be toxic to neurons (Haidet-Phillips et al., 2011). It has been found that mutant SOD1 promotes a toxic phenotype in astrocytes. More motor neurons survive after co-culturing with ALS astrocytes that have had the mutant SOD1 knocked down, compared to those which have not (Haidet-Phillips et al., 2011). Interestingly, transplantation of wild type astrocytes precursor cells into the cervical spinal cord, near respiratory motor neuron pools of mutant SOD1 rats, increases motor neuron viability, extends survival, and slows down functional decline (Lepore et al., 2008). It has been suggested that the neuroprotection observed in this model is mediated by enhanced glutamate uptake, performed by glial precursor cells (Lepore et al., 2008).

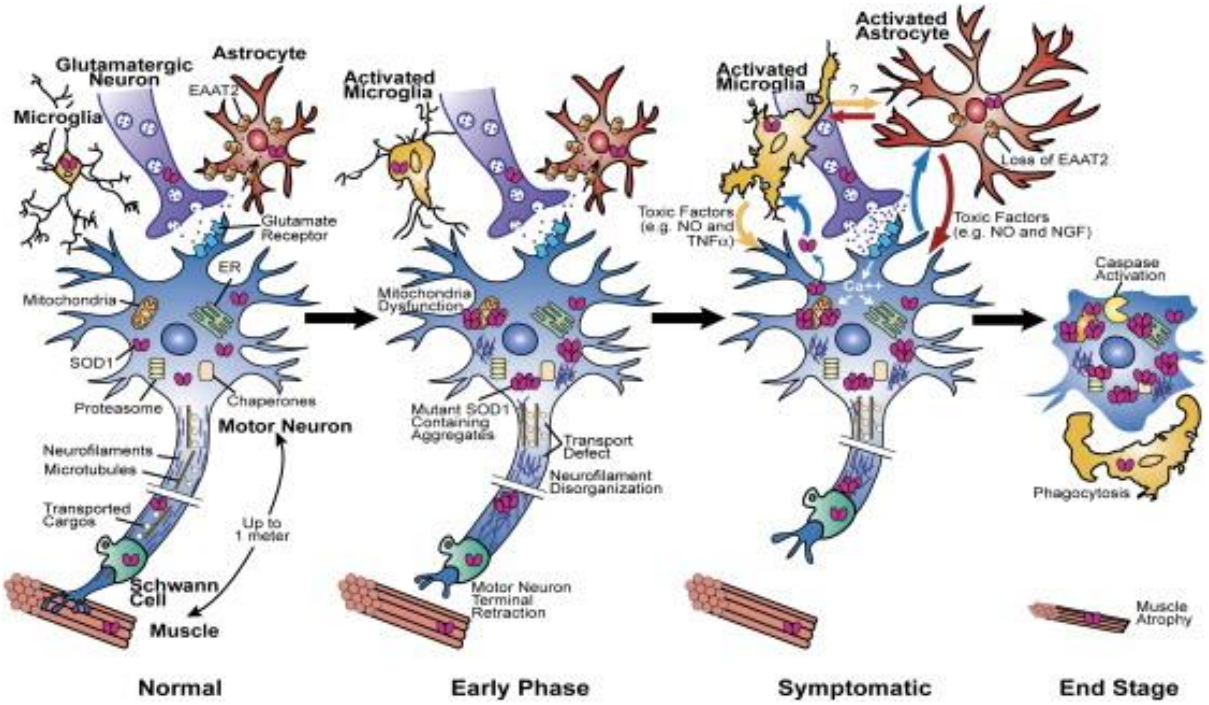


Figure 1. Schematic of the evolution of Motor Neuron Degeneration and glial activation during the course of SOD1 Mutant-Initiated ALS Disease. Four stages are defined (normal, early phase, symptomatic, and end stage). Toxicity is non-cell-autonomous, produced by a combination of damage incurred directly within motor neurons that is central to disease initiation and damage within non-

neuronal neighbours, including astroctyes and microglia, whose actions amplify the initial damage and drive disease progression and spread (Boillee et al., 2006a).

Excitotoxicity

There is evidence that excitotoxicity may play a role in the ALS disease process (Van den Bosch et al., 2006). Glutamate levels are found decreased in the spinal cord parenchyma (Malessa et al., 1991, Tsai et al., 1991, Rothstein et al., 1990, Plaitakis et al., 1988) but increased in the CSF of patients with ALS (Rothstein et al., 1990, Shaw et al., 1995b). In addition, there is evidence of increased expression of NAALADase, an enzyme involved in the synthesis of glutamate (Rothstein et al., 1990). In addition, there is evidence of decreased synaptic removal of glutamate in the spinal cords of ALS patients (Shaw et al., 1994). This reduction appears to be secondary to the selective loss of EAAT2/GLT1 glutamate transporter from astroglia in the spinal cord of ALS patients (Rothstein et al., 1995, Sasaki et al., 2000a, Fray et al., 1998). In addition, a loss in EAAT2 transporter has also been observed in the ventral horn of mutant SOD1 mice (Bendotti et al., 2001b, Bruijn et al., 1997). Genetic overexpression of EAAT2 resulted in delayed disease onset in mutant SOD1 mice (Guo et al., 2003). In addition, pharmacological stimulators of EAAT2 expression significantly increased lifespan and prevented motor neuron death in mutant SOD1 mice (Rothstein et al., 2005). Furthermore, there is evidence of a change in glutamate-AMPA receptors on motor neurons in the spinal cord of ALS cases and mutant SOD1 mice, which is associated with increased vulnerability to excitotoxicity mediated by enhanced calcium permeability (Tortarolo et al., 2006, Kawahara et al., 2003, Takuma et al., 1999). Functionally, pharmacological inhibition of AMPA receptors is associated with increased lifespan of SOD1 mutant mice (Canton et al., 2001, Van Damme et al., 2003, Tortarolo et al., 2006). Furthermore, Riluzole, the only therapeutic agent for human ALS, is reported to act by preventing the release of glutamate and blocking its action on postsynaptic AMPA receptors (Doble, 1999, Gurney et al., 1996). Similarly, pharmacological inhibition of glutamate production by carboxypeptidase II increases the lifespan of mutant SOD1 mice (Ghadge et al., 2003).

There is evidence of increased levels of intracellular calcium in the motor nerve terminals of biopsied human ALS cases and mutant SOD1 mice (Siklos et al., 1996, Siklos et al., 1998). In addition to overstimulation of excitatory neurotransmitter receptors in ALS, it has been suggested that dysregulation in calcium homeostasis is the result of mitochondrial dysfunction and endoplasmic reticulum stress (Grosskreutz et al., 2010). Associated with the increase in intracellular calcium there is also evidence of the activation of calcium dependent proteases, calpains, in mutant SOD1 mice (Stifanese et al., 2010, Gou-Fabregas et al., 2014, Stifanese et al., 2014). Calpains have important regulatory roles in many cellular processes but their prolonged activation has been implicated with neurodegeneration (Vosler et al., 2008). Functionally, inhibition of calcium entry and calpain activity reduces motor neuron degeneration in vitro (Tran et al., 2014, Tradewell and Durham, 2010).

Mitochondrial dysfunction

Histopathological studies of ALS cases demonstrate that degenerating motor neurons contain dilated and vacuolated mitochondria that have disorganised cristae and membranes (Hirano et al., 1984a, Hirano et al., 1984b). Similarly, mitochondria with multi-membrane containing vacuoles can be found in mutant SOD1 mice in presymptomatic stages before any motor neuron loss can be observed (Dalcanto and Gurney, 1994, Higgins et al., 2003, Kong and Xu, 1998). Interestingly, SOD1 protein, which is normally cytosolic, is found enriching mitochondria in both ALS cases and SOD1 mutant mice (Bergemalm et al., 2006, Deng et al., 2006, Liu et al., 2004). It has been suggested that this might drive the mitochondria changes found in the disease (Liu et al., 2004). It is however unclear whether these morphological changes are associated with mitochondrial dysfunction. There is evidence that calcium buffering capacity of mitochondrial is impaired in mutant SOD1 models (Damiano et al., 2006). In addition, some evidence points to motor neuronal energy deficits secondary to the effect of mutant SOD1 on the electron transport chain (Mattiazzi et al., 2002, Browne et al., 2006). In this vein, elevating presumed energy deficits by providing creatine in the diet of mutant SOD1 mice prolonged their survival (Klivenyi et al., 1999). However, human clinical trials have failed to show any benefit for ALS patients (Groeneveld et al., 2003, Shefner et al., 2004).

High levels of oxidative stress markers, protein carbonyl, lipid peroxidation and protein glycoxidation have been reported in areas affected by ALS disease in human cases and SOD1 mutant mice (Shaw et al., 1995a, Shibata, 2001, Shibata et al., 2002, Andrus et al., 1998, Bogdanov et al., 1998, Liu et al., 1998, Liu et al., 1999, Liu et al., 2004). A possible role of oxidative stress in the pathogenesis of ALS was strongly suggested by the fact that the gene for superoxide dismutase-1(*SOD1*) is found mutated in 20% of familial cases of ALS (Boillee et al., 2006a). Superoxide is a natural by-product of respiration which can have cytotoxic effects through its interaction with nitric oxide and the subsequent formation of peroxinitrite, a potent free radical which oxidises lipids, proteins and nucleic acids within cells (Rosen et al., 1993). Since SOD1 detoxifies the superoxide anion by its dismutation to water and hydrogen peroxide, a loss of SOD1 function was the first proposed mechanism of ALS pathogenesis (Rosen et al., 1993). However, mice expressing dismutase active (Wong et al., 1995, Gurney et al., 1994, Howland et al., 2002) and inactive forms (Bruijn et al., 1997, Ripps et al., 1995, Jonsson et al., 2004, Wang et al., 2003) of ALS associated mutant SOD1 develop similar pathology to that observed in ALS patients. In addition, deletion of endogenous SOD1 gene in mice does not cause ALS like pathology (Reaume et al., 1996) and expression of high dismutase active SOD1 mutants does not attenuate the ALS disease process (Bruijn et al., 1998, Deng et al., 2006, Jaarsma et al., 2000). This evidence strongly suggests that mutations in SOD1 cause ALS disease via a gain of toxic function and not through a loss of its normal dismutase function. In this vein, a number of mechanisms for aberrant oxidative chemistry have been suggested for mutant SOD1 (Beckman et al., 2001, Carroll et al., 2004). However, these putative mechanisms have been refuted by in vivo experiments (Subramaniam et al., 2002, Son et al., 2007). Thus, it is likely that oxidative stress is not the primary process driving ALS disease but rather a byproduct.

Pathological inclusions and failure of proteostasis

Neuropathology of ALS protein inclusions

Early post-mortem studies of FALS and SALS cases identified three main types of proteinaceous inclusions within degenerating motor neurons: 1) Bunina bodies (BB) which are found in around 90% of all ALS cases; 2) Lewy body-like hyaline inclusions (LBHI), observed in 15% of cases (mostly familial) and 3)

Small round basophilic inclusions (BI), identified in 12% of ALS cases (mostly sporadic juvenile onset) (Bunina, 1962, Hirano et al., 1967, Chou, 1978, Rowland, 2009, Matsumoto et al., 1992, Ince et al., 1998). Two more types of ALS inclusion were discovered with the use immunohistochemistry (IHC) for ubiquitin, a marker of protein proteosomal degradation, which stained previously unrecognised skein-like (SI) and rounded hyaline (RHI) inclusions (Lowe et al., 1988). In addition, ubiquitin immunoreactivity is also found in the previously described LBHIs, but not in BBs or BIs (Matsumoto et al., 1992). Interestingly, ubiquitin positive inclusions (UBI) are the most common inclusions found in ALS, occurring in over 95% of cases (Piao et al., 2003).

Electron microscopy reveals that all known ALS inclusions contain at least some filamentous (suggestive of neurofilaments) and granular structures (suggestive of organelle fragments) (Murayama et al., 1989). Immunohistochemical studies confirmed the presence of phosphorylated neurofilament in ubiquitin positive LBHIs (Murayama et al., 1989). The highly granular BBs are not immunoreactive for ubiquitin or neurofilament but instead contain Cystatin C, a vesicular protein involved in the inhibition of cysteine proteases (Okamoto et al., 1993). In addition, the discovery of the association between superoxide dismutase 1 (SOD1) gene mutations and familial ALS (Rosen et al., 1993) led to the identification of SOD1 in the LBHIs of SOD1 associated FALS cases (Shibata et al., 1996). Thus some of the constituent proteins of BBs and LBHIs have been identified for many years now. However, the proteins making up the ubiquitin positive SIs and RHIs and the ubiquitin negative BIs were not discovered until quite recently.

The relationship between ALS and frontotemporal dementia shed light on the composition of SIs, RHIs and BIs. SALS can be associated with frontotemporal dementia (ALS-FTD) (Giordana et al., 2011). In addition, some forms of non-SOD1 FALS, present with a family history of FTD, ALS-FTD, or both (Giordana et al., 2011). Post-mortem studies of cases of ALS with cognitive impairment (ALSCi) demonstrate marked frontal lobe atrophy, accompanied by the presence of ubiquitin positive inclusions or Tau positive, ubiquitin negative neurofilamentous inclusions (Ferrari et al., 2011). These observations have resulted in FTD being divided into two major subtypes; FTD with tau-positive

inclusions (FTD-tau), and FTD with ubiquitin-positive (FTD-U) but tau-negative inclusions (Ferrari et al., 2011). Interestingly, some of the ubiquitin positive inclusions found in FTD-U are identical to those observed in ALS (SI/RHIs and LBHIs) and ALS-FTD, but just as for ALS, the identity of their constituent proteins were unknown until recently (Ferrari et al., 2011). The group led by Virginia Lee and John Trojanowski created novel monoclonal antibodies (mAbs) against FTD-U protein inclusions and with the use of mass spectrometry identified transactivating DNA binding protein-43 (TDP-43), a protein involved in gene transcription and translation, as a constituent of those inclusions (Neumann et al., 2006). Immunolabelling with antibodies specific for TDP-43 demonstrated that all ubiquitin-positive inclusions in FTD-U were found to be immunoreactive for TDP-43 (Neumann et al., 2006). Similarly, immunohistochemical analysis revealed that the ubiquitin-positive inclusions (SI, RHI and LBHIs) in ALS which had no constituents previously identified, were also all immunoreactive for TDP-43 (Neumann et al., 2006). Importantly, mutations in the TDP-43 gene were then associated with ALS and FTD (Sreedharan et al., 2008, Borroni et al., 2009).

Recently, studies have gone on to demonstrate, that TDP-43 positive inclusions are found in more than 90% of ALS (Tan et al., 2007, Mackenzie et al., 2007) and around 60% of FTD cases (Seilhean et al., 2011). Interestingly, however, TDP-43 immunoreactivity is not found in SOD1-positive LBHIs, Cystatin-positive BBs or in BIs (Tan et al., 2007, Mackenzie et al., 2007). Researchers were guided to the identification of the constituent proteins of BIs by the discovery that mutations in the FUS gene are associated with ALS (Kwiatkowski et al., 2009, Vance et al., 2009). FUS is another protein which has function in RNA regulation at both the transcription and translation level (Kwiatkowski et al., 2009, Vance et al., 2009). FUS immunoreactivity was found in the ubiquitin- and TDP-43-negative BIs characteristic of juvenile ALS cases (Kwiatkowski et al., 2009, Vance et al., 2009) and in the majority of cases of tau- and TDP-43-negative frontotemporal lobar degeneration (Neumann et al., 2009). Interestingly, recent studies have gone on to demonstrate that FUS protein is found in the skein-like inclusions of a large proportion of non-SOD1 ALS cases

and that it can often colocalise with TDP-43 in such inclusions (Deng et al., 2010).

Dysregulation of translation in ALS

The identification of the involvement of the RNA binding proteins TDP-43 and FUS in ALS has suggested that the disease may represent a failure in RNA processing within motor neurons (Droppelmann et al., 2014). In support with this hypothesis, other genes involved in RNA metabolism have recently been discovered to associate with ALS (Droppelmann et al., 2014). These include: ARHGEF28 (encoding Rho Guanine Nucleotide Exchange Factor; RGNEF), HNRNPA1 (encoding Heterogeneous Nuclear Ribonucleoprotein A1), TAF15 (encoding TATA-binding protein-associated factor 2N) and EWS (encoding Ewing Sarcoma) (Droppelmann et al., 2014). In addition, many proteins involved in RNA metabolism are detected in the pathological inclusions of postmortem tissues from individuals with ALS (Bentmann et al., 2013, Dormann et al., 2010, Kim et al., 2013, Liu-Yesucevitz et al., 2010, Collins et al., 2012, Keller et al., 2012). Furthermore, a hexanucleotide GGGGCC repeat expansion in the noncoding region of the C9ORF72 gene has been recently found to be the most common genetic abnormality in ALS, being found in around 40% of all cases of FALS and 8% of SALS (DeJesus-Hernandez et al., 2011, Majounie et al., 2012, Renton et al., 2011). The hexanucleotide repeats in C9ORF72 have been shown to generate RNA foci which give rise to aberrant translation that generates protein species which sequester proteins, including those involved in RNA regulation, (Donnelly et al., 2013, Lee et al., 2013b), and thus may provide a basis for the formation of protein inclusions.

Dysregulation of proteostasis

The presence of intracellular protein inclusions in ALS indicates that there is a failure in proteostasis in the disease (Robberecht and Philips, 2013). Normally, the unfolded protein response, ubiquitin proteasome system, and autophagy-lysosome system function to monitor protein quality and protect cells from dysfunctional, misfolded or denatured proteins (Blokhuis et al., 2013).There is evidence that failure in these systems may be driven by the aberrant aggregation of mutant proteins and that this process is at the centre of the pathogenesis of ALS (Saxena and Caroni, 2011, Bendotti et al., 2012).

Endoplasmic reticulum stress

The ER is the first cellular compartment where proteins are synthesized and folded. For this process, a large and efficient network of chaperones, foldases, and co-factors are expressed at the ER to promote folding and prevent abnormal aggregation of proteins. When the function of this organelle is affected and abnormal oxidative protein folding occurs at the ER lumen “ER stress” occurs (Matus et al., 2013b). ER stress activates the unfolded protein response (UPR), a signalling pathway that increases the protein folding capacity and quality control mechanisms of the ER to promote proteostasis (Matus et al., 2013b). However, chronic ER stress can result in cell death by a number of mechanisms (Matus et al., 2013b).

The protein folding chaperone machinery, which includes heat-shock proteins (HSPs), assists protein folding under physiological circumstances, and prevent protein misfolding and aggregation during cellular stress (Blokhuis et al., 2013). Heat shock proteins are found upregulated in the motor neurons of the spinal cord of ALS cases (Anagnostou et al., 2010) and within protein inclusions (Basso et al., 2009, Watanabe et al., 2001). However, it has been reported that there is an overall decrease in chaperone activity in mutant SOD1 mice (Tummala et al., 2005), which may be due to the preferential binding and inhibition of heat shock proteins by mutant SOD1 protein (Okado-Matsumoto and Fridovich, 2002). Functionally, pharmacological activation of HSPs increases the lifespan in mutant SOD1 mice (Kieran et al., 2004). Similarly, upregulation of HSPs in vivo and in vitro increases solubility of and reduces toxicity of mutant SOD1 (Crippa et al., 2010), FUS (Miguel et al., 2012), TDP-43 (Crippa et al., 2010, Gregory et al., 2012).

Ultrastructural studies of motor neurons in ALS cases demonstrate evidence of endoplasmic reticulum fragmentation (Oyanagi et al., 2008). Post mortem immunohistochemical studies have demonstrated the induction of the UPR in cases of ALS. There is evidence of enhanced phosphorylation of eIF2α and increased levels of XBP1s, ATF4, CHOP, PERK, ATF6, IRE1 and the ER foldases PDIA1 and ERp57 (Ilieva et al., 2007, Atkin et al., 2008, Hetz et al., 2009, Ito et al., 2009).

Mutant SOD1 mice demonstrate accumulation of mutant SOD1 protein in the endoplasmic reticulum (ER) (Kikuchi et al., 2006, Urushitani et al., 2008, Tobisawa et al., 2003) and the presence of ER chaperones such as protein disulfide isomerase (PDI) in mutant SOD1 protein inclusions (Atkin et al., 2006). In fact PDI is also found in SOD1, TDP-43 and FUS inclusion in human cases of ALS (Ilieva et al., 2007, Honjo et al., 2011, Farg et al., 2012). There is evidence of endoplasmic reticulum stress in early stages of the disease process in mutant SOD1 mice (Saxena et al., 2009). This has been demonstrated by the upregulation of genes and proteins involved in the unfolded protein response (UPR): PERK, ATF4 and phosphorylated eukaryotic initiation factor 2alpha (Pi-eIF2 alpha) (Saxena et al., 2009). Importantly, the UPR appears to have a functional role in the disease as its pharmacological inhibition, by preventing the dephosphorylation of Pi-eIF2 alpha, delays disease and reduces motor neuron degeneration in mutant SOD1 mice (Saxena et al., 2009) In this vein, haploinsufficiency of the PKR-like ER kinase (perk), the enzyme responsible for the phosphorylation of Pi-eIF2 alpha, exacerbates neurodegeneration in mutant SOD1 mice (Wang et al., 2011). In addition, reducing the expression of GADD34, which normally dephosphorylates p-eIF2 and allows recovery from the global suppression of protein synthesis, markedly ameliorates disease in SOD1 mice (Wang et al., 2014).

Ubiquitin-proteasome system

The ubiquitin-proteasomal system (UPS) is essential for the clearance of aberrant proteins in the cellular environment (Finley, 2009). The degradation of a protein via the UPS involves two steps: 1) the tagging of the target protein with multiple ubiquitin molecules and 2) degradation of the tagged protein by the 26S proteasome (Finley, 2009).

In addition to the presence of ubiquitin positive inclusion, changes in the expression of UPS-related proteins have been found in the motor neurons of ALS cases and in animal models of the disease (Cheroni et al., 2005, Urushitani et al., 2004, Urushitani et al., 2002). Furthermore, some of the mutations associated with ALS affect genes of proteins which either directly interact with the UPS or which are targets of degradation by it (Bendotti et al., 2012).

Mutant SOD1 protein misfolds and forms insoluble aggregates in SOD1 FALS cases and mutant SOD1 mice (Basso et al., 2006). In addition, Wild-type SOD1 protein when oxidised also misfolds, is aggregation prone and has neurotoxic effects (Ezzi et al., 2007, Bosco et al., 2010, Rakhit et al., 2002). Interestingly, immunolabelling using an antibody specific for the oxidised form of SOD1 has recently demonstrated the presence of SOD1 protein inclusions in a subset of sporadic ALS patients (Bosco et al., 2010). Protein inclusions in SOD1 ALS cases and mutant SOD1 mice are immunoreactive for proteins that are members of the UPS, including dorfin, CHIP, Gp78, NEDL1, SUMO, UCHL1 and MITOL (Bendotti et al., 2012). Interestingly, although some of the mutant SOD1 protein in the inclusions found in SOD1 mice is ubiquitinated, the majority is not (Basso et al., 2006). This suggests that there may be an underlying failure of the UPS driving the formation of SOD1 protein inclusion within neurons (Basso et al., 2006). In vivo, the expression of proteasome subunits decreases as the intracellular accumulation of SOD1 protein increases in the motor neurons of SOD1 mutant mice (Cheroni et al., 2005, Kabashi et al., 2008, Urushitani et al., 2002, Cheroni et al., 2009). Failure of the UPS has been demonstrated in SOD1 transgenic mice that expressed a fluorescently tagged reporter substrate of the UPS (Cheroni et al., 2009). These mice accumulated the fluorescent reporter protein within motor neurons suggesting that UPS is indeed impaired in SOD1 mutant mice (Cheroni et al., 2009). Functionally, activation of UPS in mutant SOD1 mice appears to be neuroprotective, as transgenic overexpression of dorfin, a ubiquitin ligase, in these mice decreases mutant SOD1 protein and ameliorates neurological deficits (Sone et al., 2010).

Genetics have also provided evidence that failure in the UPS may drive the disease process in ALS. Mutations in the ubiquilin-2 gene (UBQLN2) have been associated with X-linked FALS or FALS-FTLD (Deng et al., 2011). UBQLN2 is a member of the ubiquitin-like protein family which delivers proteins to the proteasomal degradation machinery (Ko et al., 2004). In vitro, cells expressing ALS-linked UBQLN2 mutants demonstrate an impairment of the UPS (Deng et al., 2011). In addition, UBQLN2 has been found in the SIs, which also contain TDP-43, in the spinal cord of humans with ALS (Deng et al., 2011). Furthermore, it has been found in inclusions of SALS and FTLD cases (in the

absence of disease-linked UBQLN2 mutations) (Deng et al., 2011) and of patients with FUS mutations (Robberecht and Philips, 2013).

Autophagy

Autophagy is a process by which cellular contents are degraded in a controlled fashion through the formation of intracellular vesicles (autophagosomes) and fusion with lysosomes (Mizushima, 2007). The UPS is involved in the degradation of short lived proteins while autophagy is responsible for the degradation of long-lived proteins and intact organelles or proteins complexes (Mizushima, 2007). Autophagy depends on microtubule transport and proteins associated in this process, including dynein, histone deacetylase 6 (HDAC6) and p62 (Lee et al., 2010, Pankiv et al., 2007). Importantly, dysregulation of autophagy can result in an specific type of cell death, termed autophagy cell death (type II cell death), which is characterised by the accumulation of autophagosomes and the lack of nuclear condensation (Scarlatti et al., 2009).

Neuropathological studies demonstrate increased number of autophagosomes in the spinal cords of ALS patients and SOD1 mutant mice (Sasaki, 2011, Morimoto et al., 2007, Li et al., 2008). However, it is not clear whether the increase in autophagosome number indicate autophagy induction or autophagy flux impairment in ALS (Chen et al., 2012). On the one hand, in vitro induction of autophagy by rapamycin treatment decreases mutant SOD1 protein aggregates and associated toxicity (Kabuta et al., 2006). On the other hand, activation of autophagy by treatment of SOD1 mutant mice with rapamycin augmented motor neuron degeneration (Zhang et al., 2011). SOD1 can be degraded by autophagy and the UPS in neuronal cells (Kabuta et al., 2006). It has been demonstrated that this process is dependent on the interaction between SOD1 and the autophagy related protein p62 (Gal et al., 2009). Evidence suggests a similarly relationship between mutant TDP-43 and autophagy. TDP-43 inclusion co-localise with the maker of autophagy LC3 and the adaptor protein p62 (Wang et al., 2010, Brady et al., 2011). It has been demonstrated that TDP-43 protein degradation occurs through both autophagy and the UPS (Wang et al., 2010). Activation of autophagy in cellular models of mutant TDP-43 pathology results in reduced protein aggregation and neurotoxicity (Caccamo et al., 2009). In vivo, treatment of transgenic TDP-43

mice with the pharmacological activator of autophagy reduces motor deficits and neuronal loss (Wang et al., 2012b).

Genetics has supported a role of autophagy in ALS further. Mutations in the sequestosome 1 gene (SQSTM1; also known as p62), which encodes for a key adaptor protein involved in autophagy, has also been associated with ALS (Fecto et al., 2011). In addition, its protein has been found in the ubiquitin-/TDP-43-/FUS-positive inclusions of a large portion of both familial and sporadic ALS cases (Deng et al., 2010). In addition, mutations in the gene coding for charged multivesicular body protein 2b (CHMP2B) have been found in FTD and ALS cases (Parkinson et al., 2006, Cox et al., 2010). CHMP2B protein is a subunit of endosomal sorting complexes required for transport-III (ESCRT-III), which is necessary for the formation of multivesicular bodies in autophagy (Filimonenko et al., 2007). It has been reported that cells depleted of ESCRT subunits or expressing ALS associated mutant CHMP2B show inhibited autophagic protein degradation, leading to the accumulation of ubiquitinated proteins which include TDP-43 and p62 (Filimonenko et al., 2007). Similarly, mutations in the gene coding for valosin-containing protein (VCP) have been associated with ALS. VCP is a protein that interacts with various ubiquitin-binding partners to regulate many ubiquitin-dependent processes including autophagy (Ju et al., 2008). Loss of VCP or expression of mutant VCP results in accumulation of autophagosomes and in their failure to mature in vitro (Ju et al., 2009, Ju et al., 2008). Furthermore, mutations in the optineurin (OPTN) gene have been associated with ALS (Maruyama et al., 2010). Optineurin protein has been found in inclusions containing TDP-43, SOD1 and FUS in ALS cases (Ito et al., 2011, Maruyama et al., 2010). Optineurin is a ubiquitin-binding protein that is involved in autophagy (Wild et al., 2011) and which is a target of the UPS (Shen et al., 2011). Functionally, overexpression of wild type or ALS –linked mutant optineurin results in the inhibition of UPS and the activation of autophagy in vivo and in vitro (Shen et al., 2011).

Dysregulation of axonal transport

There is evidence that neuromuscular denervation and axonal degeneration are the earliest pathological changes observed in ALS (Fischer et al., 2004). It has been suggested that these changes are in part due to changes at the level of

the neuromuscular junction (NMJ) and due to defects in axonal transport (Dadon-Nachum et al., 2011).

Electrophysiological and histological assessment of NMJ function in human ALS cases and mutant SOD1 mice has demonstrated evidence of denervation, which begins prior to the onset of muscle weakness and which is initially compensated by axonal sprouting at the NMJ (Felice, 1997, Hayworth and Gonzalez-Lima, 2009, Gordon et al., 2004, Fischer et al., 2004). At the molecular level, there is reduced muscle expression of Cdk5, a protein normally upregulated in muscle during denervation, in mutant SOD1 mice (Park and Vincent, 2008). There is also evidence of increased expression of Sema3A in the muscle in mutant SOD1 mice, which has been suggested to repel the terminal axon from the NMJ it innervates (Pasterkamp and Giger, 2009). In addition, Sema3A has also been found secreted by terminal Schwann cells in mutant SOD1 mice (De Winter et al., 2006). The axon guidance protein Nogo-A is also found upregulated in muscle of ALS cases and mutant SOD1 mice early on the disease (Jokic et al., 2005). In addition, there is evidence of reduced expression of trophic factors, such as Glial cell line-derived neurotrophic factor (GDNF) and Insulin-like growth factor (IGF-I) in the muscle of ALS cases (Yamamoto et al., 1996, Lunetta et al., 2012). Importantly, silencing the expression of mutant SOD1 protein in lower motor neurons, by injecting the muscle with a lentiviral vector expressing RNAi molecules specifically targeting the human SOD1 gene, ameliorated disease in mutant SOD1 mice (Ralph et al., 2005).

There also changes in Schwann cells in ALS cases and mutant SOD1 mice. Histological studies on ALS cases have described myelin alterations along the peripheral nerves which are most likely secondary to axonal degeneration in the disease (Perrie et al., 1993). Schwann cells in mutant SOD1have been shown to play an active role in the ALS disease process (Lobsiger et al., 2009, Wang et al., 2012d). They express axon repellent molecules such as semaphorin 3A and CD44 (Gorlewicz et al., 2009, De Winter et al., 2006). Importantly, specific deletion of mutant SOD1 from Schwann cells in mutant SOD1 mice ameliorates disease and extended survival (Wang et al., 2012d).

Neuropathological studies demonstrate abnormal accumulation of phosphorylated neurofilaments within motor neurons of ALS cases (Hirano et al., 1984b, Hirano, 1991) and mutant SOD1 mice (Sasaki and Iwata, 1996, Sasaki et al., 2005). In addition, neurofilament binding proteins such as alpha-internexin, kinesin-associated protein 3 (KAP3), peripherin and tau are also found in protein aggregates in ALS post-mortem cases (Page et al., 2011, Gros-Louis et al., 2004, Corrado et al., 2011, Strong et al., 2006, Tateno et al., 2009). Phosphorylation of neurofilament by p38 alpha stress-activated protein kinase is involved in the accumulation of neurofilament with motor neurons of ALS cases and mutant SOD1 mice (Ackerley et al., 2004, Ackerley et al., 2003). In addition, p38MAPK is a component of the intracellular inclusions found in human amyotrophic lateral sclerosis and mutant SOD1 Transgenic mice (Bendotti et al., 2004).

Genetic studies have found mutations in the neurofilament heavy (NF-H) gene in around 1% of sporadic ALS cases but not in familial cases of ALS (Al-Chalabi et al., 1999, Figlewicz et al., 1994, Tomkins et al., 1998). In addition, mutations in the gene for the intermediate filament subunit protein, peripherin, are found in cases of sporadic ALS (Gros-Louis et al., 2004, Corrado et al., 2011, Leung et al., 2004). Interestingly, a variant within the gene of the kinesin-associated protein KIFAP3 has been identified to decreased KIFAP3 expression and increased length of survival for patients with sporadic ALS, has been identified (Landers et al., 2009). Similarly, mutations in the profilin gene, an important mediator of actin dynamics, have also been found to account for a small portion of inherited ALS cases (Wu et al., 2012).

There is evidence of delayed anterograde axonal transport in mutant SOD1 mice, which is present in very early stages of the disease (Williamson and Cleveland, 1999, Zhang et al., 1997, Collard et al., 1995, Bilsland et al., 2010, Parkhouse et al., 2008). In vitro, both oxidised (found in sporadic cases of ALS) and ALS-associated mutant SOD1 protein have been shown to inhibit both fast retrograde and kinesin-based anterograde axonal transport (Bosco et al., 2010, De Vos et al., 2007). Similarly, mutant SOD1 protein has been found to interact with the dynein complex which is involved in retrograde transport (Zhang et al., 2007). In addition, mutant SOD1 protein sequesters kinesin-associated protein

3 (KAP3), a protein involved in anterograde axonal transport (Tateno et al., 2009).

The effect of neurofilament protein burden on axonal transport also appears to be important in neuronal viability. Mice overexpressing neurofilament proteins (NFL-H or NFL-L) develop age dependent motor neuron degeneration (Cote et al., 1993, Xu et al., 1993). Similarly, the presence of dominant negative point mutation in one of the neurofilament genes (NFL-H) results in fatal progressive paralysis in mice characterised by motor neuron degeneration (Lee et al., 1994). Interestingly, deletion of neurofilament genes NFL-H, NFL-M and NF-L prolongs survival in mutant SOD1 mice (Nguyen et al., 2001, Williamson et al., 1998). It has been suggested that this effect is secondary to a decrease in the burden neurofilaments place on the axonal transport machinery (Boillee et al., 2006a). There is evidence that the largest motor neurons, those which have the largest calibre axons and innervations ratios, are the most vulnerable to ALS degeneration in human cases and mutant SOD1 mice (Bendotti et al., 2001a, Fischer et al., 2004, Feinberg et al., 1999, Hegedus et al., 2007). These motor neurons of larger size are likely to have higher demands on axonal transport and higher vulnerability to its dysfunction (Pun et al., 2006). Neurofilaments slow down axonal transport via protein interactions with the axonal transport machinery through their phosphorylated tail domain (Ackerley et al., 2003). Thus neurofilament burden on axonal transport may promote motor neuron degeneration. In line with this, SOD1 mice expressing neurofilament lacking phosphorylated tail domains demonstrate ameliorated neurodegenerative disease (Lobsiger et al., 2005). Similarly, in Legs at odd angles (Loa) mice, mutations in the motor protein dynein are associated with axonal transport defects and motor neuron degeneration (Hafezparast et al., 2003). However, mutant SOD1 mice which express one Loa mutated dynein allele demonstrate improved axonal transport and ameliorated disease (Kieran et al., 2005).

Neurotrophic factors

Trophic support is essential for neuronal viability in physiological and pathological states. There is evidence that the expression of neurotrophic factors is dysregulated in ALS and that their delivery may provide a therapeutic approach for the disease (Tovar-y-Romo et al., 2014).

Brain-derived neurotrophic factor (BDNF) is found in motor neurons at similar levels as in controls (Kawamoto et al., 1998) but the expression of its receptor, trk B, is increased in the spinal cord of ALS cases (Mutoh et al., 2000). Levels of BDNF in the CSF of ALS patients are similar to controls (Grundstrom et al., 2000). Functionally, BDNF does not appear to play a role in ALS degeneration as clinical trials of subcutaneous BDNF did not alter disease outcome in ALS patients (Kasarskis et al., 1999).

Insulin-like growth factor 1 (IGF-1) expression has been found either unaltered or increased in the spinal cord of ALS cases (Dore et al., 1996, Kerkhoff et al., 1994, Adem et al., 1994b, Adem et al., 1994a). However, the expression of IGF-1 is impaired in muscle from ALS patients (Lunetta et al., 2012). Functionally, IGF-1 was found to be protective in mutant SOD1 mice (Kaspar et al., 2003, Dodge et al., 2008). However, clinical trials of subcutaneous IGF-1 have failed to demonstrate an effect on ALS patients (Borasio et al., 1998b, Lai et al., 1997).

Increased levels of glial cell-line-derived neurotrophic factor (GDNF) have been found in the spinal cord, CSF and muscle of ALS patients (Yamamoto et al., 1996, Grundstrom et al., 2000, Grundstrom et al., 1999). In addition, there is evidence of increased expression of the GDNF receptor, RET, in the motor neurons of ALS cases (Mitsuma et al., 1999). Functionally, muscular delivery or overexpression of GDNF preserves promotes motor neuron function and prolongs survival in mutant SOD1 mice (Suzuki et al., 2008, Mohajeri et al., 1999).

Leukemia Inhibitory Factor (LIF) and Ciliary Neurotrophic Factor (CNTF) have been described as modifier genes in ALS disease (Giess et al., 2000, Giess et al., 2002). A mutation in the LIF gene, which affects its ability to bind its receptor, has been found in ALS patients (Giess et al., 2000). Similarly, mutations in CNTF, which affect its expression have been found in patients with ALS and are associated with a more severe form of the disease (Giess et al., 2002). CNTF and its receptor CNTFR-alpha are found upregulated in the spinal cord of ALS cases (Duberley et al., 1995, Schorr et al., 1996). LIF expression has not been characterised in the spinal cord but it has been found upregulated

in skin biopsies from patients with ALS (Hu et al., 1999). Functionally, genetic deletion of CNTF in mutant SOD1 mice accelerates motor neuron degeneration (Giess et al., 2002). In this vein, systemic administration of LIF improved motor function in mutant SOD1 mice but failed to protect motor neurodegeneration or prolong lifespan (Azari et al., 2003). However, human clinical trials have demonstrated no effect of subcutaneous CNTF on ALS disease (Miller et al., 1996, Penn et al., 1997).

Vascular endothelial growth factor (VEGF) levels were shown to be unaltered in spinal cord but were found raised in the serum of patients with ALS compared to controls (Nygren et al., 2002). People with mutations in the VEGF gene, which reduce its expression, are at 1.8 greater risk of developing ALS (Lambrechts et al., 2003). Reduced expression of VEGF in mice results in ALS-like disease in mice (Oosthuyse et al., 2001). In vitro, mutant SOD1 mediated neurotoxicity is ameliorated by treatment with VEGF and this neuroprotective effect is mediated by the PI3K/AKT pathway (Li et al., 2003, Lunn et al., 2009). In vivo, there is a decrease in the expression of VEGF receptors in the spinal cord of mutant SOD1 mice (Lunn et al., 2009). In addition, mutant SOD1 mice with reduced VEGF expression died earlier and had exacerbated motor neuron degeneration (Lambrechts et al., 2003). In contrast, lentiviral mediated overexpression of VEGF or its receptor prolongs survival in mutant SOD1 mice (Azzouz et al., 2004, Storkebaum et al., 2005), and intracerebroventricular delivery of recombinant VEGF results in prolonged survival in mutant SOD1 rats (Storkebaum et al., 2005).

Dysregulation of signalling pathways

There is evidence of dysregulation in a number of signalling pathways in ALS (Hu et al., 2003b, Hu et al., 2003a).

The non-receptor tyrosine kinase c-Abl, which is activated by Src kinase mediated phosphorylation, is found upregulated in human cases of ALS and activated in mutant SOD1 mice (Katsumata et al., 2012). Abl is involved in cytoskeletal rearrangement and migration (through Rac/Wave1/2/Arp2/3 and cortactin), receptor endocytosis (through ERK5, STAT1/3, Rac/JNK), proliferation (through Rac/Nox or MK4/7/JNK/Myc) and apoptosis (through SAPK/JNK and p38 mitogen activated protein kinase (MAPK)) (Sirvent et al.,

2008, Sun et al., 2000). Importantly, c-Abl appears to have a role in ALS neurodegeneration as pharmacological inhibition of its activation ameliorates disease in mutant SOD1 mice (Katsumata et al., 2012).

GSK-3 has been found upregulated in the spinal cord of ALS cases (Hu et al., 2003b). GSK-3 affects various intracellular signalling pathways which affect neuronal survival including phosphatidylinositol 3-kinase/Akt and Wnt (Goold and Gordon-Weeks, 2003, Patel et al., 2004). GSK-3 has been shown to directly affect a number of transcription factors involved cell survival including the CCAAT/enhancer binding (CEBPB) protein, NFAT, Myc, HSTF-1, the cyclic AMP response element binding protein (CREB), activator protein-1 (AP-1), β-catenin, NFkB, p53, release of cytochrome c, and caspase-3 (Bijur and Jope, 2001, Grimes and Jope, 2001, Takadera and Ohyashiki, 2004, Watcharasit et al., 2003). In vitro, motor neuron cells expressing mutant SOD1 protein also show upregulation of GSK-3, and GSK-3 inhibition reduces cell death (Koh et al., 2005a, Jeon et al., 2013). In vivo, pharmacological inhibition of GSK-3 is associated with suppressed disease progression in mutant SOD1 mice (Koh et al., 2007, Sugai et al., 2004, Ahn et al., 2014, Ahn et al., 2012).

There is evidence of increased expression and activation of p38 MAPK (Mitogen-activated protein kinase) in the spinal cord of ALS patients and in the motor neurons of mutant SOD1 mice. It also forms part of intracellular protein inclusions found in both ALS cases and SOD1 mice (Hu et al., 2003b, Tortarolo et al., 2003, Ackerley et al., 2004, Bendotti et al., 2004). The p38 MAPK is implicated in various functions such as phosphorylation of cytoskeletal proteins and the synthesis of cytokines and nitric oxide (Mielke and Herdegen, 2000, Ono and Han, 2000). This kinase has been implicated in a motor neuron specific form of cell death, which involves upstream signalling from the cell death receptor Fas and death-associated protein 6 (Daxx) (Raoul et al., 2006). In addition, there is in vitro evidence that mutant SOD1 neurodegeneration is associated with p38 mediated inhibition of fast anterograde axonal transport (Morfini et al., 2013). Interestingly, pharmacological inhibition of activated protein kinases (SAPK) p38 and c-Jun-N-terminal kinase (JNK) reduces mutant SOD1 induced motor neuron death in vitro and ameliorates neurodegeneration in mutant SOD1 mice (Dewil et al., 2007).

The mammalian sterile 20 (STE20)-like kinase 1 (MST1) has been found upregulated in the motor neurons of ALS cases and mutant SOD1mice (Lee et al., 2013a). MST1 is associated with the regulation of cell growth, apoptosis, stress response, and senescence (de Souza and Lindsay, 2004, Lehtinen et al., 2006). Importantly, it has been recently associated to neuronal cell death initiated by oxidative stress (Lehtinen et al., 2006, Yuan et al., 2009). Mutant SOD1 protein is found to lead to the activation of MST1 by inducing its dissociation from Thioredoxin-1 and promoting MST1 homodimerisation (Lee et al., 2013a). Functionally, genetic deletion of the MST1 gene delays disease onset and extends survival in mutant SOD1 mice (Lee et al., 2013a). This neuroprotective effect was associated with reduced activation of p38 and improved autophagy flux (Lee et al., 2013a).

Post-mortem studies have reported an increase in Pi3K (Phosphoinositide 3-kinase) activity in the spinal cord of ALS patients (Wagey et al., 1998). In contrast, Pi3K immunoreactivity has been reported as decreased or unchanged in spinal motor neurons of mutant SOD1 mice (Warita et al., 2001, Peviani et al., 2007). The Pi3K and AKT pathway play a major role in the transduction of survival signals in neurons and in mediating protection against toxic insult (Peviani et al., 2014). In this vein, mutant SOD1 mediated cytotoxicity is ameliorated by pharmacological activation of Pi3K or AKT (Koh et al., 2005b, Nawa et al., 2008). In addition, genetic over expression of AKT3 within motor neurons prevents mutant SOD1 mediated neuronal loss in vitro and in vivo (Peviani et al., 2014). This neuroprotective effect was reported to involve the inhibition of ASK1 and GSK3 (Peviani et al., 2014).

Microsatellite analysis has identified PLCD1, the gene encoding for phospholipase Cδ1 (PLCδ1), to be associated with human ALS (Simpson et al., 2009). In addition, the expression of PLCδ1 is found upregulated within motor neurons in mutant SOD1 mice (Simpson et al., 2009). PLCδ1 is a protein which hydrolyses PIP2 to diacylglycerol (DAG) and IP3 and is involved intracellular calcium homeostasis. It is upregulated within neurons in excitotoxic conditions (Shimohama et al., 1995) and is involved in the release of calcium from the ER via the activation of ER IP3 receptors (Nakamura et al., 2003). Functionally, while overexpression of IP3 receptor accelerates disease progression in mutant

SOD1 mice (Staats et al., 2012), genetic deletion of PLCδ1 prolongs survival of mutant SOD1 mice (Staats et al., 2013).

Immunohistochemical studies have demonstrated increase expression of Jun-N-teminal kinase (JNK) in spinal cord astrocytes of ALS cases (Migheli et al., 1997). In addition, JNK expression has also been found upregulated in the spinal cord microglia of mutant SOD1 mice (Veglianese et al., 2006). Furthermore, there is evidence of aberrant retrograde axonal transport of activated JNK in mutant SOD1 within motor neurons (Perlson et al., 2009). JNKs are involved in transducing signals of different forms of cellular stress, including excitotoxicity through the phosphorylation of many substrates such as the transcription factor c-Jun (Raivich, 2008). In vitro, pharmacological inhibition of JNK at the axonal compartment of motor neurons from mutant SOD1 mice is associated with reduced activation of c-Jun at cell body and reduced cell death (Perlson et al., 2009). Similarly, pharmacological inhibition of activated protein kinases (SAPK) p38 and JNK reduces mutant SOD1 induced motor neuron death in vitro and ameliorates neurodegeneration in mutant SOD1 mice (Dewil et al., 2007).

The functional role of Janus Kinases in mutant SOD1 mediated disease has been investigated because of their involvement in inflammation, oxidative stress and apoptosis (Kiu and Nicholson, 2012). Pharmacological inhibition of JAK3 was found to increase survival of mutant SOD1 mice. However, inhibition of JAK2 did not affect motor neuron degeneration in mutant SOD1 mice despite having some effect on microglia activation (Tada et al., 2014).

Changes in gene expression

There is evidence of differential gene expression in the spinal cord of ALS patients and mutant SOD1 mice compared to controls (Heath et al., 2013). Gene expression in ALS has been assessed using microarrays on whole spinal cord, whole ventral horn, ventral horn grey matter and motor neuron homogenates (Heath et al., 2013).

Microarray analysis of motor neurons from cases with ALS demonstrates that the expression of a variety of genes is downregulated when compared to controls. These include genes associated with cytoskeleton/axonal transport

(such as dynactin and microtubule-associated proteins), transcription (EGR3), signalling (c-Abl) and cell surface antigens/receptors (TRK-C). In addition, the expression of genes involved in cell death (such as caspases-1, -3, and -9) is upregulated. However, there is also increased expression of neurotrophic factors (CNTF, GDNF and HGF) and other neuroprotective molecules (NF-κB) (Jiang et al., 2005).

Microarray analysis of the grey matter of the spinal cord of ALS patients demonstrates changes in the expression of a variety of genes when compared to controls. There are alterations in genes involved in mitochondrial function (e.g. downregulation of ATP synthase δ-subunit), oxidative stress (downregulation in transferrin and heat shock protein hsp90), inflammation (upregulation of HLA class I and cathepsin) excitotoxicity (upregulation of glutamate transporter EAAT1), apoptosis (downregulation of inhibitor of apoptosis 1 (IAP1)), cytoskeletal architecture (down regulation of dynactin, α-tubulin, and synaptobrevin), cell adhesion (down regulation in integrin-β3), RNA transcription (upregulation of FUS) and translation (down regulation in SMN), proteasomal function (upregulation of the E6-AP ubiquitin protein ligase 3A), growth (downregulation in fibroblast growth factor 4 (FGF4)) and signalling (upregulation in transducin-β2, adenylyl cyclase, and protein kinase C-δ) (Dangond et al., 2004).

Microarray analysis of motor neurons in mutant SOD1 mice demonstrates that there is little differential gene expression compared to controls at pre-symptomatic stages of the disease (Perrin et al., 2005). However, as the disease progresses the number of differentially expressed genes in mutant SOD1 mice, compared to controls, increases dramatically (Perrin et al., 2005). The expression of intermediate filament vimentin was found to consistently increase with disease progression in these mice and this was associated with the formation of cytoplasmic inclusions containing vimentin protein (Perrin et al., 2005). In addition, genes associated with neuronal cell growth or maintenance, such as the IGF binding protein NOV, matrix metalloproteinase, connexin 43 and GAP-43, were massively upregulated in mutant SOD1 mice compared to controls.

Similarly exon-centric microarray technology has been used to analyse changes in gene expression and alternate splicing in the lumbar spinal cord of mutant SOD1 mice (Chen et al., 2010). This approach demonstrates that the expression of genes involved in immunity and inflammation (e.g. CD68 upregulation); gliosis (GFAP and S100 upregulation), and lipid metabolism (LPL upregulation) is dysregulated in mutant SOD1 mice. In addition, a number of genes involved MAPK signalling pathway, T-cell receptor signalling pathway and cell adhesion molecules are both differentially expressed and alternatively spliced in mutant SOD1 mice compare to wild type control (Chen et al., 2010).

Changes in transcription factor expression and activation

There is evidence of dysregulation in the process of gene transcription in ALS disease, with aberrant changes in the expression and activation of a number of transcription factors.

Nuclear Erythroid 2-Related Factor 2 (Nrf2) is a transcription factor involved in coordinating the upregulation of cellular defences to oxidative stress. It acts on the antioxidant response element (ARE) in a number of cytoprotective genes including heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), and the catalytic subunit of glutamate cysteine ligase (GCLC) (Chan et al., 2001). There is evidence of reduced expression of Nrf2 in the spinal motor neurons of ALS cases compared to controls (Sarlette et al., 2008). Functionally, global genetic deletion of Nrf2 modestly accelerated the onset of disease but did not affect lifespan in mutant SOD1 mice (Guo et al., 2013). However, overexpression of Nrf2 specifically by astrocytes significantly delays onset of disease and extends the lifespan of mutant SOD1 mice (Vargas et al., 2008). In addition, pharmacological activation of Nrf2 enhances motor performance and prolongs lifespan of mutant SOD1 mice (Neymotin et al., 2011).

Activating transcription factor 3 (ATF-3), a basic leucine zipper transcription factor, is implicated in the cellular response to a variety of stresses. It has a wide variety of targets including genes involved in cell survival, proliferation, and death (Hai et al., 1999, Thompson et al., 2009). ATF-3 promotes neuronal survival and the regenerative response to axonal injury in vitro and in vivo (Seijffers et al., 2006, Seijffers et al., 2007). In addition, ATF-3 prevents inflammation and brain injury following transient focal cerebral ischaemia and

kainite injections in vivo (Wang et al., 2012c, Francis et al., 2004). ATF-3 expression has been found upregulated in the spinal motor neurons of mutant SOD1 mice and rats (Vlug et al., 2005, Malaspina et al., 2010). Functionally, genetic overexpression of ATF-3 delays onset of disease in mutant SOD1 mice (Seijffers et al., 2014). This effect was associated with increased neuronal survival and neuromuscular junction innervation (Seijffers et al., 2014).

Activating transcription factor 4 (ATF-4) mediates the upregulation of genes involved in the UPR and which function in amino acid and redox metabolism, autophagy, protein folding, and apoptosis (Zinszner et al., 1998, Harding et al., 2000, Harding et al., 2003). In apoptosis, ATF-4 controls the expression of the transcription factor CHOP and modulates the levels of multiple pro-apoptotic proteins such as BIM and PUMA (Galehdar et al., 2010, Puthalakath et al., 2007, Tabas and Ron, 2011). ATF-4 expression has been found upregulated in the motor neurons of ALS cases and mutant SOD1 mice (Hetz et al., 2009, Wang et al., 2011). Functionally, genetic deletion of ATF-4 reduces the rate of birth of mutant SOD1 mice. However, ATF-4 deficient SOD1 mice that are born have a delayed onset of disease and prolonged life span (Matus et al., 2013a). This protective effect is associated with abolished expression of pro-apoptotic genes, including BIM and CHOP (Matus et al., 2013a).

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcription co-activator which binds to the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) to interact with multiple transcription factors, including nuclear respiratory factors 1 and 2 and mitochondrial transcription factor A (Tfam) (Song et al., 2013). PGC-1α and PPAR-γ have roles in protecting cells from oxidative stress (Liang et al., 2007, Itoh et al., 2008). There is evidence of decreased expression of PGC-1α and the transcription factors it regulates in mutant SOD1 mice (Thau et al., 2012). In contrast, there is evidence of increased PPAR-γ activity in the spinal cord of SOD1 mice (Benedusi et al., 2012). Functionally, genetic overexpression of PGC-1α prolonged survival and decreased motor neuron degeneration in mutant SOD1 mice (Liang et al., 2011).

The transcription factor nuclear factor interleukin 3-regulated (NFIL3) has roles in the development and survival of immune cells and motor neuron (Male et al., 2012, Junghans et al., 2004). It is trans-activated by elevated intracellular Ca2+ concentration and activation of cAMP response element-binding protein (MacGillavry et al., 2009, Nishimura and Tanaka, 2001). NFIL3 expression has been found upregulated in motor neuron of mutant SOD1 mice (Tamai et al., 2014). Functionally, genetic overexpression of NFIL3 specifically within neurons delays onset of disease and reduces axonal degeneration in mutant SOD1 mice (Tamai et al., 2014).

Mitochondrial transcription factor A (TFAM), originally has important roles in the maintenance of mitochondrial DNA and its defence from oxidative damage (Larsson et al., 1997, Schonfeld and Reiser, 2006, Xu et al., 2009, Hayashi et al., 2008). TFAM has been demonstrated to play a role in neuronal survival following ischaemic brain injury (Hokari et al., 2010). Neuronal overexpression of a human TFAM transgene resulted in delayed onset of disease and improved motor scores in mutant SOD1 mice (Morimoto et al., 2012). This effect was associated with reduced motor neuron cell loss and markers of oxidative stress and apoptosis (Morimoto et al., 2012).

In addition to those described above, the expression or activation of other transcription factors has been found dysregulated in ALS tissues. However, the functional roles of these have not yet been assessed. There is evidence of increased expression and activation of c-Jun in the motor neurons of mutant SOD1 mice (Vlug et al., 2005, Jaarsma et al., 1996), but this increase is only observed in astrocytes in the spinal cord of ALS cases (Migheli et al., 1997). In addition, there is evidence of activation of STAT-3 in the spinal cord of ALS cases and mutant SOD1 mice (Shibata et al., 2009, Shibata et al., 2010). Furthermore, CCAAT/enhancer binding protein beta (C/EBP beta) expression is found upregulated in activated microglia in ALS cases and mutant SOD1 mice (Valente et al., 2012). Similarly, the expression of CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) is found upregulated in motor neurons of ALS cases and mutant SOD1 mice (Ito et al., 2009, Vlug et al., 2005). Transcription factor p53 is also upregulated in the ventral horn of the spinal cord of patients with ALS and within motor neurons in mutant SOD1 mice

(Eve et al., 2007, de Aguilar et al., 2000). The expression of FAC1 is also enhanced in motor neurons of ALS cases (Mu et al., 1997). In contrast, expression of nuclear factor kappa B (NF-kappa B) is found down regulated in the motor neurons but upregulated in the astrocytes of ALS patients (Sako et al., 2012, Migheli et al., 1997). Similarly, heat shock transcription factor 1 (HSF1) expression is downregulated in the spinal cord mutant SOD1 mice (Mimoto et al., 2012). Finally, the levels of HoxB2 expression do not change in mutant SOD1 mice but HoxB2 has been shown to bind mutant SOD1 and form part of inclusion within motor neurons in those mice (Zhai et al., 2005).

Evidence of a regenerative effort in ALS

The degenerative process in ALS is accompanied by morphological and molecular changes indicative of a regenerative effort. These changes can be observed at all anatomical levels of the neuromuscular motor unit. At the level of the cell body, histological analysis of the spinal cord of ALS cases and mutant SOD1 mice shows that degenerating motor neurons undergo the chromatolytic reaction characteristic of regenerating motor neurons (Martin, 1999, Manetto et al., 1988, Sobue et al., 1990, Sasaki et al., 2000b, Kusaka et al., 1988, Riancho et al., 2014). In addition, the increased expression of a number of regeneration associate genes and proteins, including c-Jun, AFT-3, Pi3K and GAP-43, can be observed in the motor neurons of ALS cases and mutant SOD1 mice (Malaspina et al., 2010, Parhad et al., 1992, Ikemoto et al., 1999, Wagey et al., 1998, Jaarsma et al., 1996, Lobsiger et al., 2007). At the ventral root, there is evidence of regenerating axons (Hanyu et al., 1982, Sobue et al., 1981, Fischer et al., 2004), as well as increased neurotrophic signalling (Kerkhoff et al., 1991, Lobsiger et al., 2009) in both ALS cases and mutant SOD1 mice. At the level of the muscle there is axonal terminal sprouting, collateral sprouting and reinnervation of NMJs (Tsujihata et al., 1984, Atsumi, 1981, Hegedus et al., 2008, Hegedus et al., 2007) and proregenerative gene expression by denervated muscle fibres (Gonzalez de Aguilar et al., 2008, Weis et al., 1998, Yamamoto et al., 1996, Bjornskov and Norris, 1984, Frey et al., 2000b).

These regenerative changes appear to have a functional significance in ALS disease as it has been demonstrated that muscle weakness only becomes

apparent when NMJ reinnervation fails to compensate the ALS mediated denervation (Felice, 1997, Hayworth and Gonzalez-Lima, 2009, Gordon et al., 2004). In this vein, there is evidence that ALS preferentially affects motor neurons depending on their regenerative ability. Axons innervating neuromuscular junctions have differential terminal sprouting potential (Frey et al., 2000b). Interestingly, it has reported that synapse types that fail to exhibit terminal sprouting are selectively vulnerable to ALS disease, whereas synapses undergoing robust paralysis-induced sprouting are selectively resistant (Frey et al., 2000b). In addition, interventions that enhance this regenerative effort ameliorates disease in mutant SOD1 mice. For example genetic overexpression of the regeneration associated transcription factor ATF-3 prolongs the survival of mutant SOD1 mice by enhancing terminal axonal sprouting and NMJ innervation at the muscle (Seijffers et al., 2014). In addition, pharmacological inhibition of Rho Kinase, which is associated with enhanced neurite outgrowth in vitro, improves motor performance and prolongs survival of mutant SOD1 mice (Tonges et al., 2014, Gunther et al., 2014). In contrast, deletion genetic deletion of miR-206, a microRNA required for efficient regeneration of neuromuscular synapses after axotomy, exacerbates disease and shortens lifespan of mutant SOD1 mice (Williams et al., 2009). Thus a regenerative effort mounted by degenerating motor neurons may modulate ALS disease.

Trauma and ALS

The aetiology of ALS remains elusive with multiple risk factors loosely associated with the disease (Longo et al., 2012). A link between ALS and trauma to the nervous system has been suggested for over one hundred years (Woods, 1911) and a number of retrospective epidemiological studies support this (Chen et al., 2007).

Higher incidence rates of ALS have been found in populations were trauma is more prevalent, including military veterans, American-football and soccer and players (Coffman et al., 2005, Chio et al., 2005, Abel, 2007, Chio et al., 2009). A number of case-control studies and meta-analysis have indicated that trauma to head, neck or back are more likely to have occurred to patients with ALS prior to their diagnosis than control populations (Kondo and Tsubaki, 1981, Strickland et al., 1996, Chen et al., 2007, Binazzi et al., 2009, Schmidt et al., 2010, Pupillo et al., 2012). Although these studies suggest that ALS may result from traumatic nervous injury, the majority fail to confirm any radiological or neurophysiological evidence of nervous injury in the studied ALS groups prior to diagnosis.

In traumatic brain injury the brain and spinal cord undergo shear deformation that produces transient elongation and injury of axons, which are associated with subsequent plastic cellular and molecular reactions that resemble those following peripheral nerve axotomy (Buki and Povlishock, 2006). Interestingly, a case control study demonstrates that ALS patients are more likely to have suffered radiologically confirmed myelopathy due to spinal spondylosis prior to diagnosis (Setzer et al., 2008). Furthermore, a number of cases of ALS in military personnel have been associated with preceding peripheral nerve injury (Riggs, 1993, Riggs, 2001). This suggests that axonal injury may modify the ALS pathogenic process in certain individuals.

It has been suggested that genetic and epigenetic factors may contribute to a predisposition to the development of ALS following trauma (Malaspina et al., 2010, Yip and Malaspina, 2012). This has been supported by evidence that spondylotic myelopathy prior to ALS is more common in people carrying the ApoE epsilon 4 allele (Setzer et al., 2008). In addition, a FIG4 mutation associated with ALS was first described in case were quadriplegia occurred

subsequent to trauma to the first affected limb (Chow et al., 2007, Chow et al., 2009).

Interestingly, recent molecular analysis of SOD1G93A mice after injury of the peripheral or central nervous system demonstrate defects in their normal response to such injuries (Malaspina et al., 2010, Yip and Malaspina, 2012, Mesnard et al., 2011a, Haulcomb et al., 2014, Mesnard-Hoaglin et al., 2014). SOD1G93A mice show increased expression pro-apoptotic and pro-inflammatory genes and reduced expression of pro-survival genes (Malaspina et al., 2010, Yip and Malaspina, 2012, Mesnard et al., 2011a, Haulcomb et al., 2014, Mesnard-Hoaglin et al., 2014). Thus, a defect in the response to traumatic nervous injury may play a role in ALS pathogenesis.

Peripheral Nerve Regeneration

The neuropathological changes observed in ALS resemble those following axotomy, including loss of target innervation at the neuromuscular junctions, axonal loss and motoneuron death, as well as reactive neuroinflammatory changes (Fischer et al., 2004, Raivich et al., 2004). In addition, there is evidence of a regenerative effort mounted by the nervous system in ALS disease. Thus, analysis of the physiological response of the nervous system to acute axonal injury may shed light on factors which play a role in the pathological process observed in ALS.

Morphological changes

Cell body response

Following peripheral nerve injury, the injured neuronal cell body exhibits a number of ultrastructural changes. Accumulations of rough endoplasmic reticulum, known as Nissl bodies, are found randomly dispersed in the neuronal cytoplasm, rather than in the normal linear arrangement (Lieberman, 1971). These structures are visible under light microscopy when stained with basophilic dyes and their dispersion is termed ‘chromatolysis’ or ‘chormatolytic reaction’ (Nissl, 1894). The neuronal cell body swells, the nucleus moves to the cellular periphery and synaptic terminals retract (Lieberman, 1971). There is also an increase in intracellular organelles, such as mitochondria and ribosomes. These changes are consistent with an increase in cellular metabolism and protein synthesis, which are necessary for axonal growth and repair (Raivich and Makwana, 2007) (Fig.2B). If axotomised neurons fail to connect with their peripheral target, the injured neurons may become atrophic, remain in the chromatolytic state or even go on to degenerate and die (Deumens et al., 2010).

Axonal response

Following axotomy, axonal changes are observed proximally and distally to the site of injury.

Distal changes

The distal nerve segment undergoes anterograde changes termed Wallerian degeneration (Perry et al., 1987) (Fig. 2B). Around 24 hours after injury, the axons bead and swell. Subsequently, granular disintegration of the cytoskeleton occurs, breaking axons into fine debris (George et al., 1995, Sievers et al.,

2003, Bignami and Ralston, 1969, Trojanowski et al., 1984). In addition, the myelin sheath partitions into fatty material (Lassmann et al., 1978). Remaining myelin debris contains molecules that are inhibitory to the growing axon including myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) (Huang et al., 2005). Schwann cells degrade the myelin and extracellular debris and are the main phagocytic cell in early time points after injury (Perry et al., 1995). Infiltrating macrophages are gradually recruited by Schwann cells, through the release of cytokines (Tofaris et al., 2002), and they take over the process of phagocytosis from Schwann cells at later time points (Tanaka et al., 1992). Schwann cells proliferate within the basal lamina tubes, where they align to form bands of Büngner. The create a pathway for the regenerating axon to reach its peripheral target and also provide necessary substrates and growth factors (Bradley and Asbury, 1970, Stoll et al., 1989).

Proximal changes

Following the resealing of the severed axoplasm, the tip of the axon swells and is inflated with smooth endoplasmic reticulum, mitochondria, and microtubules (Meller, 1987, Vallee and Bloom, 1991). These axonal swellings eventually give rise to a sheet-like expansions called a lamellapodia, from which numerous fine processes called filopodia extend; these structures form the ‘growth cone’, first described by Santiago Ramon y Cajal (1928). Growth cones are highly motile structures (Spira et al., 2003), which are steered towards the peripheral target by guidance cues and growth promoting signals found in the bands of Büngner (Ide, 1996). Once the regenerating axon has crossed the site of injury, its rate of elongation is around 1-4mm per day (Bisby and Keen, 1985, Forman and Berenberg, 1978, Gutmann et al., 1942) (Fig. 2C &D).

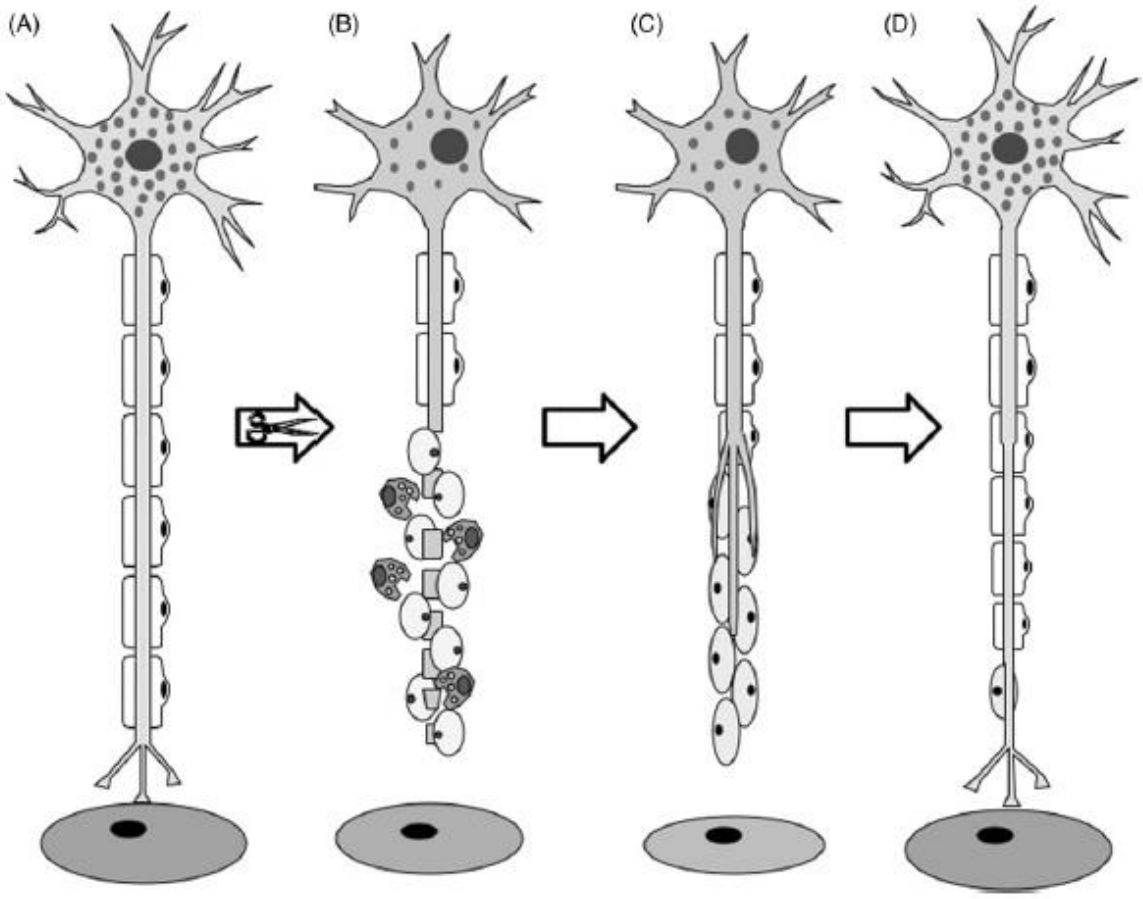


Figure 2: Schematic of the main events of degeneration and regeneration after peripheral nerve injury. (A) Normal nerve fibre, maintaining synaptic contact with target cells. (B) Transection of the fibre results in distal fragmentation of axon and myelin sheaths. Macrophages and Schwann cells phagocyte degraded materials. Chromatolysis at the neuron soma and dendritic arbour retraction occur. (C) Fine sprouts emerge from the proximal axonal end, and elongate in association with the proliferated Schwann cells in the distal segment, that line up in bands of Büngner. (D) Axonal reconnection with target cells and maturation of the nerve fibre. The regenerated axon remains of smaller calibre and with shorter internodes than normal. The neuron returns to a normal transmitting phenotype. Target cells may suffer atrophy and phenotypic changes during denervation (Navarro et al., 2007).

Axonal sprouting and functional recovery

Morphologic studies by Ramon y Cajal (1928) demonstrate that injured axons produce a number of branches or sprouts, which are tipped by growth cones. Sprouting has been observed at the nodes of Ranvier (Cajal, 1928), distal part of dendrites (Fenrich et al., 2007) and at the level of the injured neuronal cell body after axonal injury (Linda et al., 1985, Makwana et al., 2010). Aberrant axonal sprouts are removed or pruned, particularly those that regenerate through inappropriate distal pathways and innervate the wrong targets

(Brushart, 1993, Brushart et al., 1998). Importantly, functional recovery depends on the accuracy of target reinnervation and it is therefore dependent on pruning of aberrant sprouting (Brushart, 1993, Brushart et al., 1998).

In rodents, following a crush injury, axons regenerate, show accurate target innervation, and there is good functional recovery (Magill et al., 2007). An evaluation of axonal innervation of the motor endplate at the neuromuscular junction (NMJ), demonstrate that intact mice have one axon innervating one motor endplate (Magill et al., 2007). However, one week after peripheral nerve crush all of the motor endplates are denervated, at two weeks they are partially innervated, at 3 and 4 weeks they are hyperinnervated or polyinnervated, and by six weeks all endplates are singly innervated like intact controls. At six weeks after nerve crush, and not prior to that, functional recovery reaches its maximum level, suggesting that polyinnervation of motor endplates is not ideal for functional recovery (Magill et al., 2007).

Interestingly, after peripheral nerve transection, target innervation is inadequate and has been associated with a less than optimal functional recovery (Angelov et al., 2005). Following transection, motor endplates can be incorrectly reinnervated by axons that have regenerated through wrong nerve fascicles (Angelov et al., 2005). This has been associated with uncoordinated muscle activity (Angelov et al., 2005). In addition, poor functional recovery after transection has been explained by supernumerary collateral branches innervating one muscle fibre (Bendella et al., 2011). Interestingly, reducing the amount of axonal branching has been shown to yield better functional recovery after axonal injury (Angelov et al., 2005).

Finally, although axons may regenerate and innervate targets, the diameter of the regenerated axons as well as their conduction velocity and excitability has been shown to be below normal (Fields and Ellisman, 1986a, Fields and Ellisman, 1986b), which may result in incomplete or inadequate recovery of the innervated muscle (Fields and Ellisman, 1986a, Fields and Ellisman, 1986b).

Inflammatory and glial response to injury

Microglia

Following injury, microglial activation is apparent by a change in their morphology and the expression of cell adhesion molecules, cytoskeletal proteins, and antigen presentation molecules (Table 3). This molecular change occurs in a stepwise manner, which can be divided into the following stages; resting microglia in the normal brain (stage 0), state of alert (stage 1), homing (stage 2), phagocytosis (stage 3a) and bystander activation (3b) (Raivich et al., 1999).

In the normal brain (stage 0), microglia with their ramified morphology express receptors for complement fragments, and Fcγ receptors that bind to immunoglobulins (Peress et al., 1993, Williams et al., 1994). In the state of alert (stage 1), microglia increase the expression of immunoglobulin-G, thrombospondin and the αMβ2-integrin, a receptor for the complement 3ib fragment (Kloss et al., 1999, Moller et al., 1996). The cell surface receptor ligand, intracellular adhesion molecule 1(ICAM1), is also expressed. This ligand mediates the adhesion of microglia to lymphocytes, granulocytes as well as other microglia in the vicinity, by interacting with αMβ2 and αLβ2 integrins, which are found in the cell surface of these cells (Hynes, 1996).

Next, the homing stage (stage 2) is characterized by the homing and adhesion of microglia to damaged neurons or degenerating dendrites. This step in associated with an increase in the expression of α5β1 and α6β1integrins in mice (Hailer et al., 1997, Kloss et al., 1999), as well as cytoskeletal proteins like vimentin (Graeber et al., 1988, Raivich et al., 1993). A reduction in the ramification of microglial processes is also evident; this change in morphology aids mobility and adhesion properties (Angelov et al., 1995). Microglia are found to move into direct contact with injured neuronal cell bodies, where the cells displace synaptic input in a process called synaptic stripping (Blinzinger and Kreutzberg, 1968). At this stage, a decrease in the alert phase markers such as αMβ2 and ICAM1 is accompanied by an increase in the receptors for microglial mitogens such as, macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) (Raivich et al., 1998a), which are associated with an increase in the number of local microglia

(Raivich et al., 1994). In addition, microglia in stage 2 show a weak expression of major histocompatibility complex (MHC) class I molecules (Raivich et al., 1993, Streit et al., 1989) and immunoaccessory glycoproteins, like B7.2 (Bohatschek et al., 1998). Microglia can return to a non-activated state in the absence of cell death, by decreasing in density, becoming highly ramified, being territorially distributed and having normal levels of complement receptors, Fcγ-receptors and IgG immunoreactivity (Raivich et al., 1999).

In the stage of phagocytosis (stage 3a), neuronal death mediates the transformation of microglia into phagocytic cells that remove debris, such as axons and myelin in Wallerian degeneration (Moller et al., 1996, Streit and Kreutzberg, 1988). When the removal of large debris like degenerating pyramidal neurons is required, microglia form clusters or nodules of 3 to 20 microglia (Moller et al., 1996, Streit and Kreutzberg, 1988). This change is associated with a strong upregulation in the expression in α5β1 and α6β integrins, MHC1 antigen-presenting complex, as well as the de-novo expression of MHC2. In addition, the B7.2, ICAM1 and αXβ2 integrin, which may serve as stimuli to activate T-cells in the presence of a specific antigen, are also strongly expressed. Finally, bystander activation (stage 3b) is also observed in the presence of phagocytosis. Microglia in the vicinity of phagocytic microglia, show an increase in MHC1, B7.2, α4β1, αMβ2 and ICAM1 (Bohatschek et al., 1998, Kloss et al., 1999, Raivich et al., 1998b). In addition, when lymphocytes infiltrate they aggregate in a concentric distribution around microglial nodules (Raivich et al., 1998b), which suggests the presence of a soluble cytokine produced by phagocytotic microglia.

Table 3: Molecular markers of microglial activation. Abbreviations: w: weak, m: moderate and s: strong expression as identified using immunoreactivity. Edited from Raivich et al.,(1999).

Grading	Type of response	Specific markers (mouse)
stage 0	Normal brain	FcγR (w), IgG (w), αMβ2 (w)
stage 1	State of alert	IgG (m), αMβ2 (s), ICAM1 (s)
stage 2	Homing	α5β1 (s), α6β1 (s), M-CSFR (s), MHC1(w), B7.2(w)
stage 3a	Phagocytosis	MHC1 (s), B7.2 (s), αXβ2 (s), αMβ2 (s), α5β1 (s), α6β1 (s), ICAM1 (s), IgG (s)
stage 3b	Bystander activation	MHC1 (m), B7.2 (m), α4β1(m), αMβ2 (m), ICAM1 (m)

During the process of activation, microglial cells secrete pro-inflammatory cytokines including tumour necrosis factor-alpha (TNFα), Interferon-γ, and interleukin 1β; and upregulate the expression of oxidant molecules such as NO, which can all have neurotoxic effects (Welser-Alves and Milner, 2013, Kawanokuchi et al., 2006, Eriksson et al., 1998, Schluter et al., 1997, Sheng et al., 1997, Zhang et al., 1998, Minghetti and Levi, 1998, Ding et al., 1997) However, microglia are also involved in neuronal protection following injury. Microglia are well known for producing trophic factors including brain derived neurotrophic factor (BDNF), and insulin-like growth factor 1 (IGF1) (Gomes et al., 2013, Suh et al., 2013).

Astrocytes

In response to neuronal injury, astrocytes rapidly increase the synthesis of GFAP (Tetzlaff et al., 1988). This is followed by transformation of protoplasmic astrocytes into stellar, fibrillary astrocytes (Graeber and Kreutzberg, 1986). This change is controlled by the cytokine transforming growth factor-beta 1 (TGFb1), as the absence of this cytokine results in the decrease of GFAP-positive fibrillary actrocytes following brain injury in mice (Jones et al., 1998). In the severely injured brain, protoplasmic astrocytes express the cell adhesion molecule CD44 and demonstrate a velate morphology (Raivich et al., 1999).

T-cells

The presence of T-cells is evident following CNS injury (Raivich et al., 1999). The extent of T-cells being recruited to the brain depends on the severity of injury. After mild or indirect neuronal injury, only a fewer T-cells are seen one

day after axotomy (Raivich et al., 1998b). However, the numbers of T-cells dramatically increase in the presence of cell death, with 100-fold increase in T-cell influx in injured tissue compared to the unaffected tissue (Raivich et al., 1998b). The population of T-cells entering the brain is mixed, with CD3-, CD4- as well as CD8-positive T-cells being present. T-cells in the brain aggregate around phagocytic microglia that express MHC1, B7.2 and αXβ2, which are important molecules involved in antigen presentation (Raivich et al., 1999).

Molecular changes

Following peripheral nerve injury, stress signals induces the expression of transcription factors, adhesion molecules, growth associated proteins and structural components within axotomised neurons (Raivich and Makwana, 2007).

Injury cues to axotomised neurons

There are three main cues that inform the neuron of injury (Patodia and Raivich, 2012b): 1) the deprivation of neurons from neurotrophic factors following axonal injury leads to the disinhibition of regeneration processes within 12 -24 hours following injury (Raivich et al., 1991); 2) Proteins from the extracellular environment and neighbouring cells enter through the tip of the axon and are carried retrogradely to the neuronal cell body (Schmied et al., 1993, Hanz et al., 2003); 3) There is rapid influx of extracellular ions such as calcium and sodium through the transiently opened axonal membrane (Yoo et al., 2003), subsequently followed by an increase in injury-mediated action potentials that cause an elevation of intracellular calcium and cAMP, which can activate various downstream pathways (Berdan et al., 1993) (Berdan et al., 1993).

Signalling cascades

Injury signals are propagated within the neuron via signalling cascades, which use a network of interacting proteins to influence cellular processes (Raivich and Makwana, 2007). One such signalling pathway, collectively known as the Mitogen-activated protein (MAP) kinase cascade, uses signals from growth factor receptors to sequentially stimulate protein kinases (Raivich and Makwana, 2007). This phosphorylation cascade activates regulatory molecules in the cytoplasm and cell nucleus to initiate, within the injured neuron, processes that may lead to neuronal regeneration (Raivich and Makwana, 2007). In vivo, the kinases ras, raf, the mitogen-associated protein kinase kinase (MEK) and the group of extracellular signal-regulated kinases 1 and 2 (erk1, erk2), are up-regulated after axonal injury (Kiryu et al., 1995). In vitro, neurite outgrowth and local protein synthesis is dependent on the kinases Erk1 and 2, p38 and caspase-3 (Campbell and Holt, 2003, Verma et al., 2005), as well as cAMP, and the cAMP-dependent protein kinase A (PKA) (Chierzi et al., 2005). Additionally, the ras, raf, mitogen-associated protein kinase kinase

(MEK) and the group of Erk 1 and 2 molecules, have been shown to promote survival and neurite growth of cultured embryonic neurons (Borasio et al., 1989, Ihara et al., 1997). Furthermore, neurite outgrowth of cultured adult neurons is dependent on the translocation of Erk from the axon to the cell body (Perlson et al., 2005).

The phosphatidylinositol-3 kinase-Akt (PI3K-Akt) pathway is also involved in axonal regeneration following injury. Expression of the PI3K gene (Ito et al., 1996) and the subsequent activation of protein kinase B or Akt are observed following axotomy (Owada et al., 1997, Murashov et al., 2001). Mice with altered PI3K catalytic ability have reduced signalling through Akt, dampened axonal regeneration and impaired functional recovery after injury (Eickholt et al., 2007). Additionally, in neonates, Akt overexpression promotes neuronal survival following hypoglossal nerve injury (Namikawa et al., 2000). In adults, Akt overexpression promotes accelerated axonal regeneration following axotomy (Namikawa et al., 2000).

Jun-N-terminal kinases (JNK) also participate in the neuronal response to axotomy. An increase in activated JNK is found in the rat dorsal root ganglion following peripheral nerve injury (Kenney and Kocsis, 1998). JNKs carry out the N-terminal phosphorylation of the transcription factor c-Jun (Waetzig et al., 2006). After peripheral nerve injury there is an increase in JNK activation, as well as phosphorylated c-Jun (Waetzig et al., 2006). Inhibition of JNKs blocks neurite outgrowth in cultured adult neurons (Lindwall et al., 2004). In addition, neuron specific genetic deletion of JNK1 and JNK2 delays functional recovery after facial nerve injury in mice (Ruff et al., 2012).

Transcription factors

Transcriptional factors contribute to the changes in neuronal gene expression after injury. The activation of signalling pathways in injured neurons leads to the increased expression and nuclear translocation of some transcription factors such as, c-Jun, Jun D, ATF3 (cyclic AMP-dependent transcription factor), STAT3, P311, p53, CREB (cAMP response element-binding), Sox11 (sex-determining region Y box-containing 11) and C/EBP (CCAAT/enhancer binding protein) β, δ. (Schwaiger et al., 2000, Raivich et al., 2004, Nadeau et al., 2005,

Di Giovanni et al., 2006, Jankowski et al., 2009, Moore and Goldberg, 2011, Ruff et al., 2012, Patodia and Raivich, 2012b). In contrast, the expression of other transcription factors such as islet-1, Fra-2, ATF2, and TDP-43, is downregulated (Doyle and Hunt, 1997, Herdegen et al., 1997, Hol et al., 1999, Moisse et al., 2009b, Sato et al., 2009). These transcription factors bind to complementary DNA promoter regions and increase or repress the expression of target genes (Patodia and Raivich, 2012b).

Regeneration associated genes

Changes in transcriptional regulation are subsequently followed with altered expression of a number of regeneration associated genes after injury, which can be grouped into the following categories; adhesion molecules, guidance molecules, neuropeptides and cytoskeletal adaptors (Patodia and Raivich, 2012a).

Adhesion molecules

Adhesion molecules on the surface of the axonal growth cone, guides the axon along the bands of Büngner towards its target (Grumet, 1991, Shapiro et al., 2007). Following injury, axons express integrins such as α7β1, as well as receptors for laminin, fibronectin and paxillin, which are molecules found on the surface of Schwann cells and the inner lining of the neural tube basal membrane (Kloss et al., 1999, Werner et al., 2000, Vogelezang et al., 2001, Vogelezang et al., 2007, Ekstrom et al., 2003, Wallquist et al., 2004, Gardiner et al., 2005).The expression of the receptor CD44, which binds to hyaluronic acid (Jones et al., 2000), and galectin-1, a receptor for galactoside residues (Horie and Kadoya, 2000, Akazawa et al., 2004), are also upregulated. In addition, axons express molecules involved in homophilic binding such as ninjurin (Araki and Milbrandt, 2000) and gicerin/CD146 (Hiroi et al., 2003). Furthermore, enzymes such as urokinase and plasminogen activator (PA), metalloproteinases (MMP) 2, 3 and 9 (Demestre et al., 2004, Shubayev and Myers, 2004), and damage-induced neuronal endopeptidase (DINE) (Kiryu-Seo et al., 2000) are also expressed by the regenerating axons.

Thus far, the adhesion molecules that have been functionally characterised in vivo include α7β1, and the β2-microglobulin (β2-MG). A moderate reduction in

axonal regeneration was found in mice deficient of β2-MG (Oliveira et al., 2004). A decrease in the speed of axonal regeneration and reinnervation of the peripheral tissue was observed in mice deficient of α7β1 (Werner et al., 2000). Although the function of CD44 in regeneration has not yet been investigated in vivo, it has been demonstrated that antibody inhibition of CD44 affects the routing of retinal axons in brain slice preparations (Lin and Chan, 2003).

Neuropeptides

Specific neuronal populations express neuropeptides after axonal injury and these neuropeptides can be found in the most distal part of the growing axon known as the growth cone (Raivich and Makwana, 2007). Following injury, subpopulations of spinal and motor neurons can upregulate the neuropeptides calcitonin gene-related peptide (CGRP), galanin, and pituitary adenylate cyclase activating peptide (PACAP) (Moore, 1989, Raivich et al., 1995). Functionally, when the PACAP gene is deleted the promotion of the initial axonal growth that is seen within 24 hours after injury and reinnervation of the peripheral target is delayed (Armstrong et al., 2008). The deletion of galanin or its receptor results in a reduction in speed of regeneration after sciatic nerve crush (Holmes et al., 2000). Reducing the production of intra-axonal CGRP with short interfering RNA (siRNA) decreases axonal growth across a conduit between the proximal and distal nerve stumps (Toth et al., 2009). In addition, blocking the receptor for CGRP in Schwann cells with siRNA, results in decreased axonal growth, which suggests that axon interaction with Schwann cells, is important for neurite growth (Raivich et al., 1992, Toth et al., 2009).

Guidance molecules

Guidance molecules allow elongating axons to navigate to the correct destination by inducing axonal attraction or repulsion signals. Many guidance molecules that promote growth have been identified, for example, Bex1, an intracellular adaptor molecule that interacts with p75NTR, and neuropilin-2, a transmembrane receptor protein that binds to semaphorins, are strongly up regulated after axonal injury (Lindholm et al., 2004). Mice deficient of Bex1 (Khazaei et al., 2010) or neuropilin-2 (Bannerman et al., 2008) showed significantly slower functional recovery than controls. However, deleting their complementary receptors does not have such a strong effect. The deletion of

the Bex1 receptor, p75NTR, fails to alter neuronal survival or speed of functional recover (Khazaei et al., 2010). In addition, deleting the semophorin recpetors 3B, 3C and 3F, which bind to neuropilin-2, has no effect on functional recovery, but reduces the density of the light neurofilament-positive axons in the distal nerve (Bannerman et al., 2008).

Molecules that inhibit growth are also involved in axonal regeneration, for example, the G-protein RhoA and the Rho-associated kinase (Hiraga et al., 2006, Madura et al., 2007, Cheng et al., 2008). After injury, the G-protein RhoA and the Rho-associated kinase are strongly expressed (Hiraga et al., 2006, Madura et al., 2007, Cheng et al., 2008). Inhibition of RhoA GTPase signalling through RhoA-associated kinase (ROCK) accelerated functional recovery and increased the density of both myelinated and unmyelinated axons after peripheral nerve injury (Hiraga et al., 2006, Madura et al., 2007).

Cytoskeletal adaptors

The growth cone is a motile structure at the distal tip of the elongating axon, which interacts with the surrounding cells and the extracellular substrate to find its target. This process as well as being dependent on adhesion molecules, guidance molecules, and neuropeptides, is also dependent on the adaptor molecules within the growth cone (Baas and Ahmad, 2001, Ellezam et al., 2002, Zhang et al., 2003, Madura et al., 2004, Bouquet et al., 2004). The major adaptor proteins expressed in the growth cone are the GAP43/neuromodulin and cytoskeleton-associated protein 23 (CAP23) (Goslin and Banker, 1990, Bomze et al., 2001). These molecules are important in cytoskeletal remodelling of the growth cone. Depleting cultured neurons of GAP-43, resulted in abnormal growth cone adherence andmobility, failure to express f-actin, reduced axonal branching and enhanced sensitivity to inhibitory stimuli (Aigner and Caroni, 1995). In vivo, GAP-43 is important in path-finding, as the axons of mice deficient of GAP-43 enter inappropriate tracts during development (Strittmatter et al., 1995). Mice lacking CAP23 fail to produce sprouting at the neuromuscular junction following a muscular injection of botulinum toxin A (Frey et al., 2000a). Alternatively, the over expression of GAP43 or CAP23 promotes excessive neuromuscular sprouting (Caroni, 1997). Neuronal deletion of the CAP23 gene

dramatically reduces the level of innervation of the mouse whisker pad, following transection of the facial nerve (Werner et al., 2000).

Cell death signals

The cell surface molecules that mediate neuronal cell death following axotomy include Fas, TNFR 1 and 2 (Terrado et al., 2000b, Raivich et al., 2002, Ugolini et al., 2003), and even p75NTR (Ferri et al., 1998, Terrado et al., 2000a, Gschwendtner et al., 2003). Apart from TNF-2, these receptors exert their proapoptotic effects through FADD (Fas-Associated protein with Death Domain). Animals lacking FADD demonstrated enhanced neuronal protection to axotomy induced cell death signal due to Fas or TNFR (Ugolini et al., 2003). Tumor suppressor p53 and its downstream cascade also influence neuronal cell death (Kiryu-Seo et al., 2005). Mice that are deficient for p53 show a strongly elevated amount of neuronal survival after hypoglossal nerve transection in mice. This is associated with a reduced expression of Bcl-2 homology domain 3 (BH3)-only protein Noxa, the actin-binding protein Coronin 1b and the GTPase Rab13, all of which regulate neurite outgrowth. Deficiency of p53 decreases neurite outgrowth and axonal regeneration (Di Giovanni et al., 2006). Furthermore, in neonates, axotomy induced cell death has been ameliorated by deletion of bax and by inhibiting caspase 3 or the whole family of caspases (Sun and Oppenheim, 2003, Chan et al., 2003).

Proteins studied in this thesis

In this thesis three ALS associated proteins, namely TDP-43, FUS and alsin, and the regeneration associated transcription factor c-Jun were studied in the context of motor neuron degeneration and regeneration.

TDP-43

Autosomal dominant mutations in TARDBP, the gene coding for TAR DNA-binding protein 43 (TDP-43), have been found in around 3% of familial and 1.5% of sporadic cases of ALS (Lagier-Tourenne and Cleveland, 2009). In addition, TDP-43 is a major constituent of the intracellular protein inclusions found in ALS cases at post-mortem (Tan et al., 2007, Mackenzie et al., 2007). TDP-43 is a 414 amino acid protein, member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. It contains five functional domains: two RNA recognition motifs (RRM1 and RRM2), a nuclear export signal (NES), a nuclear localization signal (NLS) and a glycine-rich sequence at the C-terminal. Each of the RRM domains has two highly conserved hexameric ribonucleoprotein 2 (RNP2), and octameric ribonucleoprotein (RNP1) regions, which are involved in DNA and RNA binding; the NES and NLS enable TDP-43 to shuttle between the nucleus and the cytoplasm; and glycine-rich C-terminal mediate protein::protein interactions (Lagier-Tourenne and Cleveland, 2009). Most of the ALS associated mutations identified so far are localized in the C-terminal region (Lagier-Tourenne and Cleveland, 2009). TDP-43 is ubiquitously expressed across different cell types and normally localised to nucleus of cells (Ou et al., 1995). However, in the presence of cellular stress, TDP-43 translocates to the cytoplasm (Ayala et al., 2008b). This is particularly apparent in the neurons and glia of ALS cases, where TDP-43 is absent from the nucleus and instead found in cytoplasmic inclusions (Neumann et al., 2006, Dickson et al., 2007). In ALS, TDP-43 undergoes aberrant post-translational modifications; it is ubiquinated, hyperphosphorylated and cleaved producing C-terminal fragments (CTFs) of around 25 kDa (Neumann et al., 2006, Arai et al., 2006).

TDP-43, as a member of the hnRNP family, has roles in multiple levels of RNA processing including transcription, splicing, transport and translation (Buratti and Baralle, 2008). Within the nucleus TDP-43 has been found to localise to sites of transcription and cotranscriptional splicing (Casafont et al., 2009).TDP-

43 has been described to repress transcription by binding to: TAR DNA sequence of human immunodeficiency virus type-1 (HIV-1) (Ou et al., 1995), the mouse SP-10 gene promoter and GT repeats in the target gene sequence of cyclin-dependent kinase 6 (Cdk6) (Abhyankar et al., 2007, Lalmansingh et al., 2011, Ayala et al., 2008a). In addition, TDP-43 plays an important role in the regulation of mRNA splicing (Buratti et al., 2001). TDP-43 associates with a number of proteins involved in splicing through its glycine rich C-terminal domain (Buratti et al., 2005, Freibaum et al., 2010). Recent work using cross-linking and immunoprecipitation coupled with high-throughput sequencing has identified TDP-43 binding sites in the RNA of 6,304 genes in the mouse brain (Polymenidou et al., 2011). In addition, massively parallel sequencing and splicing-sensitive junction arrays have revealed that levels of 601 mRNAs are changed and 965 altered splicing events occur after depletion of TDP-43 from mouse adult brain using antisense oligonucleotides (Polymenidou et al., 2011). The mRNAs affected by TDP-43 depletion included those from genes involved in neurodegeneration such as FUS, progranulin and the progranulin receptor sortilin (Polymenidou et al., 2011). Furthermore, TDP-43 is also involved in micro-RNA (miRNA) processing (Buratti et al., 2010). It is found in perichromatin fibres (Casafont et al., 2009) a nuclear region specifically associated with miRNA biogenesis (Lin et al., 2006) and within the microprocessor protein complexes involved in mRNA biogenesis and maturation (Gregory et al., 2004, Kawahara and Mieda-Sato, 2012). Functionally, experiments where TDP-43 expression is knocked down have demonstrated that the expression of a number of miRNAs is under the control of TDP-43 (Buratti et al., 2010, Kawahara and Mieda-Sato, 2012). Importantly, these miRNAs are involved in neuronal function, differentiation and synapse formation (Li et al., 2013b, Kawahara and Mieda-Sato, 2012).

TDP-43 binds a number of RNA targets in the cytoplasm through their three-prime untranslated region (3´UTR) (Tollervey et al., 2011). It interacts with proteins involved in RNA transport (Freibaum et al., 2010), is trafficked in neurons (Fallini et al., 2012, Wang et al., 2008a) and can be detected in presynaptic membrane of axon terminals and dendrite terminals (Narayanan et al., 2013). Functionally, mutations in TDP-43 impair mRNA transport in vivo and

in vitro (Alami et al., 2014). In addition, TDP-43 is a component of RNA stress granules, microscopically visible cytoplasmic bodies consisting of mRNA and RNP complexes, which stall translation under conditions of cellular stress (Freibaum et al., 2010, Anderson and Kedersha, 2008, Anderson and Kedersha, 2009). Functionally, TDP-43 binds to other protein within SGs and it contributes to the formation and maintenance of SGs (McDonald et al., 2011).

It is unclear whether mutations in TDP-43 cause toxicity via a loss of normal function or a toxic gain of aberrant function (Lagier-Tourenne et al., 2010). Genetic deletion of both TDP-43 alleles is embryonically lethal in mice (Wu et al., 2010, Sephton et al., 2010, Kraemer et al., 2010). However, mice with a single TDP-43 allele knocked out have been reported to be developmentally indistinguishable from control littermates (Wu et al., 2010, Sephton et al., 2010, Kraemer et al., 2010). Nonetheless, aged heterozygous TDP-43 knockout mice develop motor dysfunction in the absence of detectable neurodegeneration (Kraemer et al., 2010). In line with an essential role in cellular viability, knockdown of TDP-43 induces cell death in cultured tumour cells and differentiated neurons (Ayala et al., 2008a, Iguchi et al., 2009). However, wild-type TDP-43 overexpression also results in neurotoxicity in vitro associated with increased cytoplasmic localisation of TDP-43 protein (Barmada et al., 2010). Cytoplasmic accumulation and toxicity is worsened by disrupting the NLS motif and by ALS associated mutations in TDP-43 (Barmada et al., 2010), suggesting that cytoplasmic translocation of TDP-43 may mediate toxicity. However, TDP-43 transiently moves from the nucleus to the cytoplasm as a normal physiological response to axonal injury (Moisse et al., 2009b, Sato et al., 2009).

In ALS, TDP-43 is aberrantly cleaved by caspase-3, resulting in 25 kDa CTFs being present in the brain and spinal cord of cases with the disease (Zhang et al., 2009). These CTFs accumulate in protein inclusion found in ALS cases (Neumann et al., 2006, Arai et al., 2006). Whole TDP-43 and CTFs in ALS protein inclusion are hyperphosphorylated and polyubiquitinated (Neumann et al., 2006, Arai et al., 2006). Interestingly, expression of 25 kDa CTFs in cells results in cytotoxicity associated with the CTFs being accumulated cytoplasmically, forming insoluble aggregates and being subject to hyperphosphorylation and polyubiquitination (Igaz et al., 2009, Zhang et al.,

2009). In vivo, mice overexpressing human wild-type or ALS associated mutant TDP-43 develop a spastic paralysis reminiscent of ALS (Wils et al., 2010, Wegorzewska et al., 2009). As in humans, these mice also demonstrate the presence of 25 kDa CTFs (Wils et al., 2010, Wegorzewska et al., 2009). However, CTFs in mice do not accumulate in the cytoplasm but in the nucleus where they form inclusions (Wils et al., 2010).

FUS

Mutations in the gene encoding fused in sarcoma/translocated in liposarcoma (FUS/TLS or FUS) account for ~3% to 5% of familial ALS (Rademakers et al., 2012). In addition, FUS protein is a component of the protein inclusion found in ALS cases at post mortem (Kwiatkowski et al., 2009, Vance et al., 2009, Deng et al., 2010). FUS is a member of the FET family of proteins, which includes TATA box-binding protein-associated factor 68 kDa (TAF15) and Ewing sarcoma (EWS), and is involved in all stages of gene expression from transcription to protein translation (Law et al., 2006, Tan and Manley, 2009).

FUS contains seven functional domains: glutamine-glycine-serine-tyrosine-rich or prion-like domain (QGSY-rich), RNA recognition motif (RRM), glycine-rich (Gly-rich) two arginine-glycine-glycine-rich motifs (RGG1 and 2), zinc finger domain (ZFD), and a nuclear localisation signal (NLS). The QGSY and Gly-rich domains are involved in protein::protein interactions, the RRM, ZFD and RGG domains are involved in RNA/DNA binding, and the NLS is involved in nucleocytoplasm shuttling (Andersson et al., 2008). Most of the ALS associated mutations identified are localized in the Gly-rich region and NLS (Lagier-Tourenne et al., 2010). FUS is ubiquitously expressed in most tissues and is predominantly localized subcellularly to the nucleus (Andersson et al., 2008) but can engage in nucleocytoplasmic shuttling (Zinszner et al., 1997) particularly under cellular stress (Sama et al., 2013). Although some nuclear clearing of FUS immunoreactivity does occur in FUS inclusion-bearing motor neurons in ALS this is less obvious than the TDP-43 nuclear clearing seen in TDP-43 inclusion-bearing motor neurons (Vance et al., 2009, Tateishi et al., 2010).

FUS directly binds to single and double stranded DNA (Baechtold et al., 1999, Liu et al., 2013), RNAPII promoters (Tan et al., 2012) and telomeres (Dejardin and Kingston, 2009, Takahama et al., 2013). FUS is involved in DNA repair in

neurons (Mastrocola et al., 2013, Wang et al., 2013); it also regulates transcription by interacting with transcription factors (Sanchez-Ramos et al., 2011, Du et al., 2011, Hallier et al., 1998, Kim et al., 2010, Sato et al., 2005, Uranishi et al., 2001), binding to gene promoters (Wang et al., 2008b), and by regulating RNA Polymerase II and III (Schwartz et al., 2012, Tan and Manley, 2009). ChIP and promoter microarrays have demonstrated that there are over 1000 genes with promoters to which FUS binds (Tan et al., 2012). These genes are involved in various cellular processes, including gene expression, cell cycle, and neuronal functions (Tan et al., 2012). However, FUS depletion caused a modest upregulation of only 275 genes and downregulation of 335 genes in mice (Lagier-Tourenne et al., 2012).

Like TDP-43, FUS is also a hnRNP, and thus has roles in RNA processing (Calvio et al., 1995). FUS binds to GGUG motifs (Lerga et al., 2001) and structural AU-rich stem loops in target RNAs (Hoell et al., 2011). Consistent with a role in splicing, FUS binds to introns within prespliced RNAs (Lagier-Tourenne et al., 2012)s. In addition, FUS associates with components of the spliceosome (Kameoka et al., 2004, Meissner et al., 2003, Yang et al., 1998). Identified FUS targets include RNAs of genes involved in actin dynamics and the cytoskeleton (Fujii et al., 2005, Ishigaki et al., 2012, Lagier-Tourenne et al., 2012, Orozco et al., 2012, Rogelj et al., 2012). In addition, recent genome-wide approaches have identified transcripts bound and potentially regulated by WT and mutant FUS (Ling et al., 2013). These have demonstrated that ALS-associated mutant FUS preferentially binds to a number of cytoplasmic RNAs involved in the UPR and ER stress (Hoell et al., 2011, Colombrita et al., 2012). Functionally, depletion of FUS results in around 3,000 exons being altered, many of which are associated with genes that have neuronal functions or are linked to neurodegeneration (Ishigaki et al., 2012). Interestingly, depletion of FUS results in the inclusion of exon 10 in MAPT mRNA, a molecular species associated with FTD and Parkinsonism (Orozco et al., 2012).

FUS is involved in the transport of RNA from the nucleus to cytoplasmic and throughout the cell (Zinszner et al., 1997, Fujii and Takumi, 2005). The NLS domain in FUS interacts with the nuclear import receptor Transportin (Dormann et al., 2010). The interaction with Transportin and nuclear export is controlled by

methylation of arginine residues in the NLS motif of FUS (Dormann et al., 2012). In addition, FUS interacts with several motor proteins including Myo5A, 6 and KIF5B (Yoshimura et al., 2006, Takarada et al., 2009, Kanai et al., 2004). In response to synaptic activation FUS translocates into dendritic spines (Fujii and Takumi, 2005) and in response to hypertonic stress it moves out of nucleus to the cytoplasm (Sama et al., 2013). Finally, like TDP-43, FUS is also involved in the SG formation (Bentmann et al., 2013). Interestingly, ALS-linked FUS mutant species, which locate to the cytoplasm, preferentially incorporate into SGs under conditions of cellular stress (Bentmann et al., 2012, Bosco et al., 2010, Dormann et al., 2010, Kino et al., 2011).

To date, it is not clear whether ALS-linked mutations cause a loss of normal FUS function or induce the protein to gain an aberrant toxic function. Genetic deletion of FUS in inbred mice results in chromosomal instability and perinatal death (Hicks et al., 2000) but only male sterility and enhanced sensitivity to radiation in outbred mice (Kuroda et al., 2000). Neither of these mouse lines demonstrates motor dysfunction or evidence of neurodegeneration (Hicks et al., 2000, Kuroda et al., 2000). Transgenic rats overexpressing ALS-associated mutant FUS develop progressive paralysis associated with motor axon degeneration and substantial loss of cortical and hippocampal neurons (Huang et al., 2011). Interestingly, overexpression of wild-type (WT) human FUS is enough to cause age dependent cognitive deficits associated with cortical and hippocampal neuronal loss in rats (Huang et al., 2011). Recently, mice overexpressing WT human FUS have been described to develop progressive limb weakness and subsequent fatal paralysis, associated with motor neuron loss and neuropathological changes reminiscent of ALS (Mitchell et al., 2013).

Alsin

ALS2, the gene encoding for alsin, is mutated in an autosomal recessive form of juvenile ALS (Yang et al., 2001, Hadano et al., 2001). Alsin is a 1657 amino acid protein with three guanine-nucleotide-exchange factor (GEF)-like domains (Yang et al., 2001). The amino-terminal regulator of chromatin condensation like domain (RLD) resembles the GEF for Ran GTPase. The middle Dbl homology (DH)- and pleckstrin homology (PH)-like domains are similar to the GEF of Rho GTPase. The carboxyl-terminal vacuolar protein sorting 9 (VPS9)-like domain is

homologous to the GEF of Rab GTPase (Yang et al., 2001). Most mutations in the ALS2 gene associated with ALS lead to premature stop codons resulting in carboxyl-terminal truncated proteins that are unstable compared with the wild-type alsin (Yamanaka et al., 2003). However, missense mutations in the RLD domain of alsin have also been identified (Eymard-Pierre et al., 2006, Panzeri et al., 2006).

Genetic deletion of ALS2 in mice produces only subtle neuropathological changes characterised by smaller motor neuron cell bodies and smaller calibre peripheral motor axons (Devon et al., 2006). In addition, there is evidence of axonal degeneration in the corticospinal tract and peripheral motor nerve of alsin deficient mice (Yamanaka et al., 2006, Gros-Louis et al., 2008). Clinically, ALS2 deficient mice have no motor deficits but are significantly less active than wild type controls (Devon et al., 2006, Yamanaka et al., 2006). However, there is also evidence that genetic deletion of ALS2 results in age-dependent deficits in motor coordination and motor learning, as well as a heightened anxiety responses in mice (Cai et al., 2005). In comparison to this mild clinical effects, knocking down ALS2 expression, with morpholinos in zebrafish, results in severe developmental abnormalities, swimming deficits and motor neuron degeneration (Gros-Louis et al., 2008). Interestingly, ALS2 mRNA splicing variants have been found in ALS2 knock out mice, and treatment with these rescue the abnormalities seen in alsin knock down zebrafish (Gros-Louis et al., 2008).

Alsin has been described as an activator of Rac1 and Rab5 small GTPases (Topp et al., 2004, Otomo et al., 2003, Kanekura et al., 2005). Rab5 is a protein involved in endocytic trafficking of neurotransmitters and neurotrophic receptors in motor neurons (Wucherpfennig et al., 2003, Brown et al., 2005, Topp et al., 2004). ALS2 deficiency has been shown to reduce trkB and IGF-1 receptor trafficking in vitro and in vivo (Devon et al., 2006). Rac1 and its downstream effectors p21-activated kinase (PAK) family kinases are involved in neurite outgrowth by regulating actin dynamics within growth cones (Etienne-Manneville and Hall, 2002, Nikolic, 2002, Li et al., 2000b). Alsin has been reported to colocalise and activate Rac1 and PAK1 within growth cones (Tudor et al., 2005), and promote neurite outgrowth (Tudor et al., 2005, Jacquier et al.,

2006, Otomo et al., 2008). In addition, alsin has been reported to interact with glutamate receptor interacting protein 1 (GRIP1) both in vitro and in vivo, and to colocalise with GRIP1 in neurons. GRIP1 transports AMPA-type glutamate receptor subunit 2 (GluR2) to dendrites and anchors them in both postsynaptic membrane and intracellular compartments (Setou et al., 2002, Song and Huganir, 2002). Cells deficient of alsin, show disrupted subcellular distribution of GRIP1 and reduced GluR2 at the synaptic/cell surface (Lai et al., 2006). This reduction in GluR2-containing AMPA receptors renders alsin deficient neurons more susceptible to glutamate receptor mediated toxicity in vitro (Lai et al., 2006).

Furthermore, alsin has been reported to interact with mutant SOD1 protein, through its RhoGEF domain and to suppress mutant SOD1 cytotoxicity in vitro (Kanekura et al., 2004). In a glia cell and motor neuron co-cultures, alsin inhibits mutant SOD1 mediated reactive oxygen species (ROS) production, Rac1 activation, secretion of TNF alpha, and activation of NF kappa B in glial cells, leading to increased motor neuron survival in co-culture (Li et al., 2011). Interestingly however, overexpression of alsin by itself in glial cells in co-culture with motor neurons promotes the production of ROS by glia and consequent motor neuron death (Li et al., 2011). In vivo, deletion of alsin exacerbates disease in one of the mutant SOD1 mouse strains (Hadano et al., 2010). Interestingly, this effect was associated with enhanced accumulation of autophagosome-like vesicles and protein aggregates containing SOD1, ubiquitin and autophagy markers (Hadano et al., 2010).

The AP-1 transcription factor c-Jun

c-Jun is a member of the activator protein -1 (AP-1) family of transcription factors. AP-1 transcription factors are characterised by the presence of a basic leucine zipper (BLZ) domain in their structure and are divided into Jun, Fos and ATF subfamilies (Angel and Karin, 1991). These proteins only display regulatory activity on transcription when they dimerize through their BLZ motifs (Whitmarsh and Davis, 2000). Fuctional dimers can be formed through hetero- and homo-dimerisation and the particular subunit composition of each dimer determines its regulatory properties (Whitmarsh and Davis, 2000). Jun transcription factors are able to homodimerise (Angel and Karin, 1991, Jochum et al., 2001) and

heterodimerise with members of the Fos and ATF family (Hai and Hartman, 2001), as well as other transcription factors that contain a BLZ, such as CBP, MyoD, NFat or c-rel (Herdegen and Leah, 1998).

The c-Jun protein has 6 functional domains: a Jun-N-terminal kinase (JNK) binding domain, transactivation domain, hinge region, basic region, and the leucine zipper domain (Raivich, 2008). The function of c-Jun is regulated by three chemical modifications: phosphorylation at the N-terminal transactivation domain (Smeal et al., 1991, Morton et al., 2003), dephosphorylation at the hinge region (Morton et al., 2003), and acetylation at the basic region (Vries et al., 2001). JNKs mediate the phosphorylation of the transactivation domain, this process has been implicated in stress mediated neuronal cell death (Yang et al., 1997, Behrens et al., 1999). The F-box&WD domain repeated 7 (FBW7) ubiquitin ligase mediates the degradation of c-Jun by interacting with its hinge region when the latter is phosphorylated (Wei et al., 2005). Interestingly, phosphorylation of the hinge region is in part mediated by glycogen synthase kinase-3 (GSK3), and therefore c-Jun degradation depends partly on this kinase (Morton et al., 2003). Finally, the acetylase p300 mediates the acetylation of the basic region in c-Jun and promotes its transcriptional activity (Vries et al., 2001, Wang et al., 2006). These modifications are themselves controlled by different upstream signalling enzymes such as MKK4 or MKK7, PI3-Kinase and ERK1&2 (Raivich, 2008).

c-Jun expression is localised in the nucleus of cells and its upregulation is associated with neuronal trauma (Herdegen et al., 1991, Jenkins and Hunt, 1991, Raivich et al., 2004), ischaemia (Kindy et al., 1991, Wessel et al., 1991), hyperexcitation (Morgan and Curran, 1988, Gall et al., 1990, Gass et al., 1993), and apoptotic cell death (Sun et al., 2005). In addition, c-Jun is also found upregulated or activated in neurodegerative disorders such as Alzheimer's disease (Pearson et al., 2006), Parkinson's disease (Oo et al., 1999, Saporito et al., 2000) and ALS (Vlug et al., 2005, Jaarsma et al., 1996, Migheli et al., 1997). Functionally, deletion c-Jun or expression of a dominant negative form of c-Jun prevents neuronal cell death following neurotrophic factor withdrawal in vitro (Ham et al., 1995, Palmada et al., 2002). This neuroprotective effect is associated with a reduction in the expression of the pro-apoptotic protein Bim

(Whitfield et al., 2001). Importantly, JNK is involved in this type of cell death, as genetic inhibition of the ability of JNK to bind and activate c-Jun prevents neuronal cell death following neurotrophic factor withdrawal (Eilers et al., 2001, Harding et al., 2001). Interestingly, general inhibition of JNK function results in neuroprotective effects of a greater magnitude in this model than those observed following specific inhibition of its c-Jun activator function (Borasio et al., 1998a, Maroney et al., 1999, Besirli et al., 2005). In vivo, JNK mediated activation of c-Jun plays a role in excitoxic neuronal cell death, as deletion of JNK3 or the genetic modification of c-Jun (*junAA*), which renders it unable to be activated by JNKs, results in reduced neuronal cell death following kainate stimuli in mice (Behrens et al., 1999, Yang et al., 1997). However, the effects of JNK and c-Jun are not always linked. While inhibition of JNK reduces tissue loss secondary to medial cerebral artery occlusion (MCAO) in mice, genetic deletion of c-Jun or *jun*AA modification does not have such an effect (Vogel et al., 2007, Brecht et al., 2005). Similarly, while deletion of neuronal c-Jun abrogates neuronal cell death following axotomy (Raivich et al., 2004, Ruff et al., 2012), deletion of JNK or *jun*AA modification do not have such an effect (Ruff et al., 2012). Thus, there appears to be three different sets of signalling effects via the JNK/c-Jun pathway: 1) JNK-independent effects of c-Jun 2) c-Jun-independent effects of JNKs; 3) effects of JNK activated c-Jun(Raivich, 2008).

The JNK/c-Jun pathway is also involved in the response of glial and inflammatory cells to neuronal injury. Upregulation of c-Jun expression is observed mainly in astrocytes in conditions, such as Alzheimer's disease (Anderson et al., 1994) and ALS (Migheli et al., 1997), as well as in models of cerebral ischemia (Kato et al., 1995), neuropathic pain (Zhuang et al., 2006), and toxin-induced degeneration of cholinergic (Rossner et al., 1997) and dopaminergic (Nakagawa and Schwartz, 2004) neurons. Functionally, JNKs have been shown to regulate the synthesis and release of neurotrophins, cytokines, ROS and matrix degrading enzymes by astrocytes, microglia and oligodendroglia, following a variety of stressful stimuli in vitro (Hidding et al., 2002, Waetzig et al., 2005, Kim et al., 2008, Tanaka et al., 2008). Studies report that JNK mediates some of these effects in glia through the activation of glial c-

Jun (Hidding et al., 2002, Zhuang et al., 2006, Kim et al., 2008). The role of c-Jun in astrocytes, microglia or oligodendrocytes has not been characterised in vivo. However, neuronal deletion of c-Jun results in reduced perineuronal inflammation following axotomy in mice, with reduced microglia and astrocyte activation, as well as diminished T-cell infiltration around axotomised neurons (Ruff et al., 2012, Raivich et al., 2004). Similarly, genetic deletion of c-Jun from Schwann cells is associated with a failure in demyelination and macrophage recruitment in injured nerves following peripheral nerve transection in mice (Arthur-Farraj et al., 2012).

Animal models used in this thesis

In this thesis three mouse models were used to analyse the involvement of TDP-43, FUS, alsin and c-Jun in motor neuron degeneration or regeneration.

Animal models of Amyotrophic lateral sclerosis

SOD1G93A mice

The most commonly used model of ALS disease is the transgenic $SOD1^{G93A}$ mouse (Fig. 3) (Turner and Talbot, 2008). This mouse model was generated and first described by Gurney et al. (1994). The transgene design was based on the G93A human SOD1 mutation associated with familial ALS, where a single amino acid is substituted from glycine to alanine at codon 93 in the SOD1 gene (Rosen et al., 1993). The transgene expression in these mutant mice is under the control of the ubiquitous expressor endogenous human SOD1 promoter (Gurney et al., 1994). The cloned transgenes were injected to B6/SJL fertilised eggs and founder mice were crossed with B6 mice for subsequent analysis (Gurney et al., 1994). Transgenesis resulted in a high transgene-copy line named B6SJL-TgN(SOD1-G93A) 1Gur (carries 18-25 transgene copies). This line is the most widely used model of ALS (Turner and Talbot, 2008) and is commonly maintained by crossing $SOD1^{G93A}$ heterozygous transgenics with B6/SJL wild type hybrids (Turner and Talbot, 2008).

Phenotypically, as $SOD1^{G93A}$ mice age they develop features typical of ALS neurodegeneration, including motor functional deficits and pathological changes. Analysis of these mice can be divided into the presymptomatic phase, ranging from birth to around 75 days of age; and the symptomatic phase, which begins from around 75 days and lasts until death. Analysis of locomotion demonstrated that at three to four months of age mice show signs of hind limb weakness, tremor, hyper-reflexia and weight loss (Turner and Talbot, 2008). Ultimately these mice die prematurely, commonly at around 4 months (Gurney et al., 1994, Turner and Talbot, 2008).

Pathological features can be seen at the level of the muscle, nerve and neuronal soma in these mice. Neuromuscular degeneration begins at 25 (Gould et al., 2006) to 47 days of age (Fischer et al., 2004, Pun et al., 2006). Retrograde axonal degeneration is evident at 80 days and coincides with motor impairment. At 100 days a strong reduction in lower motor neuron number is

observed (Fischer et al., 2004). Ultrastructurally, motor neurons demonstrate mitochondrial vacuolisation (Dalcanto and Gurney, 1995), Golgi apparatus fragmentation (Mourelatos et al., 1996), neurofilament-positive inclusions (Tu et al., 1996) and cytoplasmic SOD1-immunoreactive aggregates (Johnston et al., 2000). A non-neuronal reaction characterised by microglial and astrocyte activation is also evident around the neuronal soma in the spinal cord at around the onset of disease (Hall et al., 1998b). Similar pathological features can be seen in cranial nuclei including that of the trigeminal, facial and hypoglossal nerve (Niessen et al., 2006).

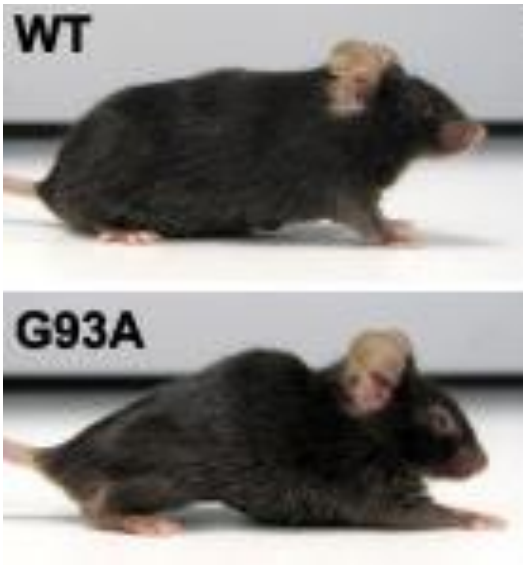


Figure 3. The phenotype of SOD1G93A mutant mice. Mice carrying the mutant form of the human $SOD1^{G93A}$ gene develop functional and pathological features of ALS. Among these features, SODG93A mice (G93A) appear to be thin along the flanks compareed to wild type mice (WT), which suggests muscle wasting. Photograph obtained from Turner and Talbot (2008).

The onset and progression of disease in $SOD1^{G93A}$ mice can be clinically established and monitored through different measures, including: weight, neurological scores, rotarod performance, grip strength wire hanging, gait analysis and wheel distance (Scott et al., 2008, Ludolph et al., 2010). To prevent unnecessary suffering, length of survival is measured using number of humane artificial clinical end points. These include: loss of self-righting ability within 10 to 30 seconds of being placed on the back, weight loss of 30% for 72

hours and onset of bilateral hind limb paralysis (Scott et al., 2008, Solomon et al., 2011).

The clinical and pathological progression of disease in SOD1^{G93A} mice depends on the number of transgene copies expressed in the transgenic mice (Gurney et al., 1994, Gurney, 1997, DalCanto and Gurney, 1997). Although the number of transgene copies carried in the high copy SOD1^{G93A} line is stable (18 to 25 copies), sporadic drops in copy number have been described due to intra locus recombination events during meiosis (Gurney, 1997). Such an event resulted in a SOD1G^{93A} transgenic mouse line with a reduced number of copies from the original high copy transgenic mice (Gurney, 1997). This mouse line carries only 8 SOD1^{G93A} transgene copies and has been designated B6SJL-TgN(SOD1-G93A)^{dl}1Gur. The low transgene copy number SOD1^{G93A} (^{dl}1Gur) mouse line demonstrates delayed onset of disease and an increase in lifespan compared to high copy number (1Gur) carriers (Gurney, 1997, Alexander et al., 2004, DalCanto and Gurney, 1997, Chiu et al., 1995).

Although widely used, the major weakness associated with the SOD1^{G93A} mouse model of ALS has been its poor predictive value in terms of potential therapies (Scott et al., 2008). However, the differences in efficacy of treatments form mice to humans may be due to the inherent differences in experimental design and control of confounding variables (Scott et al., 2008). Nonetheless, it remains to be demonstrated whether the findings in these mice are applicable to other animal models or humans and forms of familial or sporadic ALS (Robberecht and Philips, 2013).

TDP-43 mice

Transgenic mice over-expressing human TDP-43 transgene containing the ALS associated A315T mutation were used in this project (Wegorzewska et al., 2009). These mice were generated by pronuclear microinjection of a transgene construct comprising full length human TDP-43 A315T cDNA under the control of the mouse prion protein promoter (Wegorzewska et al., 2009). The phenotype in these mice consists of abnormalities associated with cortical and lower motor neuron degeneration (Wegorzewska et al., 2009). At 3-4 months of age mice lose weight, and develop gait abnormalities (Wegorzewska et al., 2009). On average mice survive around 154 days but some are found to die

spontaneously (Wegorzewska et al., 2009). This spontaneous death is due to bowel obstruction secondary reduced gut motility, which results from degeneration of the myenteric nervous system (Herdewyn et al., 2013). Assessments using electromyography demonstrates changes associated with the loss of muscle fibre innervation. This coincides with pathological changes in the muscle that are characteristic of muscle deinnervation, such as the scattered and grouped atrophic muscle fibres (Wegorzewska et al., 2009). Degeneration of descending motor axons of the spinal cord lateral columns is also evident (Wegorzewska et al., 2009). Furthermore, degenerating motor neurons in the ventral horn of the spinal cord show increased ubiquitin staining. In addition to the changes associated with ALS, pathological abnormalities that are similar to that found in frontotemporal lobar degeneration are also evident in these mice (Wegorzewska et al., 2009). Increased cytoplasminc ubiquitin staining in cortical neurons, which appears either diffuse, punctate with multiple small aggregates, or in the form of large organized cytoplasmic aggregates is observed. In addition, astrogilosis and microgliosis are observed around cortical neurons (Wegorzewska et al., 2009).

Neural specific *c-jun* knock-out mouse

Mice carrying both alleles of the *c-jun* gene flanked by lox-P sites were originally created and described by Behrens et al. (2002) to study the role of c-Jun in the liver. Mice with floxed *c-jun* alleles are fertile and do not show any phenotypical or histological abnormalities (Behrens et al., 2002).

Transgenic mice expressing Cre recombinase under the control of the neuroepithelial specific Nestin promoter and enhancer [intron II; (Zimmerman et al., 1994)] were generated and first described by Tronche et al. (1999). This Nestin Cre recombinase transgene allows the specific removal of floxed genes from neuroepithelial cells including those that will develop into motor neurons ; (Zimmerman et al., 1994). Nestin Cre mice are fertile and do not show any phenotypical or histological abnormalities (Tronche et al., 1999).

Raivich et al. (2004) created a mouse line that did not express *c-jun* in cells from the nervous system. To do this, male mice carrying two floxed *c-jun* alleles (*c-jun f/f*) were crossed with heterozygous female *Nestin Cre* mice (*NesCre*). This produced *Nestin Cre* transgenic mice carrying one floxed *c-jun* allele and a

wild type one (*NesCre c-jun f/+*). Female *NesCre c-jun f/+* mice were crossed again with male *c-jun f/f* mice to produce *Nestin Cre* transgenic mice carrying two floxed *c-jun* alleles [*NesCre c-jun f/f or c-jun ΔN*(Raivich et al., 2004)]. These mice are born with Mendelian frequency, are viable and fertile, and have normal motor, learning and fear behaviour (Raivich et al., 2004). The *c-jun ΔN* mice have overall normal brain architecture and histology apart from a 19% increase in number of motor neurons in the facial nucleus compared to their *c-jun* competent littermates (*c-jun f/f*; (Raivich et al., 2004). In addition, as described earlier, neural *c-jun* deficient mice show a major defect in peripheral nerve regeneration and central axonal sprouting response after peripheral nerve injury (Raivich et al., 2004, Makwana et al., 2010).

The facial nerve injury model

The rodent facial nerve axotomy model is widely used to study the regeneration and degeneration of the peripheral nervous system in vivo (Moran and Graeber, 2004). Motor neurons of the facial motor nucleus send axons around the abducens nucleus that then exit through the bony part of the skull (Fig.4A) to innervate the muscles that control the movements of the whiskers, lips and eyes (Fig.4B) (Semba and Egger, 1986). There are a number of benefits associated with this model. Firstly, the surgical procedure of injuring the nerve is simple (Figure 4B). Different injury severities can be tested, from the mild form of injury, nerve crush lesion, to a more severe one such as nerve cut. Secondly, injury to the facial nerve at the level of the stylomastoid foramen, keeps the CNS intact, causes no disruption of the blood-brain barrier, and reduces the chance of the nerve nucleus becoming contaminated by blood-borne elements (Streit and Kreutzberg, 1988). Thirdly, functional analysis can be easily carried out. Whisker function on the intact/uninjured side can be used to compare the extent of functional recovery on the injured side. Fourthly, an internal control nucleus located on the other side of the brainstem abolishes any effects that may be associated with inter-animal variations. For the above reasons the model is an effective tool when the quantification of the motor neuron and non-neuronal responses to axonal injury is required.

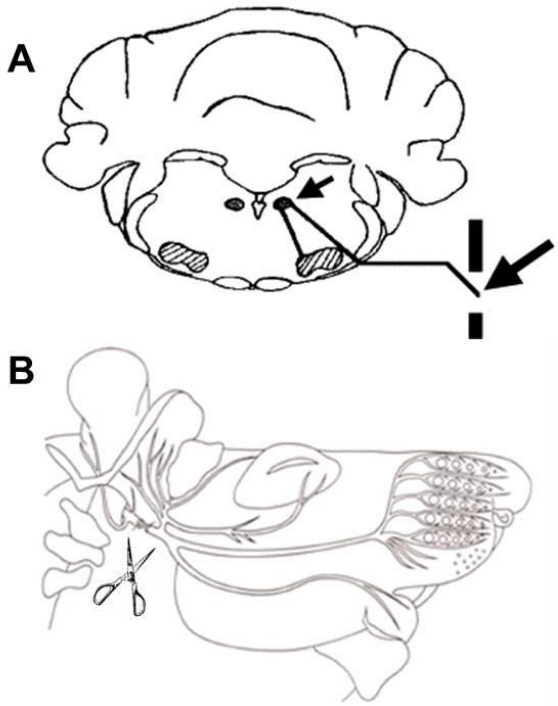


Figure 4. The facial nerve injury model. A) Diagram shows the trajectory of the facial nerve from the facial motor nucleus (hatched area), to the abducens nucleus (balck cicle: arrow), and finally exiting the skull (large arrow). Edited from Moran and Graeber, (2004). B) Sketch showing the primary branching of the facial nerve distal to the stylomastoid foramen innervating the eye lips and whisker pad. At the stylomastoid foramen a nerve injury can be performed. Edited from Angelov et al., (2011).

Aims

The expression of c-Jun is found upregulated in astrocytes in ALS cases and within motor neurons in mutant SOD1 mice (Migheli et al., 1997, Jaarsma et al., 1996). However, it is unclear whether c-Jun plays a functional role in the disease. The previously described central role of c-Jun in motor neuron death and perineuronal neuroinflammation following peripheral nerve injury strongly suggests a possible functional role in ALS motor neuron degeneration (Raivich et al., 2004). This thesis aims to determine the functional role of c-Jun in the neurodegenerative changes observed in the SOD1G93A mouse model of ALS.

In addition, as there is previous evidence of a regenerative effort mounted within the ALS disease process (Tsujihata et al., 1984, Atsumi, 1981, Hegedus et al., 2008, Hegedus et al., 2007), and c-Jun plays a central role in axonal regeneration (Raivich et al., 2004), this thesis aims to characterise further the ALS associated regenerative response in degenerating motor neuron pools of SOD1 G93A mice.

Furthermore, because the morphological and molecular changes observed in ALS and following peripheral nerve injury resemble each other (Raivich et al., 2004, Fischer et al., 2004), and a number of ALS associated protein and genes have function relevant to axonal regeneration (Narayanan et al., 2013, Tudor et al., 2005, Wang et al., 2008a), another aim of this thesis is to determine whether the pattern of expression of ALS associated proteins TDP-43, FUS and alsin changes within axotomised motor neurons after facial nerve injury.

Finally, since peripheral nerve injury results in changes in the pattern of expression of TDP-43 (Moisse et al., 2009a) and ALS associated mutations affect its normal function (Alami et al., 2014), this thesis aims to determine whether the overexpression of ALS mutated A315T TDP-43 in mice can affect the normal physiological process of axonal regeneration following facial nerve transection.

Chapter 2: Materials and Methods

Animals

All experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, and were approved by the UK Home Office and the University College London local ethics committee. Animals were housed with a 12 hour light-dark cycle, at 21-23ºC, an average humidity of 60%, and had access to food pellets and water at libitum.

Wild Type Mice

This study used C57/BL6 mice (Charles Rivers) at 4-15 weeks of age.

Genetically Modified Mice

The transgenic animals used for experimentation included, $jun^{f/f}$ & $jun^{\Delta N}$ (Axel Brehens, Cancer Research, UK), SOD1G93A (Axel Brehens, Cancer Research, UK) and A315T mutant TDP-43 (Christopher Shaw, King's College London).

The role of c-Jun in SOD1 G93A mediated motor neuron disease

Genetically modified mice were used to investigate the role of the transcription factor c-Jun in $SOD1^{G93A}$ mediated motor neuron disease. Three mutant mouse lines were used to generate a mouse model of $SOD1^{G93A}$ ALS where the *c-jun* gene may be deleted from cells in the nervous system. The three genetically modified mouse lines used in this study were: high copy $SOD1^{G93A}$ transgenic mouse model of ALS (Gurney et al., 1994), mice carrying floxed *c-jun* alleles (Behrens et al., 2002) and mice expressing cre-recombinase under the control of neuroepithelial specific nestin promoter and enhancer (Tronche et al., 1999). Interbreeding of these three mouse lines allowed for the identification changes in $SOD1^{G93A}$ mediated motor neuron disease that are dependent on neural *c-jun*.

$SOD1^{G93A}$ mouse model of ALS

High copy $SOD1^{G93A}$ transgenic mice in a B6JLF background [B6SJLTgN(SOD1-G93A)1Gur] were obtained from Jackson Laboratory and maintained by breeding hemizygous male carriers to B6/SJLF hybrids in CRUK.

$SOD1^{G93A}$ mice were originally generated and described by Gurney et al. (1994) and are the first and most commonly used model of ALS. These mice are hemizygous carriers of 18 to 25 copies of a human mutant SOD1 transgene

containing a single amino acid substitution of glycine to alanine at codon 93 (Gurney et al., 1994). This transgene was designed to replicate the $SOD1^{G93A}$ mutation found in human cases of ALS and its expression in the SOD^{1G93A} mice is driven by the human SOD1 promoter (Gurney et al., 1994).

$SOD1^{G93A}$ mice are fertile, develop progressive limb paralysis and die prematurely in adulthood. In addition, they demonstrate progressive pathological changes including the degeneration and death of motor neurons and activation glial and inflammatory cells in the nervous system (Gurney et al., 1994).

The floxed *c-jun* mouse

Mice carrying both alleles of the *c-jun* gene flanked by lox-P sites were originally described by Behrens et al. (2002). These mice are fertile and do not show any phenotypical or histological abnormalities (Behrens et al., 2002). They were originally in a mixed 129/B6/FBV background (Behrens et al., 2002) but were subsequently bread with B6 mice for at least 5 generations to establish the colony in CRUK.

Nestin Cre-Recombinase mouse

Transgenic mice expressing Cre recombinase under the control of the neuroepithelial specific Nestin promoter and enhancer [intron II; (Zimmerman et al., 1994)] were generated and first described by Tronche et al. (1999). This Nestin Cre recombinase transgene allows the specific removal of floxed genes from neuroepithelial cells including those that will develop into motor neurons. Nestin Cre mice are fertile and do not show any phenotypical or histological abnormalities (Tronche et al., 1999). This transgenic mouse line was maintained by breeding hemizygous female carriers to B6 mice in CRUK.

Nestin Cre mediated *c-jun* knock out

To create a neural specific *c-jun* knock out animal, male mice carrying two floxed *c-jun* alleles (*c-jun f/f*) were crossed with heterozygous female *Nestin Cre* mice (*NesCre*). This produced *Nestin Cre* transgenic mice carrying one floxed *c-jun* allele and a wild type one (*NesCre c-jun f/+*). Female *NesCre c-jun f/+* mice were crossed again with male *c-jun f/f* mice to produce *Nestin Cre* transgenic mice carrying two floxed *c-jun* alleles (*NesCre c-jun f/f or c-jun ΔN*). These mice were born with Mendelian frequency, were viable and fertile, and

had normal motor, learning and fear behaviour (Raivich et al., 2004). The *c-jun ΔN* mice had overall normal brain architecture and histology apart from a 19% increase in number of motor neurons in the facial nucleus compared to their *c-jun* competent littermates (*c-jun f/f*; (Raivich et al., 2004).

SOD1^{G93A} *c-jun* knock out

To determine the role of *c-jun* in SOD1^{G93A} mediated disease a two-step breeding strategy was used. First, mice carrying two floxed *c-jun* alleles (*c-jun f/f*) were crossed with heterozygous transgenic SOD1^{G93A} mice. This produced SOD1^{G93A} transgenic mice carrying one floxed c-jun allele and a wild type one (SOD1^{G93A} *c-jun f/+*). These SOD1^{G93A} *c-jun f/+* mice were then crossed again with *c-jun f/f* mice to produce SOD1^{G93A} transgenic mice carrying two floxed c-jun alleles (SOD1^{G93A} *c-jun f/f*). Second, SOD1^{G93A} *c-jun f/f* male mice were crossed with female *NesCre c-jun f/f* (*c-jun ΔN*) mice. This breeding strategy resulted in littermates that were all homozygous for floxed *c-jun* and in a Mendelian frequency expressed the SOD1^{G93A} and *Nestin Cre* transgenes. Thus, the effects of neural specific deletion of *c-jun* on SOD1^{G93A} mediated disease and SOD1^{G93A} independent effects of *c-jun* deletion could be determined between littermates.

Peripheral nerve regeneration in TDP-43 A315T model of ALS

To study the effect of overexpression of ALS associated mutant *TDP-43* protein on peripheral nerve regeneration in mice, the *TDP-43 A315T* transgenic mouse model of ALS was employed (Wegorzewska et al., 2009).

TDP-43 A315T transgenic mice were obtained from Jackson Laboratory in a B6/SJLF background but hemizygous male carries were bread with B6 mice for at least 5 generations to establish a colony at Kings College London.

TDP-43 mutant mice were originally generated and described by Wegorzewska et al. (2009) and are the first TDP-43 mouse model of ALS. These mice are hemizygous carriers of multiple copies of a human mutant TDP-43 transgene containing a single amino acid substitution of alanine to threonine at codon 315 (Wegorzewska et al., 2009). This transgene was designed to replicate the TDP-43 mutation found in human cases of ALS and its expression in *TDP-43 A315T* mice is driven by the mouse prion protein promoter (Wegorzewska et al., 2009).

Genotyping

Polymerase chain reaction (PCR) was carried out, using chloroform extracted DNA from mice tails, in order to recognize the genotypic identity of the mice used in this study.

SOD1G93A jun f/f and SOD1G93A jun ΔN

The primers used for SOD1 G93A PCR were: CATCAGCCCTAATCCATCTGA forward and CGCGACTAACAATCAAAGTGA reverse. Those used for floxed c-jun were: CTCATACCAGTTCGCACAGGCGGC forward and CCGCTAGCACTCACG TTGGTAGGC reverse. For the nestin CRE the primers were: CGGTCGATGCAACGAGTGATGAGG forward and CCAGAGACGGAAATCCATCGCTCG reverse. All PCRs were carried out under the same following conditions: 3 minutes at 94ºC then 40secs at 94ºC, 40secs at 57ºC and 60 secs at 72ºC for 35 cycles ending with 10minutes at 72ºC. A 1% ethidium bromide agarose gel, ran in a 10X TAE buffer, was used to resolve and visualise the products of the PCRs.

A315T mutant TDP-43

Genotyping for these mice was carried out in Kings College London, by the Shaw group. Genomic DNA was extracted and PCR amplified. PCR primers used are listed as follows: human TDP-43, 5′-GGATGAGCTGCGGGAGTTCT-3′ and 5′-TGCCCATCATACCCCAACTG-3′ (400 bp); Internal positive control, 5′-CAAATGTTGCTTGTCTGGTG-3′ and 5′-GTCAGTCGAGTGCACAGTTT-3′ (200 bp).

Surgical procedures

Animals were anaesthetised with a mixture of 5% Isoflurane and oxygen (Merial, UK) and then maintained with 3% Isoflurane and oxygen. Before surgery animals were checked for the depth of anaesthesia by using the paw withdrawal reflex.

Facial Nerve Axotomy

Mice received a 1 mm cut posterior to the right ear, at the level of the mastoid process. Muscles were bluntly dissected to reveal the facial nerve. For the facial nerve axotomy experiments the main branch as well as the retro-auricular branch were transected at the stylomastoid foramen, using microscissors (Fig. 1). For

facial nerve crush experiments, only the main branch of the facial nerve was crushed. Blunt forceps were used to grip the nerve approximately 1mm away from the stylomastoid foramen. The nerve was crushed for 10 seconds before releasing it. For both nerve cut and crush, the skin was closed using wound clips (Autoclip, USA). The animal was allowed to recover in a heat chamber for (32°C) ten minutes. Animals that had successful facial nerve transections or crush were identified by the lack of whisker function ipsilateral to the injury.

If retrograde labelling was required, to identify successfully reinnervating neurons, animals under anaesthesia had a piece of Gelfoam, (Johnson and Johnson, Skipton, UK) soaked with 30µl of Fluorogold (Fluorochrome, Denver, USA) diluted from stock to 2% with distilled water, inserted under the left and right whisker pads two days prior to sacrifice. Animals were sacrificed at 30 days (Fig. 1).

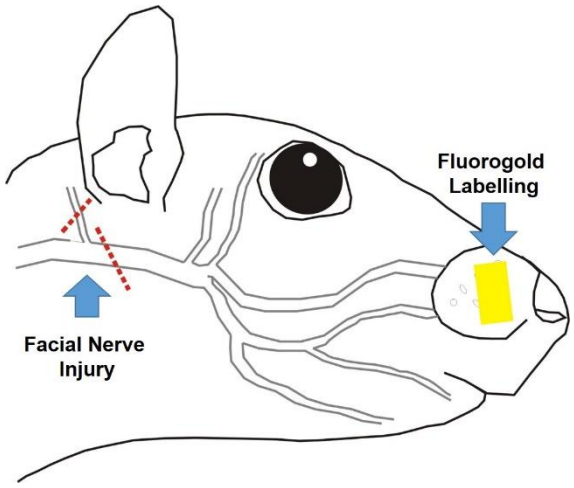


Figure 1. Simplified diagram demonstrating the right facial nerve in mice as it exits from the stylomastoid foramen. The facial nerve is drawn in grey. The main branch as well as the retro-auricular branch are transected in facial nerve axotomy experiments (red dashed lines), while in nerve crush experiments only the main branch is manipulated. For retrograde labelling of reinnervated neurons, fluorogold pads can be inserted into the whisker pads (yellow rectangle). Edited from Wang et al, (2012a).

Perfusion Fixation and Tissue Collection

Mice were terminally anaesthetised with an overdose of 2,2,2-tri-bromoethano (Avertin) (Sigma-Aldrich, Germany). This was carried out by means of an intraperitoneal injection of Avertin at 16.6 µl/g of body weight. The paw withdrawal reflex was used as an indication of deep anaesthesia. To fix the central nervous system of animals studied, transcardial perfusion/fixation was carried out. A 16-gauge needle attached to a perfusion pump (Gilson, USA) was inserted into an aperture in the apex of the heart and the vena cava was transected to allow exanguination. To wash out residual blood, 10mM phosphate buffer in 0.9% saline (PBS; pH=7.4) was perfused at a rate of 0.02L/min for 5 minutes; this was followed by 10 minutes in 4% paraformaldehyde (PFA) in 10mM phosphate buffered saline solution (PBS; pH=7.4) at the same flow rate.

Facial nerve injury experiments

For histology and immunohistochemistry, wild type or TDP-43 mice that underwent a unilateral facial nerve transection were killed between 0 to 42 days, and those that received a facial nerve crush were killed between 7 to 14 days. Here, the hindbrains were dissected using a scalpel blade.

SOD^{G93A} project

SOD^{G93A} mice were killed at two different time points in the progression of disease: day 70 (before the onset clinical symptoms) or day 104-116 (End stage, which due to ethical reasons, is taken as the onset of complete bilateral hind-limb paralysis) (Figure 2). Here, hindbrains, spinal cords, ventral roots and gastrocnemius muscle were dissected.

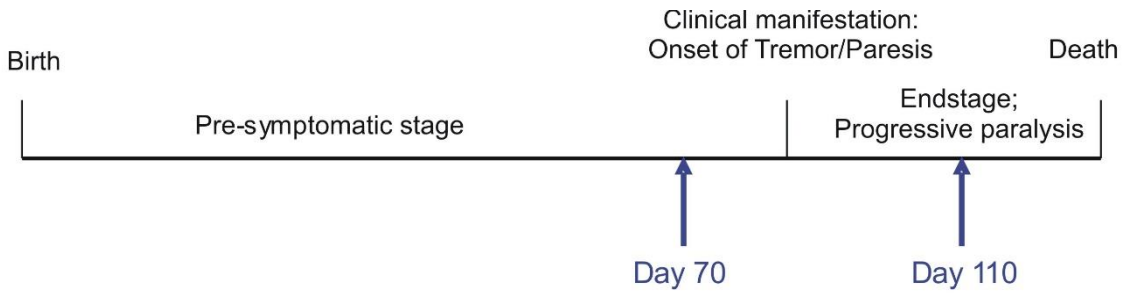


Figure 2. Time line of SODG93A disease progression, indicating in blue the time points at which animals were sacrificed.

Tissue Post-fixation and Cryoprotection

Hindbrain, spinal cord, and muscle tissue were separately immersed in 2% PFA/PBS (pH=7.4). Tissue was placed to rotate at 8 rpm for 1 hour. For cryoprotection, tissue was immersed in 30% sucrose in 0.1 M PBS buffer (pH=7.4); tissue was left to rotate at 8 rpm for 48 hours. Unless otherwise stated, tissue was embedded in OCT and frozen with dry ice. All tissue samples were stored at -80˚C prior to use.

Tissue sectioning

Unless otherwise stated, tissue was sectioned using a cryostat (Leica, Germany) with the chamber set at -20˚C. Tissue was thaw-mounted on warm, 0.5% gelatine-coated, slides (MECK). Sections were then refrozen on dry ice and stored at –80˚C until further processing.

Hypoglossal nucleus

Coronal sections through the hindbrain were cut caudal to rostral, with a thickness of 25um. Sections were collected on the appearance of the perinuclear white matter and collection stopped when the 4th ventricle became enlarged. Fifty consecutive sections were collected.

Facial motor nucleus

Coronal sections through the hindbrain were cut caudal to rostral, with a thickness of 20um. Prior to collection, the nucleus ambiguus was used to visually make the left and right side of the hindbrain symmetrical. Fifty consecutive sections were collected, from the start of the facial motor nuclei until the nuclei disappeared.

Spinal Cord

For immunohistochemistry studies, spinal cords were dissected at the cervical region. Fifty consecutive transverse sections through the cervical enlargement of the spinal cord were collected at a 40um thickness. For Hematoxylin and Eosin (H&E) stain, the spinal cord was dissected from the start to the end of the lumbar 5 segment. The L5 region of the spinal cord was then embedded in paraffin wax. Sections throughout the L5 region were cut using a microtome at 5um thickness.

Ventral roots

L5 ventral roots were placed in Barnett Fix (2.5% gluteraldehyde/PBS) for 6 days, and subsequently underwent osmication and dehydration so that they can be embedded in Araldite (Fluka, Basel/Sitzerland). Using a microtome, 1µm transverse sections were taken from the ventral root and placed on SuperFrost Plus slides (VWR International).

Gastrocnemius Muscle

Four 50um longitudinal sections per gastrocnemius were obtained at the cryostat. Using a 24-well plate, two sections of muscle were placed per well containing distilled water. Sections were kept at 4 degrees for further analysis.

Histology

Immunohistochemistry (IHC)

Tissue sections were rehydrated in distilled water and spread under a dissecting microscope. After being left to dry for 5 minutes, the sections were fixed in a solution of 4% formaldehyde in 100mM PB (pH=7.4) for 5 minutes. Sections were transferred into a cuvette containing 100mM PB (pH=7.4) for 5 minutes. The sections were then defatted by placing them in different concentrations of acetone solutions (50% for 2 minutes; 100% for 2 minutes; 50% for 2 minutes), before being washed twice for 2 minutes in PB solution and once for 2 minutes in 0.1% bovine serum albumin (PB/ BSA: Sigma,-Aldrich).

A block of 5% goat serum (Vector: containing goat IgG) in 100mM PB was pipetted (90µl) onto the sections at room temperature for a minimum of 30 minutes. Subsequently, the sections were washed in PB/BSA (0.1%) PB (100mM), PB (100mM) and PB/BSA (0.1%) before being incubated overnight at 4°C with 90µL of the optimally diluted primary monoclonal or polyclonal antibody against the specific antigen required. Table 1 shows the antibodies and concentrations used.

The sections were then washed in 3 Falcon tubes each containing 0.1% PB/BSA; 100mM PB or 100mM PB, before being incubated with a 1:100 diluted biotinylated secondary antibody at room temperature for 1 hour. Depending upon the source of the primary antibody, the corresponding secondary

antibodies used included, goat anti-rabbit, Goat anti-Hamster or Goat anti-Rat (Table 1).

The sections were once again washed in 3 Falcon tubes and incubated for 1 hour with 90 µL of the ABC reagent (avidin- biotinylated horseradish peroxidase complex;Vector) at a dilution of 1:100. Subsequently, to allow visualization of the detected antigen, the sections were washed in 100mM PB before being placed in a diluted solution of diaminobenzidine/H_2O_2 (DAB/H_2O_2; Sigma) for approximately 2 minutes.

Sections that were immunostained for CD3 with DAB were enhanced by the addition of 0.25g/l Cobalt chloride (CoCl2) and 0.2g/l Nickel sulphate (NiSO4). The slides were then washed in distilled water, dehydrated in alcohol and xylene solutions before finally being mounted with DPX (BDH,UK).

Table 1. Primary and secondary antibodies used in this study. The table lists the antibodies and their specific antigens and dilution used as well as the company they were acquired from and catalogue numbers.

Antigen	Antibody (code and type)	Dilution	Source
Primary antibodies			
Alsin	C-term-1644a.a, Rabbit polyclonal	1:1600	Everest Biotech catalogue number EB05316
Alpha 7	Anti-alpha7, Rabbit polyclonal	1:5000	Ulrike Meyer, UEA Norwich, United Kingdom
Alpha M	5C6, Rat monoclonal	1:6000	Serotec, United Kingdom, catalogue number 5C6
Alpha X	Anti-Alpha X, Hamster polyclonal	1:400	Chemicon, United Kingdom,
β1-integrin	MB1.2, Rat monoclonal, IgGa, Kappa	1:3000	Chemicon, United Kingdom, catalogue number MAB1997
B7.2	Anti-CD86, Rat monoclonal	1:3000	Pharmigen, United States, Catalogue number 553689
CD3	Anti-CD3, Rat monoclonal	1:100	Serotec, United Kingdom,
CD44	MAB2137, Rat monoclonal, IgG2b	1:5000	Chemicon, United Kingdom, catalogue number MAB2137
c-Jun	Anti-c-Jun, Rabbit polyclonal	1:200	Santacruz Biotechnology, United Kingdom
CGRP (Calcitonin gene related peptide)	Anti-CGRP, Rabbit polyclonal	1:400	Bachem, United Kingdom, T-4032.0050

FUS	400a.a-450a.a, Rabbit polyclonal		Novus, catalogue number NB100-561
Galanin	Anti-galanin, Rabbit polyclonal	1:400	Bachem, United Kingdom, T-4334.0050
GFAP (Glial fibrillary acidic protein)	Anti-GFAP, Rabbit polyclonal	1:6000	Vector, United Kingdom
TDP-43	C-term-154 a.a, Rabbit polyclonal	1:2000	Proteintech catalogue no. 12892-1-AP lot no. 1
Secondary antibodies			
Rabbit immunoglobulin (Ig)	Biot. anti- rabbit Ig, Goat polyclonal	1:100	Vector, United Kingdom
Hamster immunoglobulin (Ig)	Biot. anti-hamster Ig, goat polyclonal	1:100	Vector, United Kingdom
Rat immunoglobulin (Ig)	Biot. anti-rat Ig, goat polyclonal	1:100	Vector, United Kingdom

Haematoxylin and Eosin Stain

Lumbar spinal cords sections were deparaffinized and rehydrated by placing slides for 3 minutes in xylene, then in decreasing concentrations of Ethanol (100%, 95%, 70%, 50%), and finally in distilled water (ddH_2O). Next, slides were placed in haematoxylin stain for 2 minutes, dipped in ddH_2O to remove excess stain, and acid alcohol to differentiate sections. Acidic alcohol was rinsed with ddH_2O and neutralised with ammonia until the stain in the nucleus turned blue. Subsequently, slides were rinsed thoroughly for 8 minutes in running ddH_2O and dehydrated by dipping the slides 10 times through increasing concentrations of ethanol (50%, 70%, 95%,100%) and 100% Methanol. Slides were stained with eosin for 2 minutes and then underwent ten

dips in 100% Methanol, 100% Ethanol and Xylene before permanently affixing coverslips with resinous mounting medium DPX (Sigma).

Cresyl violet (Nissl) Stain

Hindbrain sections were rehydrated in ddH_2O, spread under a dissecting microscope, and were allowed to dry at room temperature for 5 minutes. Tissue sections were then placed in 4% Formaldehyde overnight (BDH, UK). Subsequently, the sections were changed to 70% ethanol and left overnight. Prior to staining, 4g of cresyl violet powder (BDH, UK) in 40ml of 100% Ethanol was mixed for 15 minutes to make 1% cresyl violet or Nissl. This mixture was added to 360ml ddH_2O and then filtered using Whatman size 4 filter paper. Slides were placed in Nissl solution for 6-8 minutes. Then, slides were transferred to water and increasing concentrations of Ethanol (70%, 90%, 96%) to remove excess Nissl solution. Following this, slides were immersed in 96% Ethanol containing 6 drops of glacial acetic acid (Sigma) and the level of reaction was evaluated under a light microscope. To stop this reaction, sections were transferred into 100% Ethanol, isopropanol, followed by xylene. Finally, coverslips were permanently mounted using Depex (BDH, UK).

Toluidine Blue Stain

Ventral root sections were allowed to dry under a lamp. Then, Toluidine Blue was prepared by dissolving 1g sodium borate in 100ml ddH_2O plus 1g toluidine blue (Electron Microscopy Science, Hatfield). Solution was filtered before use. Subsequently, a few drops of toluidine-blue solution were placed on the warm slide for 2-5 minutes. The excess stain was rinsed off with several washes of water. Then, slides were dehydrated with 96% and 100% Ethanol, and then passed into xylene. Sections were covered with a cover slip using Depex (BDH, UK).

Cholinesterase and Silver Stain

In order to visualize the endplates and axons at the neuromuscular junction, the muscle sections were incubated in wells with the following solutions and reagents. The incubations were carried out in the order presented in table 2 and for the time indicated. Following the last incubation in distilled water, tissue was carefully removed from wells, placed onto gelatinised slides, and spread as

needed under the microscope with fine brushes. All slides were then left overnight to dry in the oven at 37 ^{0}Cbefore being covered with a glass coverslip using DPX. The list of reagents for each solution can be found in table 3, 4 and 5. Reagents were obtained from Sigma, unless otherwise stated.

Table 2. Incubation steps for the Cholinesterase and Silver Stain

Solution	Time
Incubation Solution 1	5 mins
Distilled Water	30 secs
8mM Potassium Ferricyanide	10 mins
Distilled Water	5 mins
Absolute Ethanol	30 mins
Distilled Water	30 secs
Silver Solution *	60 mins
Reducer Solution **	4 mins
Distilled Water	30 secs

*All incubations were carried out at room temperature except for incubation in silver solution, which was at 37 ^{0}C (in oven).

** This last incubation was carried out with the plate under the microscope in order to determine extent of reaction.

Table 3. Reagents required for Incubation Solution 1 used in the Cholinesterase and Silver Stain

Regent	Volume
Maleate buffer (0.1M sodium hydrogen maleate) Maleic acid 1.16g Sodium hydroxide 0.62g Made up to 200ml with distilled water	13ml
Tri-sodium citrate 100mM	1ml

Copper sulphate (anhydrous) 30mM	2ml
Distilled water	2ml
Potassium ferricyanide 5mM	2ml
Sucrose	3g
Acetylthiocholine iodide	10mg

Table 4. Reagents required for Silver solution used in the Cholinesterase and Silver Stain. The preparation of this solution first required $CaCO_3$ to be added to distilled water and mixed gently. This solution was then filtered. Subsequently, $CuSO_4.5H_2O$ was also dissolved followed by silver nitrate. This was incubated for at least 1 hour at 37˚C prior to use.

Reagent	Volume
Distilled water	50ml
$CaCO_3$	0.05g
$CuSO_4.5H_2O$	0.025g
$AgNO_3$	5g

Table 5. Reagents required for Reducer Solution used in the Cholinesterase and Silver Stain. The reagents below were dissolved in distilled water to make up 100ml.

Reagent	Volume
Hydroquinone	1g
$NaSO_3$	10g

Immunofluorescence

Hindbrain sections were treated using the same protocol as for the immunohistochemistry, up to and including the incubation step with the secondary antibody. Subsequently, sections were washed with 1% PB/BSA and

100mM PB (pH=7.4), then sections were dabbed dry and incubated with Alexa flour 488 for 1 hour (Anti-rabbit IgG-Alexa flour 488 produced in goat; Life Technologies, UK). Excess antibody was removed using 10mM PB. A glass coverslip was mounted on top of the tissue section with VectaShield (Vector, UK). The slides were stored in the dark at 4°C until confocal scanning.

Quantifications

All quantifications were carried out by an experimenter unaware of the mouse genotype or days after facial axotomy.

Motor Function

SOD^{G93A} mice survival measure

SOD^{G93A} mice were examined for the onset of complete bilateral hind-limb paralysis. The age, in days, at which mice reached end stage was used for survival analysis.

Whisker Motor Function Assessment of TDP-43 mutant mice

Mouse whisker movement was assessed from 7 to 28 days following facial nerve injury. Whisker movement ipsilateral to the facial nerve injury was compared to that of the un-operated side and scored using a scale of 0.0 (no movement) to 3.0 (normal movement as on the un-operated side) in 0.5 increments (Raivich et al., 2004).

To obtain an average motor score, independent of time, functional recovery index (FRI) for each animal was determined by calculating the area under the curve for its whisker hair motor performance scores from day 7 to 28. The average motor score represents the average of the functional recovery indices from all animals in each group.

$$FRI = \frac{\sum(x_n - x_{n-1})(y_{n-1} + y_n)}{x_n}$$

Where x = days post injury, y = whisker hair motor performance score, and n=day post injury.

Cellular counts and immunoreactivity analysis

SOD1^{G93A} project

Neuronal Counts in the Spinal Cord

Transverse sections of L5 spinal cord stained for H&E were visualised with a Zeiss Axiolab light microscope (Carl Zeiss, UK) using a x10 objective lens. Then, the left or right ventral horns of the spinal cords were divided into two imaginary parts; ventro-medial and lateral areas (Figure 3). Motor neurons in each of these areas were counted at high power using a x40 objective lens.

Nucleated α-motor neurons in the ventral horn of the spinal cord were counted in every 10th section (by a blinded investigator). Counts across the entire L5 segment were averaged per area of interest. This average was multiplied by the total number of pf sections per L5 segment. This total number of α-motor neurons per area of interest were added together to obtain the total number of α-motor neurons in the L5 segment.

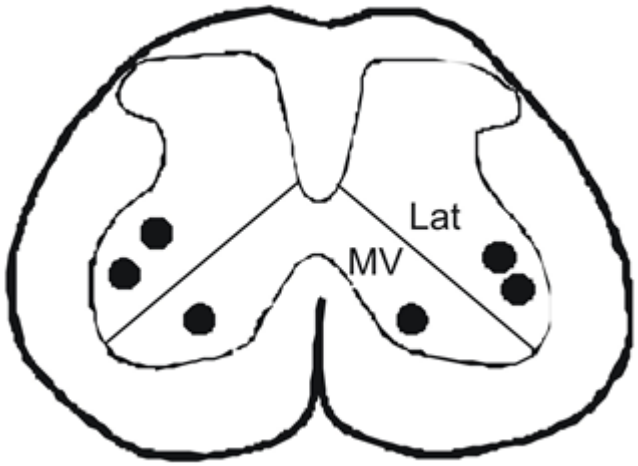


Figure 3. Schematic representation of a section through the L5 spinal cord. The thin lines resent the borders between the four areas in which motor neurons were counted. Lat: Lateral and MV: medio-ventral. The black circles represent the nucleated motor neurons that were counted.

Neuronal Cell Diameter in the Spinal Cord

Digital images of the ventral horn of spinal cord stained for H&E were obtained using a Sony 3 CCD video camera (AVT-Horn, Aachen, Germany). Optimas 6.2 software (Bothwell, WA) was used to draw around the circumference of every neuron present in the vental horn and to calculate the area of the neuron in question. Assuming the neuron is nearly spherical; the mean diameter (d) was calculated from the mean area (A) using the following formula:

$$d = \sqrt{(4\ x\ A/\pi)}$$

All α-motor neurons per slide whenre measured in every 10th slide until a total of 50 motor neurons were assessed per animal.

Cell and Cellular Nuclei Counts in the Spinal Cord

For quantifications of glial cells, T-cells, or neuronal-like cells or nuclei, 4µm-thick transverse sections trough the cervical spinal cords were immunostained for various inflammatory markers (αM, αX, GFAP or CD3) or regeneration-associated molecules (c-Jun, CGRP, CD44 and α7), were divided into the dorsal and ventral regions by drawing an imaginary horizontal line through the central canal (Fig. 4). The ventral region of the transverse spinal cord was further divided into the left and right side of the spinal cord by drawing an imaginary vertical line from the central canal down to the anterior median fissure (Fig. 4). Cells expressing inflammatory markers and regeneration associated molecules were counted in the grey matter of left and right ventral horns. The white matter of the spinal cord was also evaluated, specifically the lateral and anterior funiculi ventral to the imaginary line horizontally traversing the central canal, which will be collectively known as the ventral white matter. Cells expressing inflammatory markers were counted in the left and right ventral white matter.

The numbers of αM- or αX-positive microglial cells and microglial clusters (defined as > 3 microglial cell), GFAP-positive astrocytes with a stellar morphology, CD3-postive T-cells, c-Jun-positive neuronal-like nuclei, or CGRP-, CD44- or α7-positive cells with a neuronal morphology, were manually counted in the left and right ventral horn and/or ventral white matter of the spinal cord. Nuclei or cell counts were carried out under a light microscope at high power using a x40 objective lens. Four sections, 10 sections apart, across the cervical enlargement were sampled. The mean numbers of nuclei or cells were calculated by averaging the left and right side of the ventral horn or ventral white matter of the spinal cord, then obtaining an average per animal.

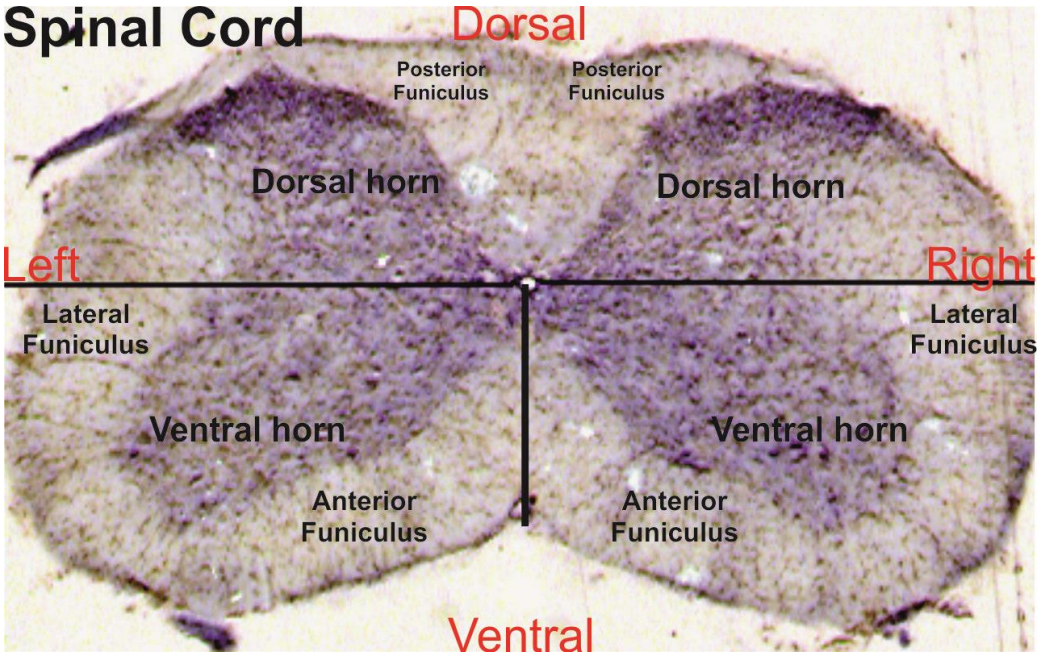


Figure 4. Quantification of non-neuronal cells in the spinal cord. Dorsal and ventral regions were divided by a horizontal line in the plane of the central canal. The ventral region was further divided into the left and right side by a perpendicular line stemming from the central canal. The spinal cord section shown here has been stained for Nissl, as the distinct Nissl staining in the grey matter allows easy discrimination of grey and white matter. The ventral horns and the ventral white matter were analysed in this project.

Immunoreactivity quantification in the spinal cord

To quantify the staining intensity of αM, GFAP or CD44 in the ventral horn and the ventral white matter, the Mean and standard deviation algorithm was used (Moller et al., 1996)(Fig 5-green outline). Three pictures were taken from the same slide containing a transverse section through the cervical spinal cord: 1) the left side of the spinal cord ventral to the central canal (Fig. 5-left red square), 2) the right side of the spinal cord ventral to the central canal (Fig. 5-right red square), 3) and an area of the glass slide without the tissue section. The images were obtained using x10 objective lens connected to a Sony 3 CCD video camera. Images were 8 bit digital, based on a 0–255 scale of optical luminosity values (OLV). Using the OPTIMAS 6.2 imaging system (Media Cybernetics Inc, Silver Spring, MD), the Mean and Standard Deviation of the optical luminosity value for the areas of interest were obtained. The ventral horn and ventral white matter were selected using the Optimas sketch tool. For these areas, the optical luminosity value was calculated by subtracting the

Standard Deviation from the corresponding Mean value. Then, the OLV (Mean-SD) for a selected area of the glass slide picture was subtracted by the OLV (Mean-SD) of the left and right ventral horns or ventral white matter. The values for the ventral horn and the ventral white matter were calculated by averaging the OLVs of the left and right side ventral horn or ventral white matter. The mean OLV of the ventral horn and ventral white matter per animal was averaged across sides, sections and animals calculated by averaging across the OLVs of ventral horn and ventral white matter of 4 slides, 10 tissue sections apart. The mean OLV per animal was used to calculate the OLV per group.

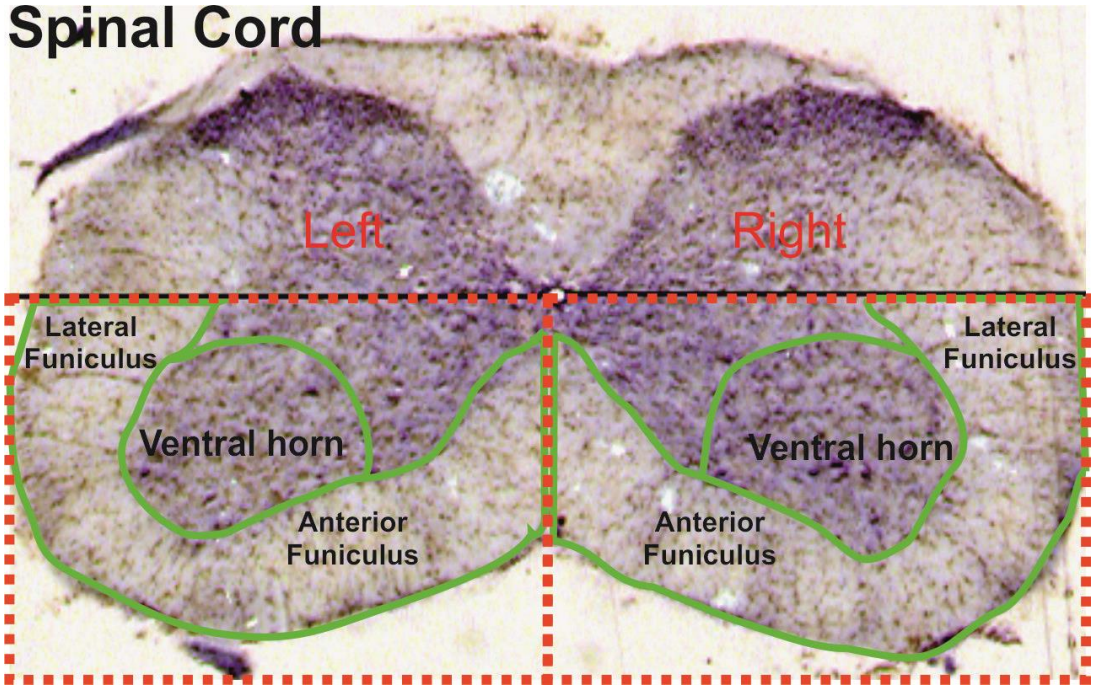


Figure 5. Quantification of immunoreactivity in the ventral horn and the ventral white matter. Pictures of the left and right spinal cord regions ventral to the central canal (red squares) were taken to quantify the staining intensity of the left and right ventral horns or the ventral white matter (green outline).

Neuronal Counts in the Hindbrain

The left and right facial and hypoglossal nuclei were analysed (Fig.6). Using a Zeiss light microscope at high power (x40 objective lens), the number of neuronal cells whithin the left and right nuclei were counted and corrected for cell size using the Abercrombie Correction coefficient (Abercrombie and Johnson, 1946).

$$N = n\,x\left[\frac{D}{D+d}\right]$$

Where N is the corrected neuronal number; n is the counted number of neurons; D is the section thickness (25um) and d is the mean neuronal diameter, which was calculated using the same method as in the spinal cord.

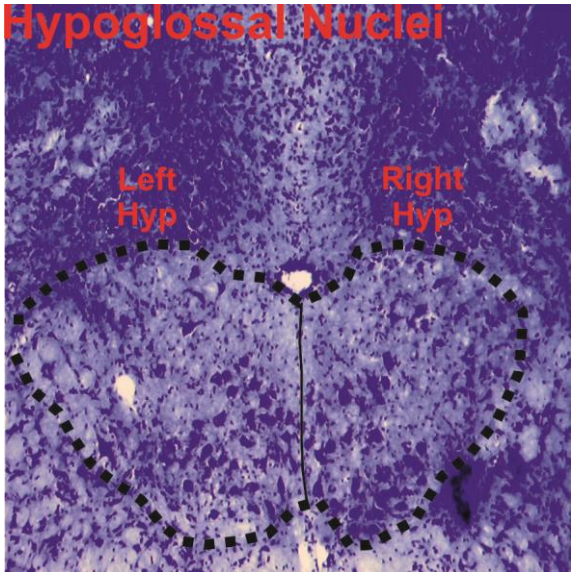


Figure 6. Quantification of motor neurons in the hypoglassal nucleus. Micrograph shows an example of a section through the hypoglossal nuclei stained with Nissl. The dashed lines show the border of the hypoglossal nucleous (Hyp). The line originating from the central canal allows the split of the left and right hypoglossal nuclei.

Non-Neuronal cell and Neuronal-like cell counts in the hindbrain

Quantification of the number of αM- or αX-positive microglia as well as GFAP- or CD44-positive astrocytes were counted in the left and right hypoglossal or facial nuclei (Fig.7), using a x40 objective lens. In addition, c-Jun-positive neuronal-like nuclei, or CD44-, α7- , β1- or galanin-positive cells with a neuronal shape were counted in left and right hypoglossal or facial nuclei using a Zeiss light microscope with a x40 objective lens. For each stain, the mean number of cells per motor nuclei, across 4 sections (200 microns away from each other), was calculated per animal.

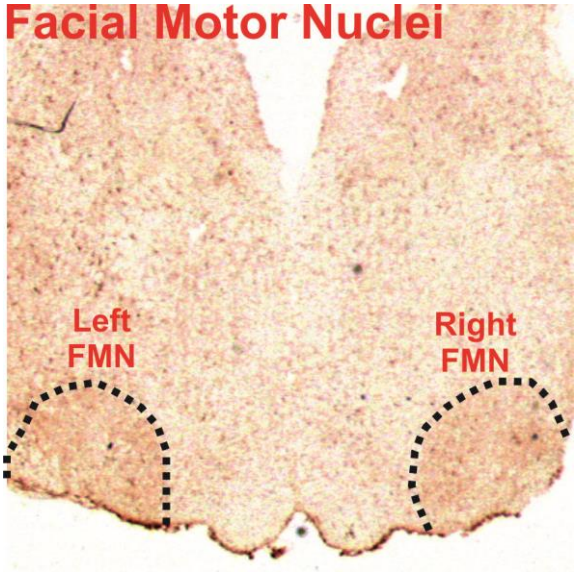


Figure 7. Quantification of non-neuronal or neuronal-like cells in the facial motor nucleous. Micrograph shows an example of a section through the facial nuclei stained for microglia. The regions outlined with dashed lines show the left and right facial motor nucleus (FMN).

Ventral Root Axon Counts

The L5 ventral roots (Fig.8) were stained with toluidine blue to visualise myelinated axons. In the ventral root, the total number of large, small, and dying axons were counted at high power using a x100 objective lens. The mean number of axons across 5 consecutive sections was calculated per animal.

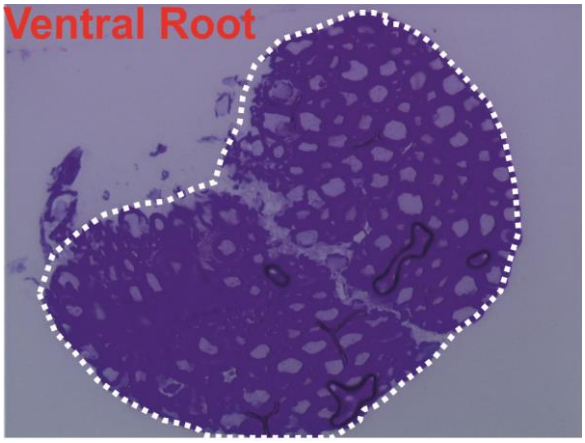


Figure 8. Quantification of axons in the ventral root. Micrograph shows an example of a transverse section through the L5 ventral root stained with toluidine blue. The dashed white line demarcates the circumference of the ventral root.

Neuro Muscular Junction Quantification

Longitudinal sections through the gastrocnemius muscle were stained using the Cholinesterase and Silver method. The total numbers of fully innervated, partially innervated or denervated neuromuscular junctions were counted. The mean number of neuromuscular junction in each of the three categories was calculated across 4 consecutive slides per animal.

Facial nerve axotomy projects

Facial motor neurons

In mice that underwent facial nerve injury, the facial nucleus ipsilateral to the facial nerve injury is known as the injured or experiment facial motor nucleus, while the contralateral nucleus is known as the uninjured or control facial motor nucleus. Using a x20 magnification lens, the total fluorogold-, Nissl- or CD-44-positive motor neurons, αM- or B7.2-positive microglial clusters, CD3-positive T-lymphocytes, as well as Galanin- or CGRP-positive sprouts, were counted in the injured and uninjured facial motor nucleus. The mean number of cells or sprouts across 5 sections, 10 slides apart, was calculated per facial motor nuclei and then per animal.

Immunoreactivity Quantification

The staining intensity was quantified by taking a picture of the left and right facial nuclei, using a Sony 3 CCD video camera. The images were 8 bit digital, based on a 0–255 scale of optical luminosity values. The immunohistochemical staining was quantified using the OPTIMAS 6.2 imaging system (Media Cybernetics Inc, Silver Spring, MD) and the Mean and Standard Deviation algorithm. The overall mean optical luminosity (OLV) value for the antibody staining intensity was determined for each facial motor nucleus, and subsequently the staining intensity of the adjacent glass was subtracted, as described above for spinal cord quantifications.

Neuronal Nuclei Quantification

To quantify the intensity of nuclear immunoreactivity the OLV (Mean-SD) algorithm was modified. The staining intensity was quantified by taking a picture of the left and right facial nuclei, using a Sony 3 CCD video camera. Using the OPTIMAS 6.2 imaging system (Media Cybernetics Inc, Silver Spring, MD) optical luminosity values were used two define a low and high immunoreactivity

(OLV) thresholds. Low optical luminosity threshold was mean plus 1 standard deviation (SD) of the axotomised image OLV. High optical luminosity threshold was calculated using the formula: (Control mean + 2SD) / (Control mean – 0.5SD) x (Axotomy mean – 0.5SD). Next, an area range was set using Object Classes Function: ArPixelCounts>200 to identify areas big enough to be a motor neuron nucleus. The use of the low optical luminosity threshold and object recognition area range allowed all motor neuron nuclei within the facial nucleus to be counted using an automatic quantification algorithm in Optimas. Next, the high optical luminosity threshold and the same object recognition area range were used to detect and quantify only the intensely labelled nuclei. The ratio between low threshold counts and high threshold counts on control side was calculated and the same was done for the axotomised side.

Image acquisition

A Sony 3CCD colour video camera was used to obtain 8-bit digital images. Digital micrographs of fluorescently labelled sections were taken using Leica TCS SP2 confocal laser microscope with the software Application Suite 2.6.0.7266 (Leica). Images were further edited using Optimas 6.2 software.

Statistical analysis

Survival curves were generated using the Kaplan–Meier method. Normal distribution was assessed using D'Agostino & Pearson omnibus normality test. Differences in survival between $SOD1^{G93A}$ jun-competent mice and those lacking neural jun was determined by log-rank (Mantel–Cox Test) and Gehan–Breslow–Wilcoxon Test and Student T-test using GraphPad Prism.

To determine the difference in immunohistochemical staining intensities, and cellular, axonal or neuromuscular junction counts between $SOD1^{G93A}$ jun-competent mice and those lacking neural jun, TDP-43 and wild type mice, or injured and intact mice, a two-tailed unpaired Student’s T-test was used.

When more than two groups were tested, for example when gender specific effects on survival were evaluated, a three-way ANOVA was used followed by a post-hoc Tukey test. The mean and standard error of the mean (SEM) was collected for all data. For all statistical analyses, significance was determined when P<5% and were carried out using Microsoft EXCEL.

Chapter 3: The role of c-Jun in amyotrophic lateral sclerosis

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterised by the degeneration of motor neurons. Approximately 10% of ALS cases are familial (familial ALS or FALS), about 20% of which are caused by mutations in the gene that encodes for cytosolic superoxide dismutase 1 (SOD1) (Bruijn et al., 2004). Transgenic mice overexpressing a variety of mutant human SOD1 associated with FALS, such as $SOD1^{G93A}$, recapitulate several aspects of ALS and have been widely used to test pharmacological and gene-based therapies (Borchelt et al., 1998). In addition to the motor neuron degeneration observed in the spinal cord, there are also substantial degenerative changes in some cranial motor nuclei (e.g. facial and hypoglossal) of ALS patients and $SOD1^{G93A}$ transgenic mice (Haenggeli and Kato, 2002, Dalcanto and Gurney, 1994). This neuronal degeneration is spatiotemporally correlated with neuroinflammatory changes, which include activation of microglia and astrocytes and the infiltration of T-cells (Alexianu et al., 2001). The molecular pathways leading to the death of neurons in the disease remain elusive. However, the transcription factor c-Jun has been found upregulated in areas or cells undergoing neurodegeneration in both ALS patients and SOD1G3A mice (Jaarsma et al., 1996, Virgo and Debelleroche, 1995).

In normal physiology, peripheral nerve transection (axotomy) causes a strong upregulation of c-Jun by injured motor neurons, and degenerative changes similar to those observed in ALS, such as loss of target innervation at the neuromuscular junctions, axonal loss and in some cases motor neuron death (Fischer et al., 2004). However, following such injury, motor neurons also mount a regenerative effort, which is characterised by the upregulation and de novo expression of axotomy-response/regeneration-related proteins (e.g. the integrins α7 and β1, the neuropeptide galanin, cytoskeletal adaptor molecule GAP-43 and the proteoglycan CD44) (Patodia and Raivich, 2012a). Such regenerative responses have also been described in ALS cases and mouse models of the disease (Malaspina et al., 2010, Parhad et al., 1992, Ikemoto et al., 1999, Wagey et al., 1998, Jaarsma et al., 1996, Lobsiger et al., 2007).

Previously in mice, deletion of the floxed c-Jun gene (*jun*) in neurons and glia, using cre::recombinase driven by the nestin-promoter (nes::cre), has been found to cause an almost complete disappearance in axotomy mediated motor facial neuron death and a reduction in reactive gliosis and T-cell infiltration (Raivich et al., 2004). However, these neuroprotective and anti-inflammatory effects were also accompanied by a severe impairment in axonal regeneration, associated with the failure of motor neurons to upregulate axotomy-response/regeneration-related proteins following injury (Raivich et al., 2004).

In ALS and mouse models of ALS there is degeneration of motor neurons in the spinal cord and brainstem, associated with inflammatory changes and the expression of regeneration associated genes in degenerating motor neurons (Malaspina et al., 2010, Parhad et al., 1992, Ikemoto et al., 1999, Wagey et al., 1998, Jaarsma et al., 1996, Lobsiger et al., 2007). In this chapter I shall examine whether c-Jun is necessary for the development of the pathological changes found in SOD1^{G93A} transgenic mice.

To determine the role of *jun* in SOD1-related ALS disease, we crossed the high copy number strain of SOD1^{G93A} transgenic mice with mice carrying floxed gene encoding c-Jun (*jun*^{f}) and those that express Cre recombinase under the control of nestin-promoter (nes::cre) that can be used to remove floxed genes in cells expressing nestin (i.e. most brain precursor cells, including those that will develop into motor neurons). In this way we generated SOD1^{G93A} *jun*$^{f/f}$ littermate mice with or without nes::cre, with the former lacking *jun* inside their brain and spinal cord (SOD1^{G93A} *jun*$^{\Delta N}$). As both groups of mice are littermates and share the same genetic background any differences between them would be only due to the nes::cre recombinase-mediated deletion of *jun*.

Results

Effects of deletion of the neuronal c-Jun gene on the lifespan of SOD1G93A mice

To investigate the effect of neuronal *jun* deletion on the survival of SOD1^{G93A} mice, transgenic SOD1^{G93A} *jun*$^{\Delta N}$ mice (n=12) and litter mate controls-SOD1^{G93A} *jun*$^{f/f}$ (n=10), were examined for the onset of complete bilateral hind-limb paralysis. For ethical reasons, this measure was defined as the end-stage

where mice underwent euthanasia. The age, in days, at which mice reached end-stage was used for survival analysis.

The Kaplan-Meier survival plot in Figure 1 shows that the life-span of the jun deficient SOD1^{G93A} *jun*$^{\Delta N}$ mutants (Fig.1-red circles) was extended in comparison to jun competent SOD1^{G93A} *jun*$^{f/f}$ control littermates (Fig.1-blue circles). Survival data from each group underwent D'Agostino & Pearson omnibus normality tests which demonstrated the data in each group to have normal distribution (SOD1^{G93A} *jun*$^{f/f}$; p=23% and SOD1^{G93A} *jun*$^{\Delta N}$; p=65%), suggesting that mean, media and mode are equal in each group. On average, SOD1G93A *jun*$^{\Delta N}$ mice survived 114±2 days which was significantly more than SOD1^{G93A} *jun*$^{f/f}$ mice which survived 104±3 days (MEAN±SEM p<2.5%, student t-test).

To investigate possible gender specific effects on survival (Scott et al., 2008), the effects of genotype, sex and combination of both was analysed, using a 3-way ANOVA. This confirmed a statistically significant effect for genotype ($F_{1,16}$=5.17, p=3%), but revealed no effect for sex ($F_{1,16}$=1.0, p=34%), or the sex and genotype combination ($F_{3,16}$=0.1, p=97%) (Data not shown).

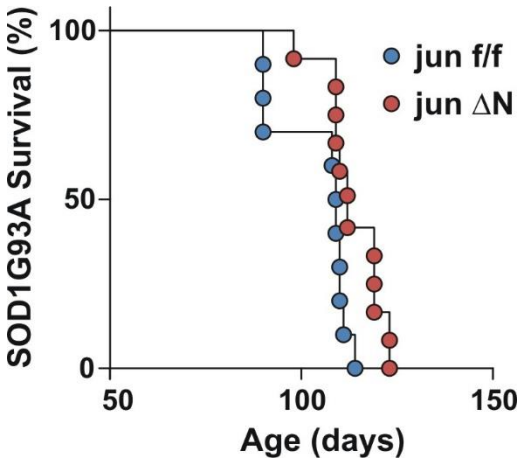


Figure 1. Neural deletion of *jun* prolongs survival of SOD1^{G93A} mice. Kaplan-Meier survival plot for SOD1G93A mice lacking neural *jun* (*jun*$^{\Delta N}$, red) and their littermate controls (*jun*$^{f/f}$, blue). On average SOD1G93A *jun* $^{\Delta N}$ mutants (n=12)

survived 10 days longer than SOD1G93A *jun* f/f controls (n=10). Gehan-Breslow-Wilcoxon, p=2.31%; Mantel-Cox log-rank, p=1.03%.

Neuroinflammation and glial activation is reduced in *jun* deficient SOD1G93A mice

SOD1^{G93A} mediated neurodegeneration in mice is characterised by a neuroinflammatory response, which begins early on, before the onset of disease, and peaks at the end, when mice die (Alexianu et al., 2001). In order to determine the effect of *jun* deletion in the early as well as late stages of this inflammatory response, we analysed the ventral horns and the ventral white matter, comprising of the lateral and anterior funiculi ventral to an imaginary horizontal line traversing the central canal, of cervical enlargement spinal cords of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice and their SOD1^{G93A} *jun*$^{f/f}$ control littermates, at two time points: a pre-symptomatic stage, where mice were allowed to survive for 70 days after birth (D70), or reach end-stage (ES), when the mice had to be culled. To study these changes immunohistochemistry was used to label cellular markers of neuroinflammation including, αM or αX (upregulated in activated microglia), GFAP or CD44 (upregulated in astrocytes), or CD3 (a pan-marker of T-cells) in sections of the spinal cord.

Microglial activation

αM

Microglia become activated in response to neuronal injury (Raivich et al., 1999). Microglial activation can be characterised by changes in cell morphology and expression of different cell membrane molecules (Raivich et al., 1999). During early activation, microglia increase the expression of the αM integrin (Raivich et al., 1999). To evaluate the effect of *jun* on early microglial activation in the ventral horn and the ventral white matter of SOD1^{G93A} mice, transverse sections through the cervical enlargements of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice and their SOD1^{G93A} *jun*$^{f/f}$ controls, at 70 days of age or end-stage, were immunostained for αM and the morphology of the microglia studied.

In mice at 70 days of age, there was little difference in the level of αM-immunostaining in the ventral white matter of the spinal cord of SOD1^{G93A} *jun*$^{f/f}$

mice (Fig. 2A) and SOD1^{G93A} *jun*$^{\Delta N}$ mice (Fig. 2B). However, a difference between groups could be observed in the morphology of microglia in the grey matter of the ventral horn of the spinal cord. Microglia in SOD1^{G93A} *jun*$^{\Delta N}$ mice (Fig. 2B) were ramified, while in SOD1^{G93A} *jun*$^{f/f}$ mice (Fig. 2A), microglia had rounded cell bodies with short processes that were strongly αM-positive. In addition, more large aggregates of αM-positive microglia could be found in the ventral horn of the spinal cord of SOD1^{G93A} *jun*$^{f/f}$ mice (Fig. 2A), than SOD1^{G93A} *jun*$^{\Delta N}$ mice (Fig. 2B). At end-stage, αM staining was strong in the spinal cords of SOD1^{G93A} *jun*$^{f/f}$ mice (Fig. 2C) and SOD1^{G93A} *jun*$^{\Delta N}$ mice (Fig. 2D). Microglia appeared to be activated, as demonstrated by presence of the short and thick processes protruding from their cell bodies in both the ventral horn and the ventral white matter. As at day 70, SOD1^{G93A} *jun*$^{f/f}$ mice (Fig. 2C) had more αM-positive clusters in the ventral horn of the spinal cord, than SOD1^{G93A} *jun*$^{\Delta N}$ mice (Fig. 2D).

To quantify the level of early microglial activation, αM immunoreactivity was measured in the ventral horn and the ventral white matter, using the Mean-SD algorithm (as described in the Material and Methods section) (Fig. 3A). It was found that αM immunoreactivity was not affected by *jun* deletion in SOD1^{G93A} mice at either of the two studied time points (Fig. 3). Since microglial cluster formation was observed, the total numbers of αM-positive clusters in the ventral horn and the ventral white matter were manually counted at high power (using a x40 objective lens). At day 70, *jun* deficient SOD1^{G93A} mice had 1±0.2 αM-positive microglial clusters in the grey matter of the ventral horn, which was significantly more than the 0.3±0.1 microglial clusters in SOD1^{G93A} *jun*$^{\Delta N}$ mice. Similarly, more microglial clusters could be found in the white matter SOD1^{G93A} *jun*$^{f/f}$ mice (Mean±SEM: 0.1±0.1), than SOD1^{G93A} *jun*$^{\Delta N}$ mice (0±0) ($p<5\%$ between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups, using unpaired Student's T-test). This difference disappeared at end-stage, when the αM-positive clusters became much more numerous in both groups; indicating that αM-positive cluster formation was only delayed, not prevented, in the *jun*$^{\Delta N}$ group.

αX integrin

In the presence of abundant neuronal debris, microglia change from a ramified to an amoeboid morphology, which is characteristic of phagocytic microglia undergoing late-phase activation (Raivich et al., 1999). Coinciding with the morphological changes, microglia express αX de-novo (Raivich et al., 1999). To evaluate the effect of *jun* on late-phase microglial activation in the ventral horn and ventral white matter of SOD1^{G93A} mice, transverse sections through the L5 spinal cords of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice and their SOD1^{G93A} *jun*$^{f/f}$ littermate controls, at 70 days of age or end-stage, were immunostained for αX.

At day 70, little to no αX-positive phagocytic microglia could be observed in transverse sections through the spinal cord of spinal cord of SOD1^{G93A} *jun*$^{\Delta N}$ (Fig.2F) mutant mice or their SOD1^{G93A} *jun*$^{f/f}$ littermate controls (Fig.2E). However, at end-stage, SOD1^{G93A} *jun*$^{f/f}$ mice (Fig.2G) had abundant αX-positive microglia with a rounded morphology and αX-positive nodules within the ventral horn. In contrast, only a few αX-positive nodules could be found in the ventral horn of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice (Fig.2H).

The number of αX-positive phagocytic microglial clusters in the ventral horn and the ventral white matter of the SOD1^{G93A} mice at day 70 and end-stage were quantified at high power (using a x40 objective lens). As observed above, the spinal cord of SOD1^{G93A} mice showed no αX-positive cluster formation at day 70. However, by end-stage there was a dramatic increase in the number of these phagocytic clusters, in both the ventral horn and the ventral white matter (Fig.3C). However, a significant *jun*-mediated effect was only observed in the ventral horn. Deletion of *jun* significantly decreased the number of αX-positive clusters found in the ventral horn at end-stage, with SOD1^{G93A} *jun*$^{f/f}$ controls having 8±1 αX-positive phagocytic microglial clusters per ventral horn, while SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice having 5±1 ($p<5\%$ between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups, using unpaired Student's T-test). This may suggest that *jun* deletion results in less neuronal debris and therefore less phagocytosis at end-stage.

Astrocyte activation

GFAP

Astrocyte activation is observed in regions of the CNS undergoing degeneration in ALS (Alexianu et al., 2001). During activation, astrocytes upregulate the expression of GFAP (Raivich et al., 1999). To evaluate the effect of *jun* on astrocyte activation in the ventral horn and ventral white matter of SOD1^{G93A} mice, transverse sections through the cervical enlargement of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice and their SOD1^{G93A} *jun*$^{f/f}$ littermate controls, at 70 days of age or end-stage, were immunostained for GFAP.

Astrocyte activation, as characterised by enhanced GFAP immunostaining, increased with disease progression in both the ventral horn and the ventral white matter of SOD1G93A mice irrespective of *jun* deletion (Fig.2I-L). At 70 days, GFAP expression was weak and there was only a few GFAP-positive astrocytes within the ventral horn and the ventral white matter (Fig.2I & J). At end-stage GFAP-immunoreactivity was strong in the ventral horn and the ventral white matter of control (Fig.2K) as well as mutant mice (Fig.2L). In addition, GFAP-immunoreactivity was more or less similar between mutant (Fig.2J & L) and control transgenic mice (Fig.2I & K) at day 70 and end-stage.

To quantify astrocyte activation, GFAP immunoreactivity (IR) was measured in the ventral horn and the ventral white matter of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice and their SOD1^{G93A} *jun*$^{f/f}$ littermate controls, using the Mean-SD algorithm (Fig. 3D) (Moller et al., 1996). It was found that the level of GFAP immunoreactivity in the ventral horn and the ventral white matter of SOD1^{G93A} mice, at both 70 days of age or end-stage, was not affected by the deletion of *jun*.

To further describe the response of astrocytes to disease progression, the number of GFAP-positive astrocytes in the ventral horn and the ventral white matter of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice and their SOD1^{G93A} *jun*$^{f/f}$ littermate controls, were manually counted at high power using a x40 objective lens (Fig.3E). As shown in Figure 3E, the number of astrocytes in the ventral white matter of SOD1^{G93A} mice, was similar at day 70 and end-stage. In contrast, the number of activated astrocytes in the ventral horn of end-stage SOD1^{G93A} mice

was 4 times more than that observed at day D70. However, astrocyte numbers in both the ventral horn and white matter were not altered in mice lacking c-Jun in neural cells, confirming the GFAP-immunoreactivity measurements.

CD44

In the presence of high levels of neuroinflammation or severe neural injury grey matter astrocytes express the proteoglycan CD44 (Jones et al., 2000, Makwana et al., 2007). To evaluate the effect of *jun* on severe astrocyte activation in the ventral horn and ventral white matter of SOD1^{G93A} mice, transverse sections through the cervical enlargement of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice and their SOD1^{G93A} *jun*$^{f/f}$ littermate controls, at 70 days of age or end-stage, were immunostained for CD44.

At the pre-symptomatic stage, CD44 staining was confined to the ventral white matter of both mutant (Fig. 2N) and control mice (Fig.2M). However, CD44 immunoreactivity at end-stage was found in both the ventral horn and the ventral white matter of both mutant (Fig. 2Q) and control mice (Fig. 2P). CD44 staining had a mottled appearance characteristic of astrocytic CD44 (Fig. 2P&Q) (Jones et al., 2000, Makwana et al., 2007). Mutant mice showed less CD44 immunoreactivity in the ventral horn (Fig. 2Q), than control mice at end-stage (Fig. 2P), but no difference in the levels of CD44 staining in the ventral white matter was observed.

To quantify this effect, CD44 immunoreactivity (IR) was measured in the ventral horn and the ventral white matter, using the Mean-SD algorithm (Fig. 3F). In line with the observations above, immunoreactivity was significantly lower in the ventral horn of *jun*-deficient SOD1^{G93A} mice (Fig. 3F-red bar) compared to *jun* competent littermate controls at end-stage (Fig. 3F-blue bar) ($p<5\%$ between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups, using unpaired Student's T-test). No other differences between the two jun genotypes were found in the white matter at end-stage or in the grey and white matter at day 70.

T-cell infiltration

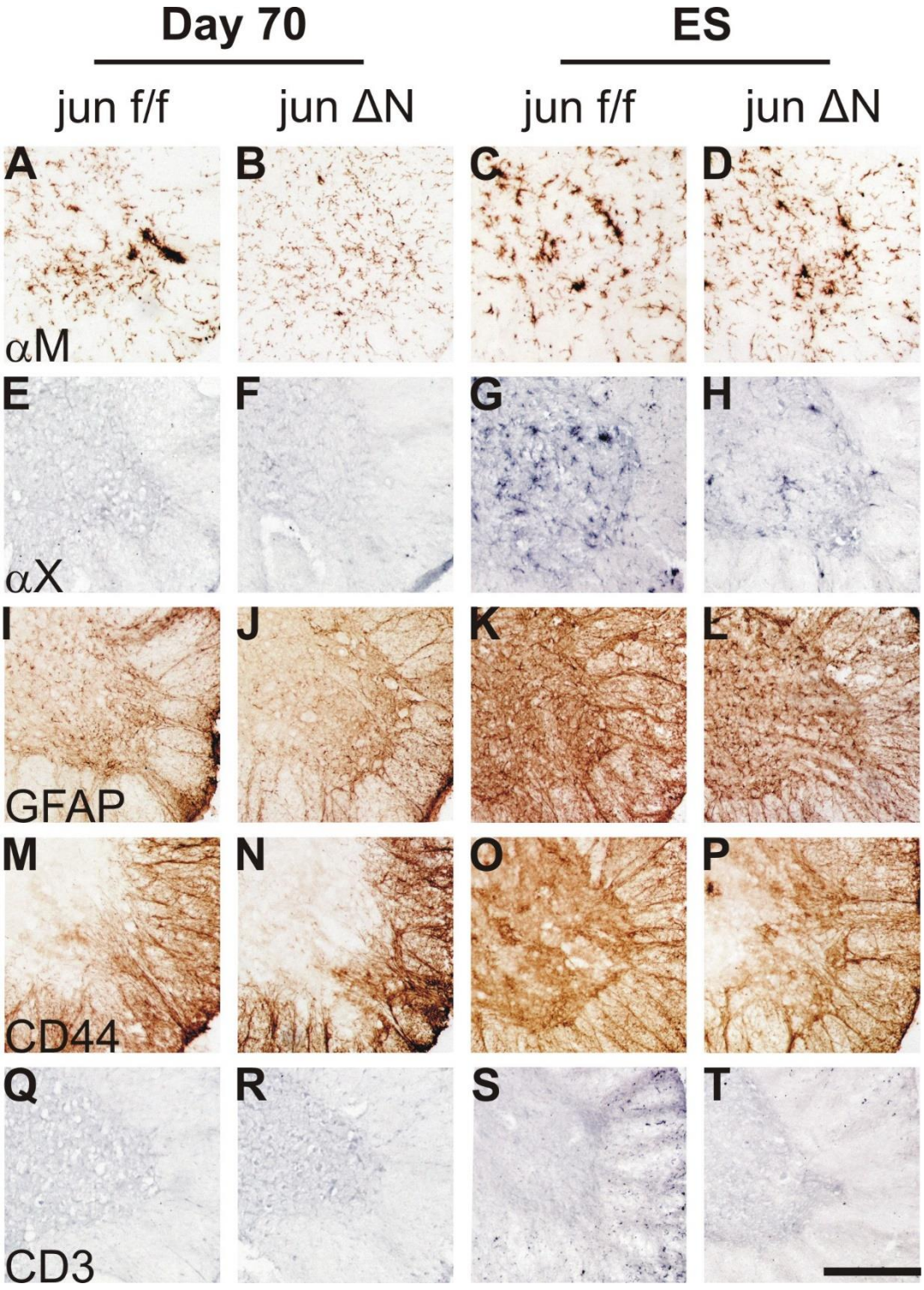
T-cell infiltration into areas undergoing neurodegeneration has been observed in ALS (Alexianu et al., 2001). To evaluate the effect of *jun* on T-cell infiltration

into ventral horn or ventral white matter of SOD1^{G93A} mice, transverse sections through the spinal cord of SOD1^{G93A} *jun*$^{\Delta N}$ mutant mice and their SOD1^{G93A} *jun*$^{f/f}$ controls, at 70 days of age or end-stage, were immunostained for the T-cell marker CD3.

At day 70, there were little to no CD3-positive T-cells in both the ventral horn and ventral white matter of SOD1^{G93A} *jun*$^{\Delta N}$ (Fig.2R) or SOD1^{G93A} *jun*$^{f/f}$ mice (Fig.2Q). However, at end-stage, T-cells appeared to preferentially infiltrate the ventral white matter of SOD1^{G93A} *jun*$^{\Delta N}$ (Fig.2T) and SOD1^{G93A} *jun*$^{f/f}$ mice (Fig.2S). Although T-cell infiltration could be seen in *jun* deficient and competent mice, there were fewer T-cells present in the *jun* deficient mice (Fig.2T) than *jun* competent littermate controls (Fig.2S).

To quantify T-cell infiltration, CD3-positive T-cells were manually counted in the ventral horn or ventral white matter at high power (using a x40 objective lens) (Fig.3G). There were little to no T-cells present in the spinal cord of SOD1^{G93A} *jun*$^{\Delta N}$ mice or SOD1^{G93A} *jun*$^{f/f}$ littermate controls at D70. However by end-stage, the deletion of *jun* was found to reduce T-cell infiltration in SOD1^{G93A} mice (Fig. 3G). There were 8±1 T-cells present per ventral white matter of SOD1^{G93A} *jun*$^{f/f}$ mice, which was significantly more than 5±1 T-cells found in the SOD1^{G93A} *jun*$^{\Delta N}$ mice ($p<5\%$ between *jun* $^{f/f}$ and *jun*$^{\Delta N}$ groups, using unpaired Student's T-test)

Figure 2. Neural deletion of *jun* reduces neuroinflammation in the spinal cord of $SOD1^{G93A}$ transgenic mice. (A-T) Immunoreactivity for inflammatory cellular markers, in the spinal cord of *jun*-competent ($jun^{f/f}$) and *jun*-deficient ($jun^{\Delta N}$) $SOD1^{G93A}$ mice at day 70 and end-stage (ES). (A-D) Alpha-M positive microglial clusters can be observed in $jun^{f/f}$ spinal cords (A), but not in $jun^{\Delta N}$ spinal cords (B), at day 70. However, at end-stage, the number of microglial clusters increased to similar levels in both *jun* genotypes (C&D). (E-H) Immunoreactivity for the phagocytic microglial marker, Alpha-X, shows many phagocytic clusters in the spinal cord of *jun*-competent (G), but only a few in *jun*-deficient (H) mice, at end-stage. However, note the absence of these clusters in both *jun* genotypes at day 70 (E&F). (I-L) Astrogliosis identified by glial fibrillary acidic protein (GFAP) immunoreactivity is not affected by *jun* deletion; low levels were observed at day 70 (I&J) and these increase dramatically to similar levels in both *jun* genotypes at end stage (K&L). (M-P) Astrocyte CD44 immunoreactivity in the grey matter of the spinal cord, normally associated with high levels of inflammation, was absent at day 70 (M&N), was present at end-stage in *jun* competent mice (O) but was visibly lower in *jun*-deficient mice (P). (Q-T) T-cells, labelled by CD3 immunoreactivity, were absent from the spinal cord at day 70 (Q&R). At end-stage, many T-cells were present in the spinal cord white matter of *jun*-competent mice (S), but less can be observed in *jun*-deficient mice (T). Scale bar 500μm.


Day 70
ES
jun f/f
jun ΔN
jun f/f
jun ΔN
A
B
C
D
αM
E
F
G
H
αX
I
J
K
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GFAP
M
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O
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CD44
Q
R
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CD3

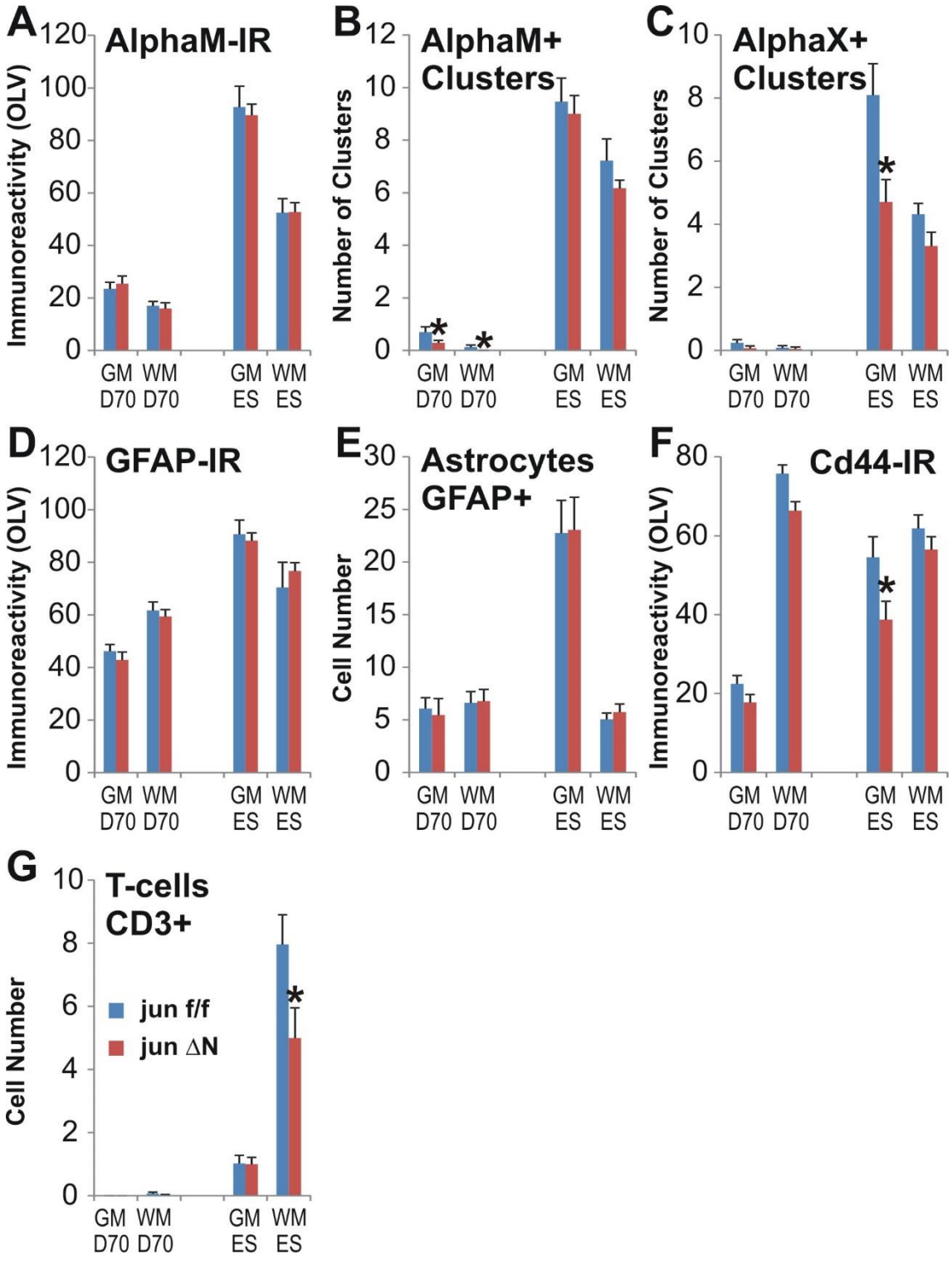

A
AlphaM-IR
Immunoreactivity (OLV)
120
100
80
60
40
20
0
GM D70
WM D70
GM ES
WM ES
B
AlphaM+ Clusters
Number of Clusters
12
10
8
6
4
2
0
*
*
C
AlphaX+ Clusters
Number of Clusters
10
8
6
4
2
0
*
D
GFAP-IR
Immunoreactivity (OLV)
E
Astrocytes GFAP+
Cell Number
30
25
20
15
10
5
0
F
Cd44-IR
Immunoreactivity (OLV)
80
60
40
20
0
*
G
T-cells CD3+
Cell Number
jun f/f
jun ΔN
*

Figure 3. Quantitation of neuroinflammatory changes in the spinal cord of SOD1G93A mice. Immunoreactivity (IR in Optical Luminosity Values-OLV) in A, D and F was quantified using the Mean+SD algorithm as described in Chapter 2. B, C, E and G show the number of microglial clusters (B&C), astrocytes (C) and T-lymphocytes (G) in the grey (GM) and white matter (WM) of the spinal cord. Compared to *jun*-competent mice (*jun* f/f), those lacking neural *jun* (*jun* $^{\Delta N}$) show a significant reduction in alpha-M-positive microglial clusters at day 70 (D70) (B), as well as a reduction in alpha-X-positive microglial clusters (C), CD44 immunoreactivity (F), and T-lymphocyte infiltration (G) at end-stage (ES). However, *jun* deletion had no effect on alpha-M (A) or GFAP (D) immunoreactivity, or in the number of activated astrocytes (E). n= 11 for *jun*$^{f/f}$ and n=8 for *jun*$^{\Delta N}$, at day 70; n=10 and 12, respectively at end-stage. *p<5% between *jun* $^{f/f}$ and *jun* $^{\Delta N}$ groups, using unpaired Student's T-test.

Neural deletion of *jun* reduces motor neuron loss without affecting cell body diameter

Motor neuron loss and atrophy are features of the SOD1^{G93A} mediated disease (Gould et al., 2006). Using the SOD1 mouse model of ALS, we aimed to examine the effect of deleting neural *jun* on the loss and atrophy of motor neurons in the ventral horn and ventral white matter of the L5 spinal cord. SOD1^{G93A} *jun*$^{f/f}$ and SOD1^{G93A} *jun*$^{\Delta N}$ mutants were analysed at 70 days of age or at end-stage. To control for the effects *jun* gene deletion on motor neuron number and atrophy, irrespective of the SOD mutation, *jun*$^{f/f}$ *and jun*$^{\Delta N}$ mice without the SOD1G93A transgene (WT (-SOD)), were also investigated. Initially, WT (-SOD) were studied at 70 or 110 days of age. However, because motor neuron counts and neuronal atrophy measurements in the WT (-SOD) mice revealed no significant difference between day 70 and day 110 (data not shown), the data was pooled into a single WT (-SOD) group (Fig. 4G & H).

Transverse sections though the spinal cord were stained with Haematoxylin and Eosin to visualise neurons in ventral horn of the spinal cord (Fig 4 A-F). Motor neurons in the ventral horn of WT *jun*$^{f/f}$ (Fig. 4A), WT *jun*$^{\Delta N}$ (Fig. 4B), SOD1^{G93A} *jun*$^{f/f}$ (Fig. 4C & E), or SOD1^{G93A} *jun*$^{\Delta N}$ (Fig. 4D & F) at day 70 and end-stage, have a triangular cell body shape, with acidophilic (red) background staining of the cytoplasm and nucleus, as well as prominent basophilic (blue) nucleolus and tigroid pattern of staining in the cytoplasm. Motor neurons at day 70 in SOD1G93A mice had a similar morphology and size as those in WT mice,

irrespective of *jun* genotype. At end-stage, in both *jun* deficient and competent SOD1^{G93A} mice motor neurons appeared smaller, had cytoplasmic vacuoles, as described in previous studies (Gurney et al., 1994), and were visibly less numerous.

Using the method described in Chapter 2, the total number of nucleated α-motor neurons in the ventral horn of the spinal cord was manually counted using a X40 objective lens (Fig. 4G). Analysis of WT (-SOD) mice, revealed that *jun* deletion had no effect on spinal motor neuron numbers; *jun*$^{f/f}$ had 1316 ±147 motor neurons per ventral horn (Fig.4G- left blue bar; n=9) and *jun*$^{\Delta N}$ had 1208+/-73 neurons per ventral horn (Fig. 4G- left red bar; p=48%; unpaired Student's T-test; n=6). Similarly, the number of spinal motor neurons at day 70, did not differ between the *jun* competent SOD1^{G93A} *jun*$^{f/f}$ mice (Fig. 4G- middle blue bar; n=20) and the *jun* deficient SOD1^{G93A} *jun*$^{\Delta N}$ (Fig. 4G-middle red bar; n=11); SOD1^{G93A} *jun*$^{f/f}$ had 1057±66 motor neurons per ventral horn and SOD1^{G93A} *jun*$^{\Delta N}$ had 1038±69 motor neurons per ventral horn (p=81%; unpaired Student's T-test). In contrast, at end-stage there were 471±46 motor neurons per ventral horn in SOD1^{G93A} *jun*$^{\Delta N}$ mice (Fig. 4G-right red bar; n=12), which was significantly more than 309+/-31 neurons per ventral horn in SOD1^{G93A} *jun*$^{f/f}$ mice (Fig. 4G-right blue bar; n=10) (p<5%; unpaired Student's T-test). Interestingly, motor neuron loss from day 70 to end-stage, was significantly ameliorated in the *jun* deficient mutants. SOD1^{G93A} *jun*$^{\Delta N}$ only lost 55% of motor neurons per ventral horn compared to the SOD1^{G93A} *jun*$^{f/f}$ controls, which lost 71% from day 70 to end-stage. To identify the effects of *jun* deletion on SOD^{G93A} mediated motor neuron atrophy, the cell body diameter of spinal motor neurons in the ventral horn was measured using the method described in Chapter 2. Figure 3G shows the cumulative distribution curves for motor neuron diameter measurements. It demonstrates that motor neuron diameter in mice not carrying the SOD1^{G93A} transgene was similar irrespective of *jun* deletion, with mean motor neuron diameter being 30.8±0.3 µm for *jun*$^{f/f}$ mice and 31.1 ± 0.4 µm for *jun*$^{\Delta N}$ mice (p>40% using unpaired Student's T-test). Likewise, SOD1^{G93A} *jun*$^{f/f}$ mice had similar motor neuron cell body diameters (29.9±1.8 µm) as *jun* deficient SOD1^{G93A} *jun*$^{\Delta N}$ mice, at day 70 (30.4 ± 0.5 µm; p=79%, using unpaired Student's T-test). At end-stage, the diameter of motor neurons in

both SOD1^{G93A} *jun* genotype groups was smaller compared to day 70; the mean motor neuron diameter significantly decreased to 21.7 ± 0.6 µm in SOD1^{G93A} *jun*$^{f/f}$ mice and to 22.0 ± 0.8 µm in SOD1^{G93A} *jun*$^{\Delta N}$ mice ($p<5\%$ using unpaired Student's T-test between day 70 and end-stage groups for each genotype). This SOD1^{G93A} mediated atrophy was independent of *jun* deletion.

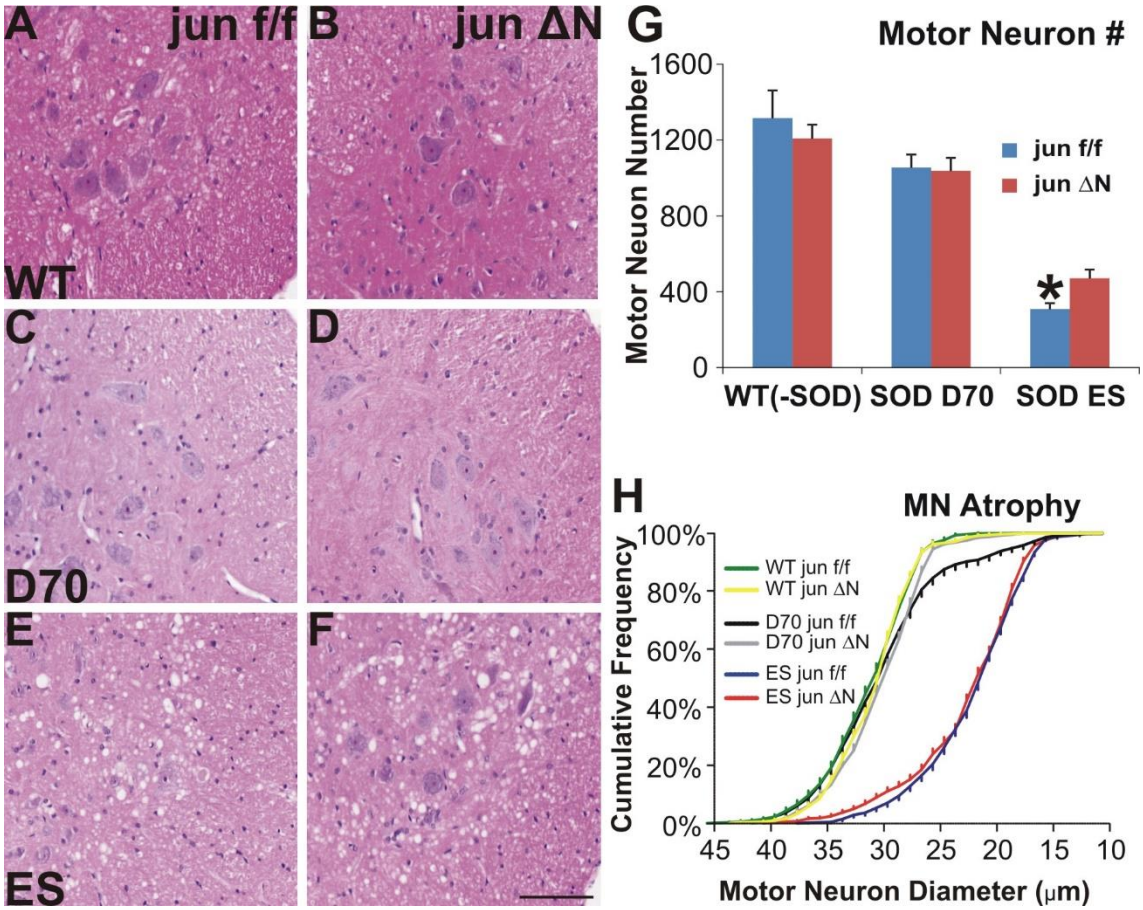


Figure 4. Neural deletion of *jun* reduces SOD1^{G93A}-mediated motor neuron loss, but does not affect neuronal atrophy. (A-F) Haematoxylin and eosin-stained ventral horns of non-transgenic wild type mice (WT, top row), and SOD1^{G93A} mice at day 70 (middle row) and end-stage (bottom row). Sections from *jun*-competent (*jun*$^{f/f}$) mice are seen in the first column and those from jun-deficient mice in the second (*jun*$^{\Delta N}$). The scale bar represents 150 μm. (G) Quantification of alpha motor neurons in the ventral horn of the 5th lumbar segment of the spinal cord. Note that neural deletion of *jun* does not affect the number of motor neurons in wild type [WT (-SOD)] mice (n=9 for *jun*$^{f/f}$ and n=6 for *jun*$^{\Delta N}$) or in SOD1^{G93A} animals at day 70 (n=20 and n=11), but SOD1^{G93A} mice lacking *jun* (n=12) reach end-stage with a significantly greater number of spinal motor neurons than those expressing *jun* (n=10). *p<5% using unpaired Student's T-test between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups. (H) Cumulative distribution of spinal motor neuron cell body diameter. (WT, n=6 and n=8; SOD1^{G93A} D70, n=7 and n=7; SOD1^{G93A} ES, n=7 and n=10 for *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups, respectively). Note the severe reduction in motor neuron diameter at end-stage which is not affected by neural deletion of *jun*.

Motor neuron loss, neuroinflammation and glial activation at the brainstem is attenuated by deletion of neural *jun*

SOD1^{G93A} mediated disease in mice involves the degeneration of some brainstem motor nuclei (Haenggeli and Kato, 2002). Here we characterised the effect of *jun* on neuronal survival, inflammation and glial activation in SOD1^{G93A} mice. The number of motor neurons, microglia and astrocytes were analysed in the hypoglossal nuclei and facial nuclei of *jun* competent or *jun* deficient SOD1^{G93A} mice.

Motor Neurons

Coronal sections through the hypoglossal nuclei or facial nuclei of WT (-SOD) *jun*$^{f/f}$ and WT (-SOD) *jun*$^{\Delta N}$, or SOD1^{G93A} *jun*$^{f/f}$ and SOD1^{G93A} *jun*$^{\Delta N}$ at 70 days of age or end-stage, were Nissl stained to visualise motor neurons. The total number of motor neurons in the hypoglossal or facial nuclei were counted at high power, using a x40 objective lens.

It has been previously reported that *jun* deletion leads to a 19% increase in the number of motor neurons in the facial nucleus of uninjured adult mice, without affecting the number of hypoglossal motor neurons (Raivich et al., 2004). However, in the current study, quantification of motor neurons in the brainstem sections of *jun*$^{f/f}$ or *jun*$^{\Delta N}$ mice (Fig. 5-blue bars), without the SOD1^{G93A} transgene, showed that *jun* deletion had no effect on motor neuron number in the hypoglossal or the facial nuclei. *jun*$^{f/f}$ mice had 1093 ± 38 motor neurons in the hypoglossal nuclei (Fig. 5-HYP), which was similar to 1041 ± 73 neurons found in *jun*$^{\Delta N}$. In addition, the number of motor neurons in the facial nuclei (Fig. 5-FAC) of *jun*$^{f/f}$ were 2411±30, which was similar to 2318±87 found in the *jun*$^{\Delta N}$ animals (p>5% using unpaired Student's T-test between *jun* $^{f/f}$ and *jun*$^{\Delta N}$ groups).

SOD1^{G93A} mice expressing *jun* showed evidence of neuronal loss in the hypoglossal nucleus, with 1078±90 hypoglossal motor neurons at end-stage (Fig. 5A & E), which was significantly less than the 1317±55 motor neurons found at day 70 (Fig. 5C & E) (p<5% using unpaired Student's T-test between day 70 and end-stage in SOD1^{G93A} *jun*$^{f/f}$ mice). In addition, there was a trend for

fewer motor neurons at end-stage (1807±114) than at day 70 (1972±44) in the facial nuclei of SOD1^{G93A} *jun*$^{f/f}$ mice (Fig.5E) (p=18% using unpaired Student's T-test between day 70 and end-stage in SOD1^{G93A} *jun*$^{f/f}$ mice). The motor neuron loss mediated by SOD1^{G93A} disease was abolished in *jun* deleted mice. The hypoglossal nuclei of SOD1G93A *jun*$^{\Delta N}$ contained 1200±68 neurons at day 70 (Fig. 5B & E), which was similar to 1295±53 motor neurons found at end-stage (Fig. 5D & E). In the same way, the facial nuclei of SOD1^{G93A} *jun*$^{\Delta N}$ had 2118±93 motor neurons at day 70, which did not differ from 2072±63 motor neurons counted at end-stage (Fig.5E) (p>5% using unpaired Student's T-test between day 70 and end-stage in SOD1^{G93A} *jun*$^{\Delta N}$ mice). The increased survival of motor neurons in the cranial motor nuclei of SOD1^{G93A} mice deficient in *jun* is reminiscent of that observed at the spinal cord.

Neuroinflammation and astrocyte activation

The effect of *jun* deletion on SOD1G93A mediated neuroinflammation in brainstem motor nuclei was investigated. To do this, coronal sections through the hypoglossal nuclei or facial nuclei of end-stage SOD1^{G93A} *jun*$^{f/f}$ and SOD1^{G93A} *jun*$^{\Delta N}$ were stained for αM, αX, GFAP or CD44 to visualise microglial and astrocyte activation. The total number of microglia or astrocytes in the hypoglossal or facial nuclei were counted, using a x40 objective lens.

Microglial activation

Corroborating the reduction in motor neuron loss at end-stage, *jun* deficient SOD1^{G93A} mice had 70% fewer αX-positive microglial clusters in the hypoglossal nuclei than *jun* competent controls (Fig. 5G). The SOD1^{G93A} *jun*$^{f/f}$ mice had 7±2 αX-positive microglial clusters in the hypoglossal nuclei, which was significantly more than 2±1 αX-positive microglial clusters found in SOD1^{G93A} *jun*$^{\Delta N}$ mice (p<5% using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$ mice). However, no difference was observed in the facial nucleus. There were 8±2 αX-positive microglial clusters in the facial nuclei of SOD1^{G93A} *jun*$^{f/f}$, which was not significantly different from the 11±5 αX-positive microglial clusters found in SOD1^{G93A} *jun*$^{\Delta N}$ (p>5% using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$ mice).

Neural deletion of *jun* had no effect on the number of αM-positive microglia found in the hypoglossal and facial nuclei. The SOD1^{G93A} *jun*$^{f/f}$ mice had 12±2 αM-positive microglial clusters in the facial nucleus, which did not differ significantly from 14±4 found of SOD1^{G93A} *jun*$^{\Delta N}$ mice. In the same way, the hypoglossal nuclei of SOD1^{G93A} *jun*$^{f/f}$ had 9±2 αM-positive microglial clusters, which was similar to 12±3 found in SOD1^{G93A} *jun*$^{\Delta N}$ mice (p>5% using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$ mice).

Astrocyte activation

The number of GFAP-positive astrocytes in the hypoglossal or facial nuclei of SOD1^{G93A} mice at end-stage was minimal and was not affected by *jun* deletion (Fig. 5H) (p>5% using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$ mice). However, as shown in Figure 5I, the number of CD44-positive astrocytes was significantly reduced in both the hypoglossal and facial nuclei of *jun*-deficient mice compared to *jun*-competent littermates. In the hypoglossal nucleus, SOD1^{G93A} *jun*$^{f/f}$ mice had 3±1 CD44-positive astrocytes, which was significantly more than 1±0.5 found in SOD1^{G93A} *jun*$^{\Delta N}$. Similarly, in the facial nucleus, the SOD1^{G93A} *jun*$^{f/f}$ had 6±1 CD44-positive astrocytes, while SOD1^{G93A} *jun*$^{\Delta N}$ only had 2±1 CD44-positive astrocytes (p<5% using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$ mice).

Thus, deletion of *jun* reduces SOD1^{G93A} mediated motor neuron loss and neuroinflammation in the brainstem as well as the spinal cord. This indicates that c-Jun has an important role in mediating SOD1^{G93A} neuropathology irrespective of the motor neuron pool studied.

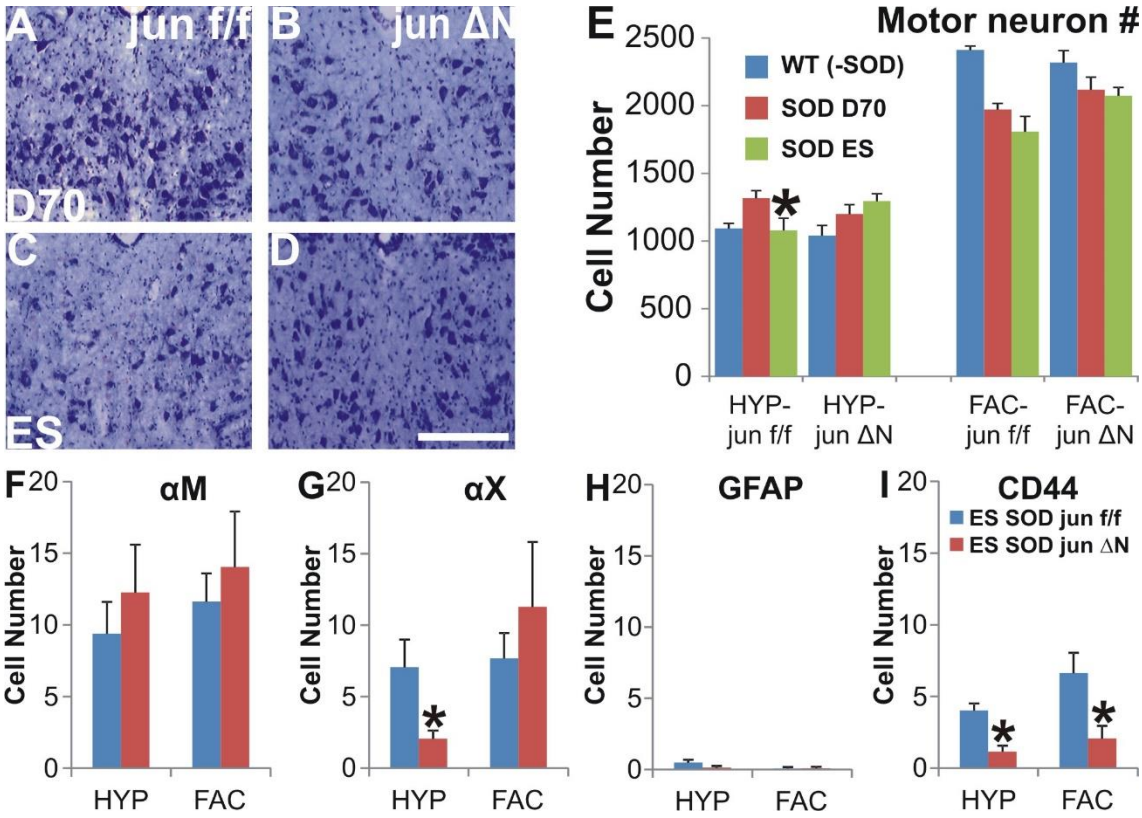


Figure 5. Neural deletion of *jun* reduces motoneuron loss and neuroinflammation in the brainstem of SOD1^{G93A} mice. (A-D) Nissl stained sections through the hypoglossal nucleus of *jun*-competent (*jun*$^{f/f}$) and jun-deficient (*jun*$^{\Delta N}$) SOD1^{G93A} mice at day 70 (D70) and end-stage (ES). Note the absence of motor neuron loss from day 70 to end-stage in *jun*-deficient mice (B&D). Scale bar 250µm. (E) Quantification of motor neurons in the hypoglossal (HYP) and facial (FAC) nuclei. Neural deletion of *jun* does not affect the number of motor neurons in wild type [WT (-SOD)] mice (n=4 for *jun*$^{f/f}$ and *jun*$^{\Delta N}$) or in SOD1^{G93A} animals at day 70 (n=11 and n=9). However, SOD1^{G93A} mice lacking *jun* reach end-stage with a significantly greater number of hypoglossal motor neurons than those expressing *jun* (n=12 and n=10). *p<5% using unpaired Student's T-test between SOD D70 and SOD ES groups. (F-I) Quantitation of microglia clusters and astrocytes in the brainstem of SOD1^{G93A} mice and the effect of *jun* deletion. Number of cellular profiles immunoreactive for the microglial markers αM (F) and αX (G), and the activated astrocyte markers GFAP (H) and CD44 (I), in the hypoglossal (HYP) and facial (FAC) nuclei of *jun*-competent (*jun*$^{f/f}$) and *jun*-deficient (*jun*$^{\Delta N}$) SOD1^{G93A} mice, at end-stage. *p<5% using unpaired Student's T-test between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups; n=10 and 12, respectively.

Deletion of *jun* protects axons in SOD1^{G93A} mice

In the SOD1^{G93A} mouse model of ALS, axonal degeneration appears to be the primary deficit, which precedes symptom onset and motor neuron death (Fischer et al., 2004, Pun et al., 2006, Gould et al., 2006). To investigate the role of *jun* on axonal degeneration of SOD1^{G93A} mice. Transverse sections through the ventral roots, from lumbar segment 5 (L5), of WT (-SOD) *jun*$^{f/f}$ and WT (-SOD) *jun*$^{\Delta N}$, or SOD1^{G93A} *jun*$^{f/f}$ and SOD1^{G93A} *jun*$^{\Delta N}$ at 70 days of age or end-stage, were stained with toluidine blue to visualise myelinated axons.

It has been confirmed that degenerative changes in axons of SOD1^{G93A} mice can be seen in the pre-symptomatic stages (Fig. 6). By 70 days of age, the large calibre myelinated axons in the L5 ventral roots of *jun* competent SOD1^{G93A} mice are surrounded by numerous small calibre axons (Fig. 6A). In contrast, in the L5 ventral roots of *jun* deficient SOD1^{G93A} mice, medium and large size axons can be seen in the vicinity of only a few small myelinated fibres (Fig. 6B). By end-stage, there are a few myelinated axons in the L5 ventral roots of *jun* competent SOD1^{G93A}, which appear shrunken and of an irregular shape (Fig. 6C). Axons in L5 ventral roots of *jun*-deficient SOD1^{G93A} mice were numerous and predominantly of a large size although they did not have a normal appearance (Fig. 6D).

Using a x100 objective lens, the total number of axons in the L5 ventral roots of WT (-SOD) *jun*$^{f/f}$ and WT (-SOD) *jun*$^{\Delta N}$, or SOD1^{G93A} *jun*$^{f/f}$ and SOD1^{G93A} *jun*$^{\Delta N}$ at 70 days of age or end-stage, were counted. As with motor neuron number, age (day 70 vs day 110) did not affect the number of axons in wild type mice without the SOD1^{G93A} transgene (data not shown), and neither did the deletion of *jun* in such mice (*jun*$^{f/f}$, 922 ± 76; jun$^{\Delta N}$, 945 ± 159) (Fig. 6E). This demonstrates that the baseline ventral root axonal counts in *jun* deficient mice are the same as controls. By day 70, SOD1^{G93A} mice expressing *jun* showed a 30% fewer in axon number compared to their *jun* deficient SOD1^{G93A} littermates. SOD1^{G93A} *jun*$^{f/f}$ mice had 692±45 axons in the ventral roots, which was significantly fewer than SOD1^{G93A} jun$^{\Delta N}$ mice that had 987 ± 98 axons ($p<5\%$ using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$ mice at day 70). By end-stage, both groups lost around 40% of their axons from their day 70 baseline. However, SOD1^{G93A} mice lacking *jun* still

had a greater number of axons compared to the *jun*-expressing mice. The ventral roots of SOD1^{G93A} *jun*$^{f/f}$ had 413±20 axons, while SOD1^{G93A} jun$^{\Delta N}$ had 550±50 (p<5% using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$ mice at day 70) (Fig. 6E). The reduction in the number of axons from day 70 to end-stage was approximately 25% in both *jun* competent and *jun* deficient SOD1^{G93A} mice.

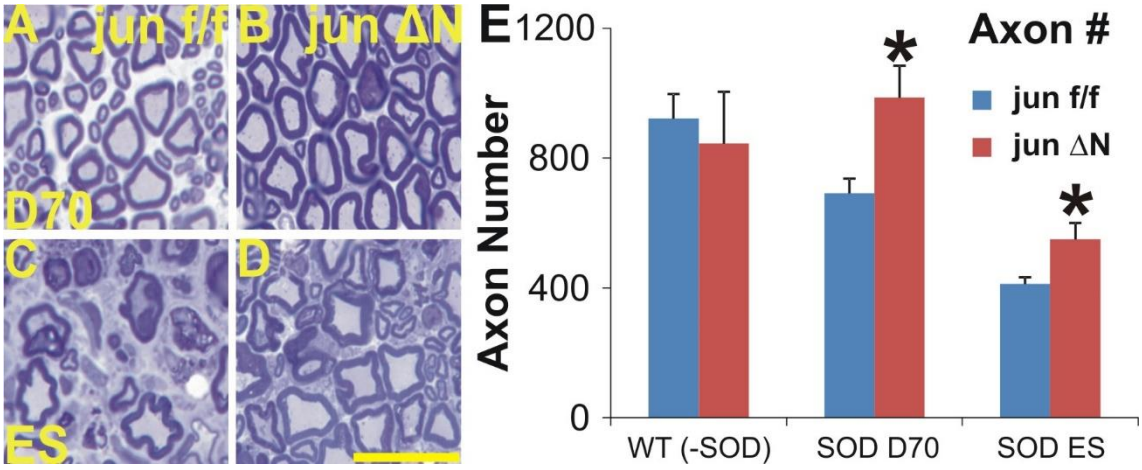


Figure 6. Axonal loss in SOD1^{G93A} mice is attenuated by neural deletion of *jun*. (A-D) Toluine blue-stained L5 ventral roots of *jun*-competent (*jun*$^{f/f}$) and jun-deficient (*jun*$^{\Delta N}$) SOD1^{G93A} mice at day 70 (D70) and end-stage (ES). Note the extensive axonal degeneration in *jun*$^{f/f}$ at end-stage, and compare to the healthier *jun*$^{\Delta N}$ ventral root. Scale bar 20µm. (E) Quantification of myelinated axons in cross sections of the 5th lumbar ventral root. Axon number in wild type mice [WT (-SOD)] is not affected by *jun* deletion (n=5 for *jun*$^{f/f}$ and n=8 for *jun*$^{\Delta N}$). However, at day 70, *jun*-competent SOD1G93A mice (n=17) have around 30% fewer myelinated axons than *jun* deficient littermates (n=8). This difference continues to end-stage, despite extensive axonal loss in both groups (n=10 and n=7). *p<5% using unpaired Student's T-test between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups.

Neuromuscular junction dennervation is not affected by deletion of *jun*

Previous studies on SOD1^{G93A} mice have demonstrated dissociation between motor neuron loss and the overall length of survival. Instead, a better correlation has been observed between length of survival and neuromuscular junction (NMJ) denervation (Gould et al., 2006). For this reason, longitudinal sections through the gastrocnemius muscle of WT (-SOD) mice competent for *jun*, or SOD1^{G93A} mice, with or without *jun*, at end-stage were stained to visualise NMJs using a combined silver impregnation and acetyl-cholinesterase histochemistry technique.

Using a x40 objective lens, the total number of fully innervated (Fig.7A), partially innervated (Fig.7B) and denervated NMJs (Fig.7C) in the gastrocnemius muscle were quantified (Fig.7D). As Figure 7D shows, almost 100% of NMJs are fully innervated in mice that do not carry the SOD1^{G93A} transgene. However, SOD1^{G93A} mice, with or without *jun*, reach end-stage with only proximally half of their NMJs fully innervated. There was a trend for *jun* deficient mice to have slightly higher percentages of fully innervated NMJs (54±2) than *jun* competent mice at end-stage (49±2) (p=9.7% using unpaired Student's T-test between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups, n=4 per group). However, when the total innervation index was calculated (=1*fully innervated + 0.5*partially innervated), the amount of innervation was similar for both SOD1^{G93A} *jun*$^{\Delta N}$ (61.2%±1.8%) and SOD1^{G93A} *jun*$^{f/f}$ mice (65.2%±1.0%) (p>5%; using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$; n=4 per group). This demonstrates that whilst *jun* deletion protected SOD1^{G93A} mice from disease-associated motor neuron and axonal loss, it did not prevent the NMJ denervation which ultimately causes paralysis and death of animal.

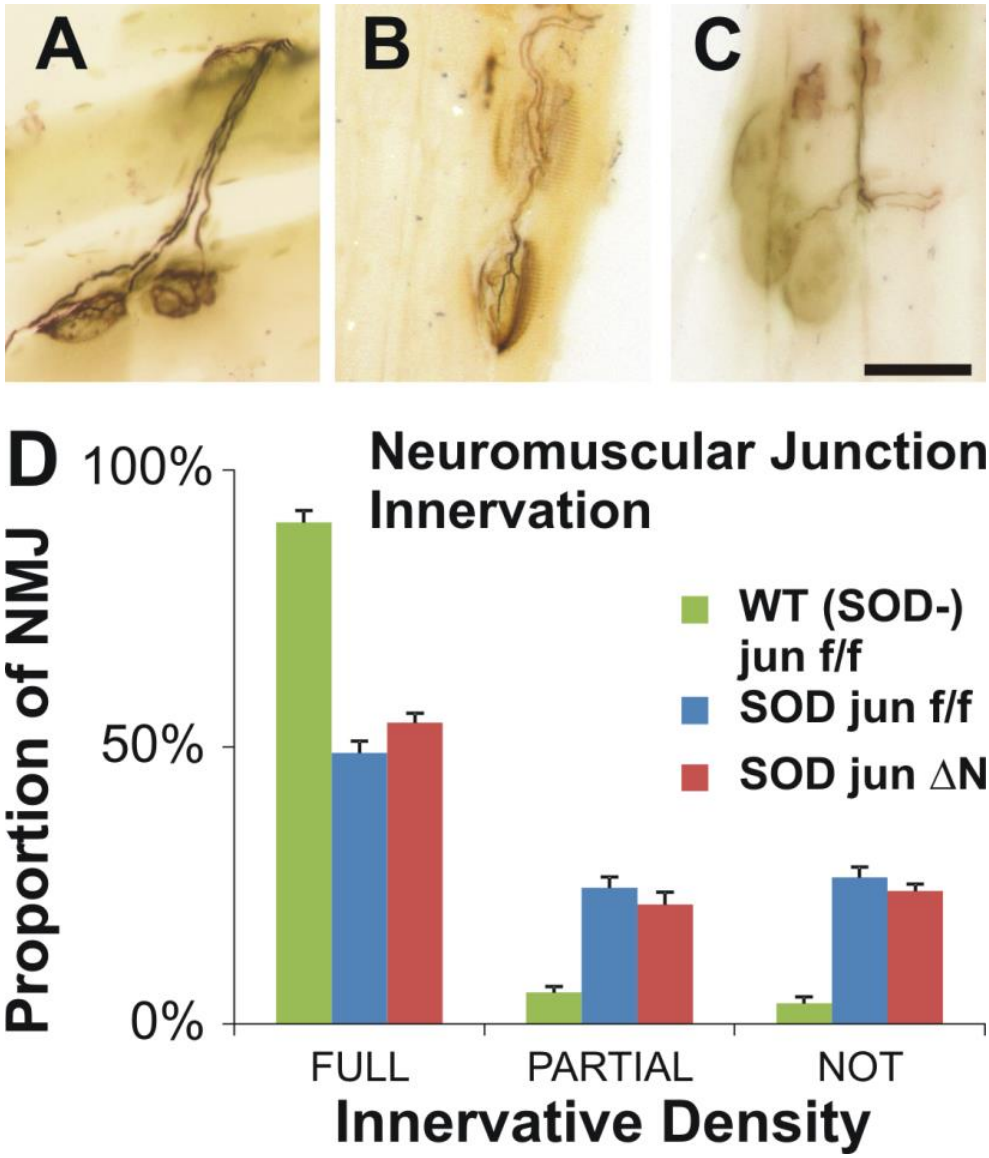


Figure 7. Neural deletion of *jun* does not protect neuromuscular junctions (NMJ) from SOD1^{G93A}-mediated denervation. (A-C) NMJs in the gastrocnemius muscle stained using silver impregnation and acetylcholinesterase (AChE) histochemistry to visualize motor axons (silver) and muscle endplates (AChE). Scale bar represents 50µm. (A) Fully innervated NMJs showing prolific terminal axon branching; (B) partial innervated, with the terminal axon passing across the NMJ and (C) denervated, with no axon inside the AChE demarcated endplate. (D) Percentage of full, partial and not innervated NMJs, demonstrates that SOD1^{G93A} mice reach end-stage with similar levels of denervation, regardless of *jun* deletion (*jun* $^{f/f}$ and *jun* $^{\Delta N}$ groups, n=4 per group).

SOD1^{G93A} mice mount a *jun*-dependent, disease-mediated axotomy response

Axonal disconnection from peripheral targets, following nerve transection, causes a retrograde chromatolytic response in the cell bodies of injured neurons (axotomy response), which involves the upregulation of transcription factors and regeneration-associated molecules (Raivich, 2011). As described in this study, SOD1^{G93A} causes axonal disconnection in the form muscle denervation and axonal loss. This suggests that a disease mediated motor neuron axotomy response might occur in SOD1^{G93A} mice. In order to determine whether such response does in fact exist, transverse sections through the cervical enlargement of *jun*-competent or *jun*-deficient SOD1^{G93A} mice, at 70 days of age or end-stage were immunostained for the regeneration associated proteins: α7, β1, galanin, CD44 as well as c-Jun itself (Figs. 8&9).

Motor neurons in the spinal cord of SOD1^{G93A} mice deficient in jun show an axotomy response at end-stage

To examine whether the nestin-driven CRE recombination effectively deletes *jun* from the motor neurons of the spinal cord of SOD1^{G93A} mice, the expression of c-Jun protein was studied in SOD1^{G93A} mice. SOD1^{G93A} *jun*$^{f/f}$ mice showed c-Jun being expressed in the nuclei of cells confined to the ventral horn, at day 70 (Fig.8A). At end-stage, the expression of c-Jun increased in the ventral horn of *jun*-competent SOD1^{G93A} mice (Fig.8B). The large size of the c-Jun-positive nuclei suggests that they belong to neurons (Fig. 8B-insert). In contrast, SOD1^{G93A} *jun*$^{\Delta N}$ mice failed to show any c-Jun-positive cell nuclei in the spinal cord at both day 70 (Fig.8C) and end-stage (Fig. 8D).

The total numbers of c-Jun-positive neuronal nuclei were counted in the ventral horn of the spinal cord, using a x40 objective lens (Fig. 9A & B). In line with the observations above, c-Jun-positive nuclei of putative motor neurons in the ventral horn of SOD1^{G93A} *jun* competent mice, doubled in number from day 70 to end-stage. There were 2±1 c-Jun-positive nuclei per ventral horn of SOD1^{G93A} *jun*$^{f/f}$ mice at day 70, which was significantly fewer than 4±1 c-Jun-positive nuclei counted per ventral horn of SOD1^{G93A} *jun* $^{f/f}$ mice at end stage ($p<5\%$; using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice at day 70 and end-stage). In contrast, the number of c-Jun-positive neuronal nuclei in the

ventral horn of the spinal cord of SOD1^{G93A} *jun*$^{\Delta N}$ mice was similar at day 70 and end-stage, with less than one c-Jun-positive neuronal nucleus per ventral horn (p>5%; using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice at day 70 and end-stage). A comparison between *jun*-competent and *jun*-deficient SOD1^{G93A} mice revealed that *jun*-competent SOD1^{G93A} mice had more c-Jun nuclei in the ventral horn of the spinal cord than *jun*-deficient SOD1^{G93A} mice at day 70 and end-stage. Nes::cre mediated deletion of *jun* significantly abolished the number of c-Jun positive nuclei by 89-98% in the ventral horn. At day 70, SOD1^{G93A} *jun*$^{f/f}$ mice had 2.5±0.6 c-Jun positive neuronal nuclei per ventral horn, while SOD1^{G93A} *jun*$^{\Delta N}$ mice at end stage had 0.3±0.2 c-Jun positive nuclei per ventral horn. Similarly, SOD1^{G93A} *jun*$^{f/f}$ mice had 4±0.7 c-Jun positive nuclei per ventral horn, which was significantly more than 0.1±0.1 c-Jun positive neuronal nuclei per ventral horn of SOD1^{G93A} *jun*$^{\Delta N}$ mice (p<1%; using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$).

As postulated above, the overexpression of the SOD1^{G93A} transgene in mice did in fact cause the de-novo expression of axotomy response markers in motor neuron like cellular profiles. At end-stage putative motor neurons were highly CD44- (Fig.8G), α7- (Fig.8K) or β1-positive (Fig.8O) in the ventral horns of the spinal cord of SOD1^{G93A} *jun*$^{f/f}$ mice (Fig.8G). In contrast, SOD1^{G93A} *jun*$^{\Delta N}$ mice had a few very faintly stained CD44- (Fig.8H), α7- (Fig.8L) or β1-positvie neurons in the spinal cord at end-stage (Fig.8P). The expression of these regeneration-associated proteins was not observed in the spinal cords of SOD1^{G93A} *jun*$^{f/f}$ (Fig.8E, I &M) or SOD1^{G93A} *jun*$^{\Delta N}$ at day 70 (Fig.8F, J &N).

Using a x40 magnification lens, the total number of CD44-, α7-, β1-, or galanin-positive neurons were counted in ventral horn of the spinal cord of SOD1^{G93A} *jun*$^{f/f}$ or SOD1^{G93A} *jun*$^{\Delta N}$ mice, at 70 days of age (Fig.9A) or end-stage (Fig.9B). Galanin-positive neurons were not present in the ventral horn of the different *jun* groups, at the two different time-points. However, at day 70, approximately one CD44-, α7-, or β1-positive neuron was counted in the ventral horn of SOD1^{G93A} *jun*$^{f/f}$ or SOD1^{G93A} *jun*$^{\Delta N}$ mice. A comparison between *jun* competent and *jun* deficient SOD1^{G93A} mice, revealed no difference in the number of neurons expressing the aforementioned proteins in the ventral horn (p>5%; using

unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$, at day 70). At end-stage, the number of CD44-, α7-, or β1-positive neurons increased in the spinal cord of SOD1^{G93A} *jun*$^{f/f}$ or SOD1^{G93A} *jun*$^{\Delta N}$ mice. The increase in the expression of these proteins appeared to be reduced by *jun* deletion. However, only CD44 expression that was significantly affected; SOD1^{G93A} *jun*$^{f/f}$ mice had 3±0.4 CD44-positive neurons per ventral horn, which was significantly more than 1±0.2 CD44-positive neurons found per ventral horn of SOD1^{G93A} *jun*$^{\Delta N}$ mice ($p<5\%$; using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$, at end-stage).

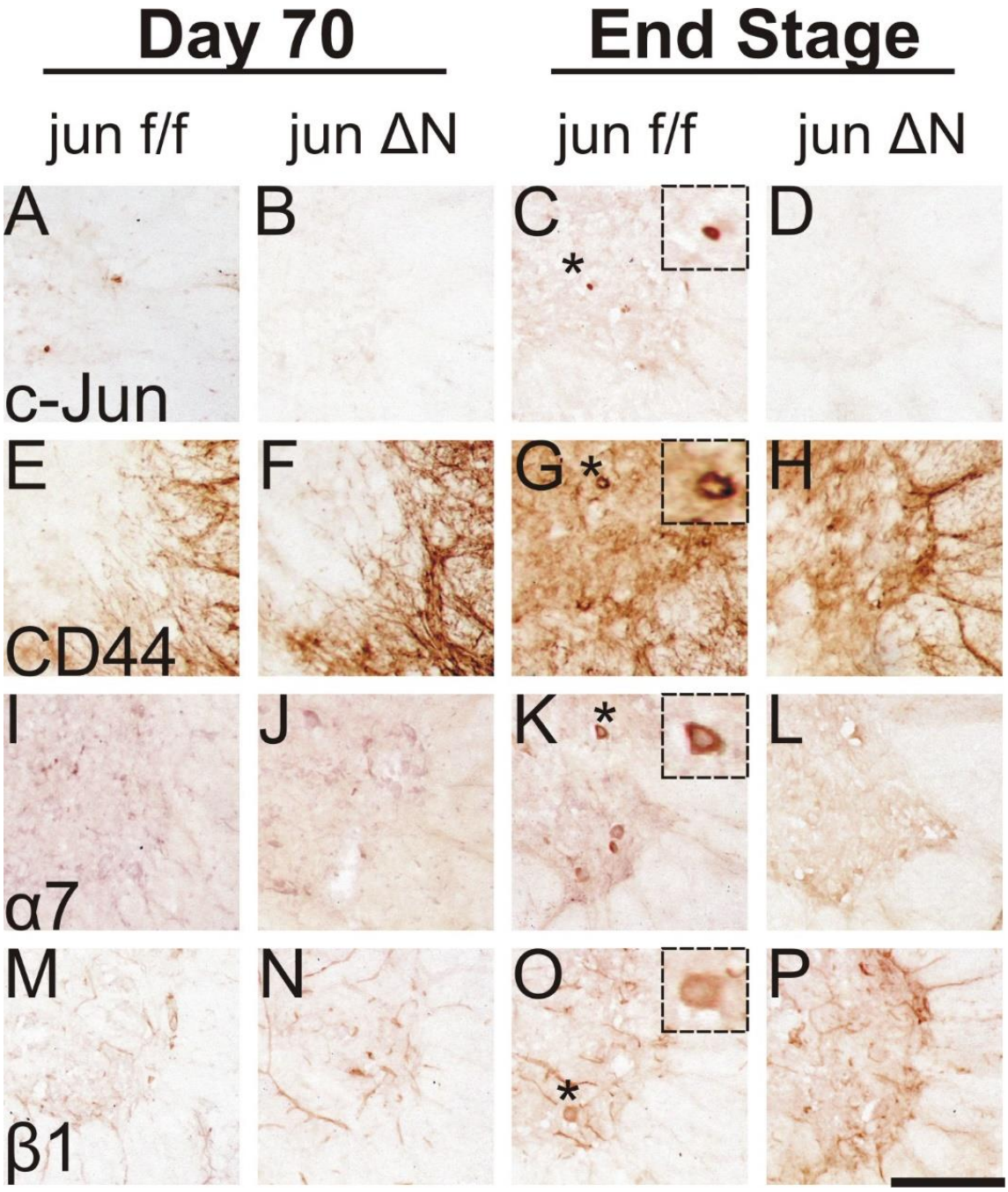

Day 70
End Stage
jun f/f
jun ΔN
jun f/f
jun ΔN
A
B
C
D
c-Jun
E
F
G
H
CD44
I
J
K
L
α7
M
N
O
P
β1

Figure 8. Spinal motor neurons in end-stage SOD1^{G93A} mice mount a *jun*-dependent disease-associated axotomy response. (A-P) Immunoreactivity for axotomy response markers c-Jun (A-D), CD44 (E-H), α7 (I-L), and β1 (M-P) in the ventral horn of *jun*-competent (*jun*$^{f/f}$) and *jun*-deficient (*jun*$^{\Delta N}$) SOD1^{G93A} mice, at day 70 and end-stage. Scale bar 300µm. Note the complete absence of c-Jun immunoreactivity from *jun*$^{\Delta N}$ spinal cords at both day 70 (B) and end-stage (D). Importantly, notice that CD44 (G), α7 (K) and β1 (O) immunoreactive motor neurons can only be observed in the spinal cord of *jun* competent SOD1^{G93A} mice at end-stage. Asterisks mark immunoreactive motoneurons magnified (x3) inset.

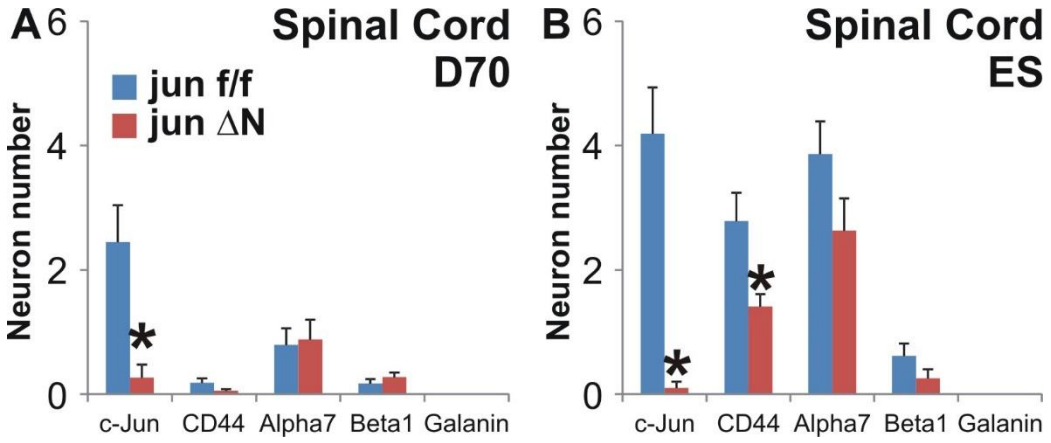


Figure 9. Quantitative description of SOD1^{G93A} mediated axotomy response and the effect of *jun* deletion. (A-B) Number of spinal motor-neuron-like cellular profiles immunoreactive for axotomy response markers in the ventral horn of the spinal cords of *jun*-competent or *jun*-deficient SOD1^{G93A} at day 70 or end-stage (*p<5% using unpaired Student's T-test between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups, n=10 for jun $^{f/f}$ and n=12 for jun $^{\Delta N}$).

SOD1^{G93A} mice deficient in jun show an axotomy response in the brainstem

Next the present project investigated whether other motor neuron pools of SOD1^{G93A} mice mounted a *jun*-dependent axotomy response, similar to that found in the spinal cord. To do this, coronal sections through the hypoglossal and facial nuclei, of *jun*-competent or *jun*-deficient SOD1^{G93A} mice at end-stage, as well as the facial nucleus of wild type animals 14 days after facial nerve axotomy, were immunostained for: α7, β1, galanin, CD44 as well as c-Jun (Figs. 10&11).

To further demonstrate that the nestin-driven CRE recombination efficiently deletes *jun* from the motor neurons of SOD1^{G93A} mice, coronal sections through the brainstem of *jun*-competent or *jun*-deficient SOD1^{G93A} mice, were stained for c-Jun, at end-stage. Strong c-jun expression was found in the nuclei of putative motor neurons of the facial (Fig.10B) and hypoglossal nucleus of *jun*-competent SOD1^{G93A} mice (Fig.10D). Like in the spinal cord, *jun* deficient SOD1^{G93A} mice failed to express c-Jun in the facial (Fig.10E) and hypoglossal nuclei (Fig.10F). Quantifications of c-Jun-positive nuclei present in the facial (Fig. 11A) or hypoglossal nucleus, revealed that c-Jun expression was significantly reduced

by 93% in the facial nucleus and 99% in the hypoglossal nuclei of SOD1^{G93A} *jun*$^{\Delta N}$ mice (Facial Nucleus: 2.0±0.8; Hypoglossal Nucleus: 0.4+/-0.4) compared to SOD1^{G93A} *jun*$^{f/f}$ mice (27.0±7.8; 27.0±4.6) (p<5%; using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$, at end-stage).

Neurons positive for axotomy response markers were found in the facial as well as hypoglossal nucleus of *jun*-competent SOD1^{G93A} mice. Coronal sections through the facial (Fig. 10G, L, Q & V) or the hypoglossal nuclei (Fig. 10I, N, S & X) revealed strongly stained CD44- (Fig.10G&I), α7- (Fig.10L&N) or β1-positve neuronal-like profiles (Fig.10Q&S). However, it was only in the facial nucleus (Fig.10V), rather than the hypoglossal nucleus (Fig.10X), where galanin-positive putative neurons could be found. In *jun* deficient SOD1^{G93A} mice, little to no neurons positive for CD44 (Fig.10H&J), α7 (Fig.10M&O), β1 (Fig.10R&T), or galanin were observed (Fig.10W&Y), in the facial (Fig.10H, M, R & W) or hypoglossal nuclei (Fig.10J, O, T & Y).

In line with the observations above, neuronal quantifications revealed that *jun* deletion reduces the expression of axotomy markers in the facial (Fig. 11A) as well as the hypoglossal nuclei at end-stage (Fig.11B). The hypoglossal nuclei of SOD1^{G93A} *jun*$^{f/f}$ mice had significantly more neurons expressing CD44 (*jun*$^{f/f}$ vs *jun*$^{\Delta N}$: 3.5± 0.9 vs 0.3±0.2), α7 (3.9±0.7 vs 0.1±0.1) or β1 (0.3±0.09 vs 0.08 ±0.08), than SOD1^{G93A} *jun*$^{\Delta N}$ mice (p<5%; using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$, at end-stage). In the same way, the facial nuclei of SOD1^{G93A} *jun*$^{f/f}$ mice had significantly more neurons expressing CD44 (*jun*$^{f/f}$ vs *jun*$^{\Delta N}$: 2.4±0.9 vs 0.3±0.6), α7 (4.8±0.8 vs 1.0±0.8), β1 (0.3±0.1 vs 0.0±0.0), or galanin (0.2±0.06 vs 0.0±0.0), than SOD1^{G93A} *jun*$^{\Delta N}$ mice (p<5%; using unpaired Student's T-test between SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$, at end-stage).

Magnitude of SOD1G93A mediated axotomy response correlates with the level of motor neuron degeneration

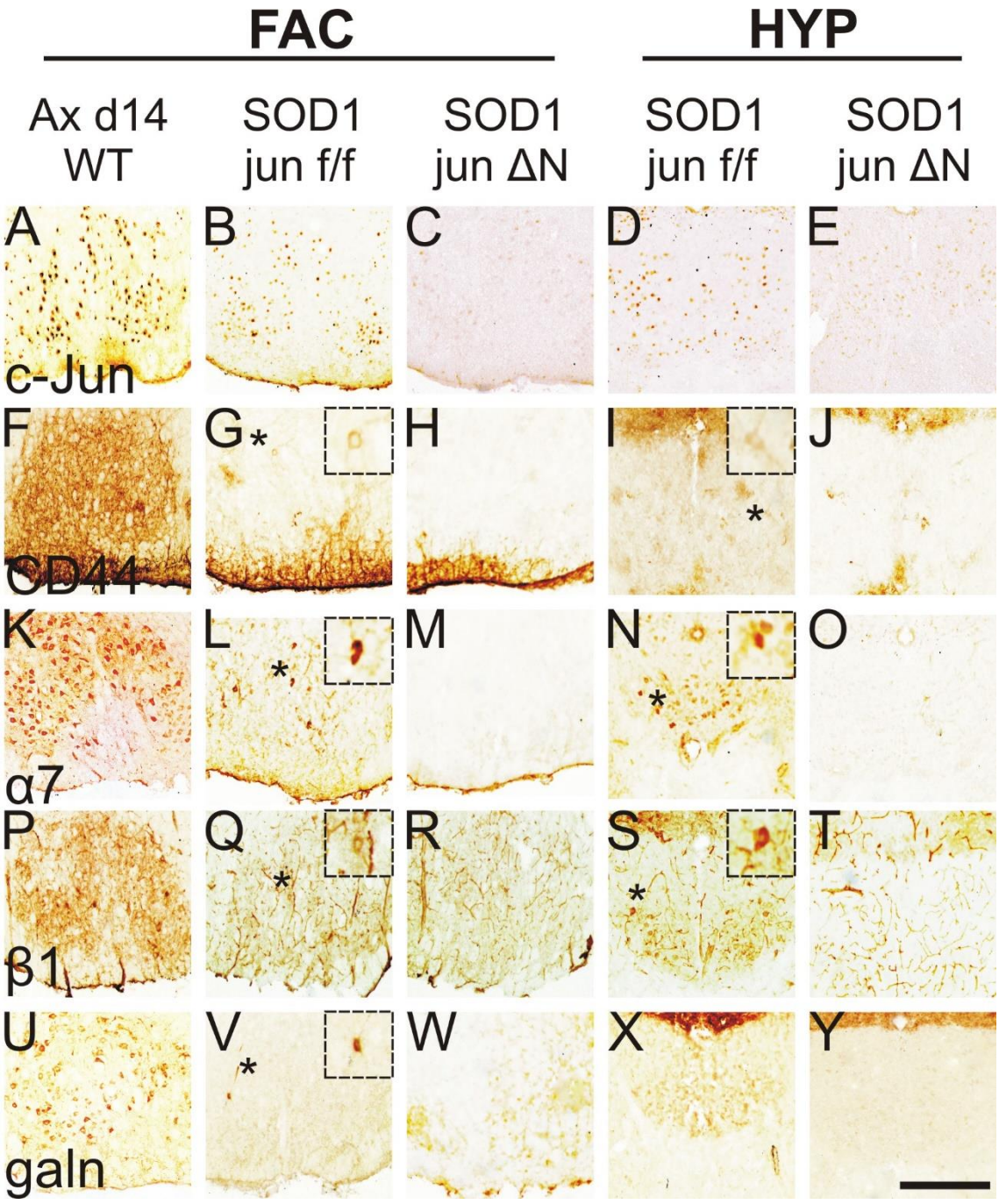
To compare the axotomy response observed in SOD1^{G93A} disease with that induced after peripheral nerve transection in wild type mice, specifically after facial nerve axotomy (FNA), a ratio of motor neurons expressing the different

axotomy response markers using the number of c-Jun-positive motor neurons as a baseline denominator, was calculated (Fig.11D).

Of the total c-Jun-positive motor neuron population, the ventral horn of *jun*-competent SOD1^{G93A} mice contained approximately 70-90% motor neurons that expressed axotomy response markers α7 and CD44, at end-stage. However, in line with previous literature (Werner et al., 2000) and our current data (Fig. 9D), in the ventral horn of *jun*-competent SOD1^{G93A} mice only a small proportion of the motor neurons undergoing an axotomy response expressed β1 at end-stage (15% of c-Jun+ population). The marker galanin was completely absent from motor neurons, as described by previous work on injured spinal motor neurons (Zhang et al., 1993).

In contrast to the ventral horn of the spinal cord (Fig. 9B), the facial and hypoglossal nuclei of *jun*-competent SOD1^{G93A} mice contained axotomy response markers – alpha7, beta 1 and CD44 – that, on average, were present on just 5-20% of the total c-Jun-positive motor neuron population (Fig. 10A&B). In addition, only the facial but not the hypoglossal nucleus of *jun*-competent SOD1^{G93A} mice contained a very low number of galanin-immunoreactive motor neurons (Figs. 10A&B). In contrast, the percentage of motor neurons expressing axotomy related markers α7 (Fig. 9K) and CD44 (Fig. 9F), 14 days after facial nerve axotomy (FNA) mice was around 90% (Fig. 9D). These numbers were similar to those observed in the ventral horn of the spinal cord of end-stage *jun*-competent SOD1^{G93A} mice, but about 5 times higher than those observed in the end-stage hypoglossal or facial nucleus of *jun*-competent SOD1^{G93A} mice (Figs. 10A-B). Thus the proportion of motor neurons undergoing a disease-mediated axotomy response at end-stage is greater in the spinal cord than in the hypoglossal and facial nuclei.

Figure 10. Cranial motor neurons in SOD1^{G93A} mice mount a *jun* dependent disease-mediated axotomy response, which was minute in comparison to that following nerve cut in wild type mice. (A-Y) Immunoreactivity for axotomy response markers [c-Jun, CD44, integrins α7 and beta 1, and neuropeptide galanin (galn)] in the facial nucleus (FAC) of: (first column) wild type mice, 14 days after facial nerve cut (Ax 14 WT), which serves as a positive control for the immunostaining for the axotomy response markers in the facial nuclei of; (second column) *jun*-competent SOD1^{G93A} mice at end-stage (SOD1 *jun*$^{f/f}$); and (third column) *jun*-deficient SOD1^{G93A} mice at end-stage (SOD1 *jun*$^{\Delta N}$). The third and fourth columns demonstrate the hypoglossal nuclei (HYP) of *jun*-competent and *jun*-deficient SOD1^{G93A} mice at end-stage, respectively. Observe the large number of facial motor neurons in WT mice, which are immunoreactive for the studied axotomy response markers, 14 days following axotomy (first column). Compare this to the very small number of immunoreactive motor neurons in the facial and hypoglossal nuclei of *jun*-competent SOD1^{G93A} mice (second and fourth column, respectively). Note that neural deletion of *jun* abolishes c-Jun immunoreactivity and the presence of axotomy markers in the facial and hypoglossal nuclei of SOD1^{G93A} mice (third and fifth column). Asterisks mark immunoreactive motoneurons magnified (x3) inset. Scale bar 300 μm.


FAC
HYP
Ax d14 WT
SOD1 jun f/f
SOD1 jun ΔN
SOD1 jun f/f
SOD1 jun ΔN
A
B
C
D
E
c-Jun
F
G
H
I
J
CD44
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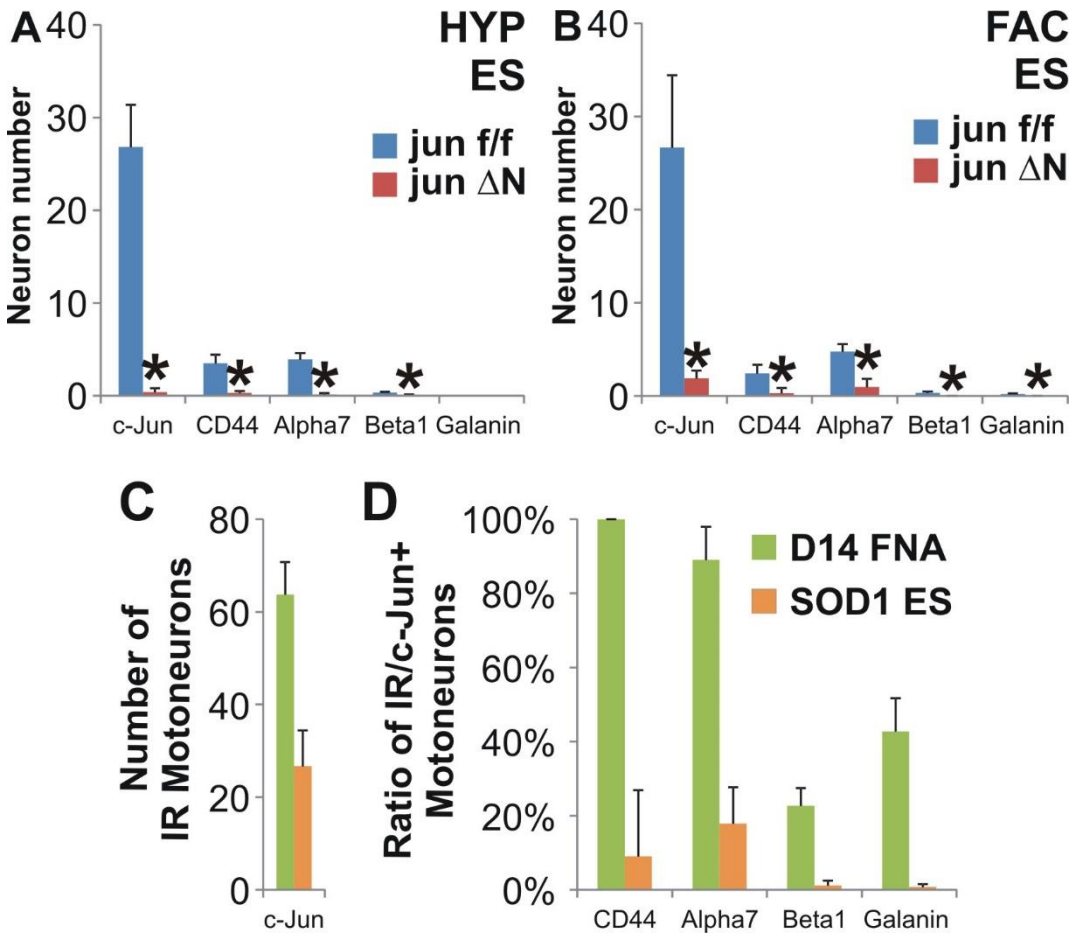


Figure 11. Quantitative description of SOD1^{G93A} mediated axotomy response and the effect of *jun* deletion. (A-B) Number of motor-neuron-like cellular profiles immunoreactive for axotomy response markers, in the hypoglossal (A; HYP) and facial nuclei (B; FAC) of SOD1^{G93A} *jun*$^{f/f}$ mice and SOD1^{G93A} *jun*$^{\Delta N}$ mice at end-stage (*p<5% using unpaired Student's T-test between *jun*$^{f/f}$ and *jun*$^{\Delta N}$ groups, n=10 for *jun*$^{f/f}$ and n=12 for *jun*$^{\Delta N}$). (C) Number of c-Jun positive motor neurons in the facial nucleus of wild type mice, 14 days after facial nerve cut (D14 FNA), and of *jun*-competent SOD1^{G93A} mice (SOD1 *jun*$^{f/f}$), at end-stage. (D) Proportion of motor neurons expressing axotomy response proteins out of the number of c-Jun positive motor neurons in: the facial nucleus of wild type mice 14 days after axotomy (green bars) and in *jun* competent SOD1^{G93A} mice at end-stage (orange bars). (C&D) n=4 for D14 FNA and n=10 for SOD1 *jun* $^{f/f}$.

Discussion

ALS is a neurodegenerative disease characterised by neuromuscular junction denervation, axonal loss, motor neuron death and associated neuroinflammatory changes (Boillee et al., 2006a). The transcription factor c-Jun has been found upregulated in motor neurons affected by the disease (Jaarsma et al., 1996). To determine the functional role of c-Jun in ALS, the current study investigated the effect of neural deletion of the c-Jun gene (*jun*) on the SOD1^{G93A} mouse model of ALS. This chapter shows that neural deletion of *jun* extends the lifespan of SOD1^{G93A} mice and reduces disease associated motor neuron death, axonal loss and neuroinflammation. In addition, it describes a disease mediated axotomy response mounted by motor neurons in SOD1^{G93A} mice that is dependent on *jun*.

Generation of SOD1G93A mice lacking neural c-Jun

The current study demonstrated that neural deletion of *jun*,mediated by the cre-lox system, driven by the nestin promoter, abolishes the expression of c-Jun protein from neurons in SOD1G93A mice.

To investigate the role of c-Jun in ALS mediated neurodegeneration triple transgenic mutant mice were generated. Three mouse strains were bred together to create a SOD1G93A mouse model of ALS which either expressed or did not express the murine *jun* gene. Each of the progenitor strains carried in their genome either: 1) multiple copies of ALS associated SOD1^{G93A} transgene, 2) endogenous murine *jun* alleles flanked by lox sites or 3) cre-recombinase gene driven by the nestin promoter. This type of genetic approach has been widely used in the past to elucidate the molecular pathways involved in ALS degeneration (Turner and Talbot, 2008).

The 28 copy number SOD1^{G93A} transgenic mouse was chosen for investigation because it is the most widely used animal model of ALS (McGoldrick et al., 2013). The clinical and histopathological changes observed in this model closely resemble those observed in ALS (Gurney et al., 1994). In addition, these mice reach end-stage relatively early in their lives compared to other SOD1 transgenic mouse models (Turner and Talbot, 2008). However, the

generalizability of this model has been criticised as the genetic mutation carried by these mice is only found in a small population of those affected with ALS (Benatar, 2007). Nonetheless, similar arguments can be made about other models of the disease e.g. ALS associate mutant TDP-43 transgenic mice. This model may be considered to be more generalizable since TDP-43 positive inclusions are found in almost all cases of ALS at post mortem (Mackenzie et al., 2010). However, the TDP-43 transgenic mouse models of ALS expresses mutations which are just as rare as $SOD1^{G93A}$ and the clinical and histopathological changes in these mice do not resemble ALS as closely as those seen in $SOD1^{G93A}$ mice (McGoldrick et al., 2013). Thus, despite the recent development of new animal models of ALS, the $SOD1^{G93A}$ mouse continues to be useful in ALS research (McGoldrick et al., 2013).

The current study made use of neural specific *jun* conditional knock out mice previously described (Raivich et al., 2004). These mice use the cre-lox system driven by the nestin promoter to specifically and effectively remove the murine *jun* gene from neural cells. Phenotypically, *jun* deficient mice have normal motor behaviour as well as normal brain and nerve architecture (Raivich et al., 2004). The conditional method used to delete *jun* is necessary for in vivo studies since global deletion of *jun* is embryonically lethal (Hilberg et al., 1993, Johnson et al., 1993, Behrens et al., 2002). Nestin, the promoter driving the deletion, is an intermediate filament protein primarily expressed by neural stem cells (Zimmerman et al., 1994). For this reason it has been widely used as a transgene expression promoter to mediate genetic changes within the nervous system (Tronche et al., 1999, Raivich et al., 2004). However, nestin is also expressed in other cell types (Zimmerman et al., 1994). Thus, the cre-lox system driven by the nestin promoter may result in the deletion of *jun* from a number of cell types in addition to neurons. These may include astrocytes, oligodendrocytes and their precursors which all have described roles in the process of ALS disease (Boillee et al., 2006a). It is therefore possible that any effects neural deletion of *jun* has on $SOD1^{G93A}$ mediated degeneration may be due to its effect on a number of cells including neurons. However, evidence from neuronal specific deletion of *jun*, driven by the synapsin promoter, suggests that the effects of neural deletion of *jun* on the response of motor

neuron to axonal injury are likely secondary to its deletion from neurons (Ruff et al., 2012).

The effect of neural c-Jun on the survival of SOD1^{G93A} mice

The current study demonstrates that genetic deletion of neural *jun* significantly prolonged the lifespan of SOD1G93A mice. This effect was modest, with *jun* deficient SOD1^{G93A} mice surviving 10 days longer than *jun* competent SOD1^{G93A} mice. For ethical reasons, the clinical end point (end-stage) was defined as the onset of complete hind-limb paralysis. Using this definition, the mean lifespan of control *jun* competent SOD1^{G93A} mice was measured to be approximately 104 days. This falls within the range of survival-measures previously reported for the 28 transgene copy SOD1G93A mice, which vary from 102 to 166 days (Heiman-Patterson et al., 2005, Heiman-Patterson et al., 2011). Nonetheless, the lifespans described in this study are at the lower end of this range.

The relatively shorter lifespan of SOD1^{G93A} mice reported in the current study may be explained by the unique genetic background of the mice used here. Genetic background affects the onset and progression of SOD1^{G93A} associated disease in mice (Ludolph et al., 2010, Heiman-Patterson et al., 2011). The crossing of SOD1^{G93A} mice in a B6/SJL background, with *jun* transgenic mice in a mainly B6 background but which originally had FVB and 129 background, might have accelerated the onset or progression of SOD1^{G93A} mediated disease. In support of this, there is evidence that ALS associated mutant SOD1^{G93A} mice in the FVB background have an average lifespan of around 107 days, close to what is reported here (Heiman-Patterson et al., 2011).

The definition of clinical end point is another factor which can affect the measure of survival. Over 20 different definitions of humane end points have been used in the past to determine SOD1^{G93A} mouse survival, and these are thought to be responsible for the wide range of reported lifespans (Scott et al., 2008, Ludolph et al., 2010). The use of a universal definition for a humane end point to measure survival in SOD1 mutant mice has been suggested (Scott et al., 2008, Ludolph et al., 2010, Niessen et al., 2006). It is suggested that end-stage should be defined as the time at which an animal cannot no longer right

itself within 30 seconds after being placed on its side (Scott et al., 2008, Ludolph et al., 2010). The majority of previously published studies, which report on survival, have used this righting reflex definition of end-stage (Scott et al., 2008, Ludolph et al., 2010). This postural reflex can be achieved by either upper or lower limbs (Tupper and Wallace, 1980). Thus it is possible that the clinical end point used in the current study may have underestimate lifespan because it took into account only lower limb function.

The current study reports that *jun* deletion extends the lifespan of SOD1^{G93A} mice. Effects of therapeutic interventions on survival can be confounded by a number of factors, including: genetic background, gender, and SOD1^{G93A} transgene copy number (Scott et al., 2008, Ludolph et al., 2010). The design of the current study controls for some of these factors. Differences in genetic background were unlikely to occur as *jun* deficient SOD1^{G93A} mice were compared to their littermate *jun* competent SOD1^{G93A} control mice. Confounding effects due to gender are also unlikely, since animal groups were gender matched. It is important to note that although previous groups have reported gender effects on survival, the presence of these depend on genetic background (Heiman-Patterson et al., 2011). In line with this, statistical analysis failed to demonstrate an effect of gender on SOD1^{G93A} mouse survival.

Drop in SOD1^{G93A} transgene copy number can occur in the mutant mice used in this study and it is known to affect survival (Scott et al., 2008, Ludolph et al., 2010). The lifespan of mice carrying a low number of transgene copies has been reported to be at least 40% to 140% greater than those carrying a high number (Ludolph et al., 2010). In the current study, transgene copy number was not quantified and potentially could have confounded the survival data. However, the difference in lifespan between the shortest-surviving and longest-surviving mouse in either group was very similar, 24 days for *jun*-competent and 25 for *jun*-deficient SOD1^{G93A} mice. This equates to a maximum increase in survival of only 27% if the longest-surviving mouse was compared to shortest-surviving mouse in each group. This is below what would be expected if transgene copy number had affected survival in at least one of the mice. Thus,

it seems unlikely that either of the groups were biased by a drop in transgene number.

The increase in measured survival due to *jun* deletion may be secondary to a delay in the onset of disease or the slowing of disease progression. Commonly, onset of disease is measured as the time by which mice develop first signs of tremor and hind-limb splay defects or a reduction in rotarod performance (Ludolph et al., 2010). However, the current study did not use a clinical measure to determine disease onset or whether prolonged survival was accompanied by effects on motor behaviour.

Inflammatory changes in SOD1^{G93A} mice deficient of c-Jun

The current study demonstrates that deletion of neural *jun* attenuates the neuroinflammation associated with disease in SOD1^{G93A} mice. This is consistent with previous studies which have shown that neural or neuron specific deletion of *jun* significantly diminishes the inflammatory response after axotomy (Raivich et al., 2004, Ruff et al., 2012). Deletion of neural *jun* reduces early and late microglia activation, as well as late astrocyte activation and T-cell recruitment around axotomised motor neurons following facial nerve transection (Raivich et al., 2004). Similar effects are observed in the current study, with reduced formation of perineuronal microglia clusters in the spinal cord of SOD1^{G93A} mice in early (day 70) and end-stages of the disease, as well as, reduced astrocyte activation and T-cell recruitment at end-stage.

It has been previously suggested (Raivich et al., 2004), that neural deletion of *jun* might affect neuroinflammation through two mechanisms, each working at different stages of the neuronal response to injury. The early stage mechanism may involve a reduced production of *jun*-dependent pro-inflammatory cytokines in the absence of tissue damage. While, the late stage mechanism may involve the reduced presence of pro-inflammatory neural debris secondary to the attenuation of *jun*-dependent neuronal cell death (Raivich et al., 2004). These two stages fit well with the description of alternative (M2) and classic (M1) activation of microglia continuum. M2 describes an anti-inflammatory microglial response that is neuroprotective and M1 a pro-inflammatory microglial response

that is neurotoxic (Buechler et al., 2000, Gordon and Martinez, 2010, Tiemessen and Kuhn, 2007).

In the current study, microglia activation was assessed by the quantification of alpha M immunoreactivity and microglia cluster formation. Although not formally assessed, these two measures may represent M2 and M1 activation. On the one hand, the early microglial activation measured by levels of alpha M immunoreactivity may best quantify M2 microglia activation at day 70 in the spinal cord of $SOD1^{G93A}$ mice were mild microglial changes with no motor neuron cell loss have occurred. On the other hand, microglia cluster counts may represent M1 activation as they dramatically increase at end stage in the spinal cord of $SOD1^{G93A}$ mice. Interestingly, alpha M immunoreactivity was not affected by neural *jun* deletion at day 70 or end stage but microglia cluster formation was at both time points. This suggests the neural *jun* may not be involved in M2 microglia activation but plays an important role in M1 activation in $SOD1^{G93A}$ mice.

In the current study, the T-cell pan marker CD3 was used to identify T-cell infiltration, however the CD4/CD8 identity of this infiltration was not assessed. As with microglia, T-cells appear to have both anti and pro-inflammatory functions in $SOD1^{G93A}$ mouse disease (Beers et al., 2011, Banerjee et al., 2008) and may be functionally divided into T helper cells ($CD4^{+}$) and cytotoxic T cells ($CD8^{+}$).The infiltration of T-cells into the ventral horn of the spinal cord of $SOD1^{G93A}$ mice occurs with increasing numbers as the disease progresses (Beers et al., 2008). In the early stages only $CD4^{+}$ cells can be found in the spinal cord of $SOD1^{G93A}$ mice. By end stage an influx of CD8+ cytotoxic T-cells is found in the spinal cord of $SOD1^{G93A}$ mice (Beers et al., 2008). However, it is important to recognise that the majority of T-cells found in the spinal cord of $SOD1^{G93A}$ mice at all stages of the disease are $CD4^{+}$ (Beers et al., 2008).

$CD4^{+}$ T-cells have been subdivided into two groups: regulatory (tregs; $CD4^{+}CD25^{High}$FoxP3) and effector (teffs; $CD4^{+}CD25^{-}$). Interestingly, tregs have been found to influence ALS disease progression in human cases and mice, with reduced number of tregs being associated with rapidly progressing disease in both species (Beers et al., 2011, Henkel et al., 2013). Recent studies have

shown that tregs directly steer the differentiation of macrophages and microglia toward the M2 activation state and that teffs promote the M1 microglial activation state (Li et al., 2010, Liu et al., 2011, Mahnke et al., 2007, Reynolds et al., 2009). Furthermore, in SOD1^{G93A} mice the number of tregs decreases while the number of teffs increases as disease progresses (Beers et al., 2011).

As both late (M1) microglia activation and T-cell infiltration are decreased by neural *jun* deletion in SOD1^{G93A} mice, it may be important to characterise the immunopathological signature in more detail. Subtyping the T-cells infiltrate, characterising the microglia activation state (M1/M2), and analysing pro and anti-inflammatory gene expression in the spinal cord of SOD1^{G93A} *jun* deficient mice, may help identify the mechanism by which the deletion of neural *jun* mediates reduced neuroinflammation in SOD1^{G93A} mice.

Motor neurons survive in the CNS of c-Jun deficient SOD1^{G93A} mice

The current study demonstrates that neural deletion of *jun* reduces motor neuron loss in SOD1G93A mice. This is line with previous studies which show that neural or neuronal *jun* deletion protects motor neurons from axotomy induced death (Raivich et al., 2004). However, the protective effect of *jun* deletion on SOD1^{G93A} mediated motor neuron loss, reported here, was only 28%, which is smaller than the 72% neuroprotective effect reported following axotomy (Raivich et al., 2004). This suggests that the mechanism driving motor neuron cell loss in SOD1^{G93A} mice is different from that of promoting motor neuron death following axonal injury. Axotomy induced motor neuron death is associated with the induction of the TNFR1 death receptor pathway in wild type mice (Haulcomb et al., 2014). In contrast, axotomy in SOD1^{G93A}, which results in exacerbated axotomy induced death, activates the Fas death pathway more strongly than the TNFR1 pathway (Haulcomb et al., 2014). Activation of the Fas pathway has been suggested as a mechanism specific for motor neuron cell death in ALS (Raoul et al., 2002; Holasek *et al*., 2005). In addition, genetic inhibition of the Fas pathway increases survival and reduces motor neuron cell death in SOD1^{G93A} mice (Petri *et al*., 2006). It is important to note that c-Jun regulates expression of the Fas gene (Ivanov et al., 2002). Thus, the

neuroprotection mediated by neural deletion of *jun* may be secondary to inhibitory effects on the Fas pathway.

The neuroprotective effect of *jun* deletion was modest, demonstrating that motor neuron loss is not completely dependent on *jun* expression. Gould et al. (2006) reported that SOD1^{G93A} mediated motor neuron death is completely abolished by genetic deletion of the BAX gene in mice. Thus, the neuroprotection provided by *jun* deletion is likely to represent an upstream effect on the BAX pathway, that delays, rather than prevents motor neuron cell loss. In support of this, c-Jun is known to regulate the expression of Bim, which itself regulates BAX (Whitfield et al., 2001). However, c-Jun is only one of the many regulatory proteins of the BAX pathway (Whitfield et al., 2001).

The ALS disease process is associated with progressive motor neuron atrophy (Gould et al., 2006, Kiernan and Hudson, 1993). In the current study, neural deletion of *jun* failed to protect motor neurons from SOD1^{G93A} disease associated atrophy. This suggests that the motor neuron atrophy in SOD1^{G93A} mice is not driven by *jun*. It has been previously suggested that motor neuron atrophy is driven by the loss of peripheral trophic support due to the denervation of motor neuron targets (Farah et al., 2003, Dasuri et al., 2013). Neural deletion of *jun* prevents axonal regeneration and target reinnervation following axotomy and therefore is unlikely to prevent atrophy in SOD1^{G93A} mice (Raivich et al., 2004). In fact it is important to note, that the neuroprotective effect of *jun* deletion following axotomy is associated with marked atrophy of surviving neurons (Raivich et al., 2004). Therefore, *jun* deletion could have exacerbated motor neuron atrophy in the SOD1^{G93A} mouse. However, although *jun* deficient SOD1^{G93A} mice reached end-stage with a greater number of motor neurons than *jun* competent mice, surviving motor neurons in both groups had similar cell body diameters.

Axonal loss in c-Jun-deficient SOD1^{G93A} mice

In the current study, *jun* deletion was associated with reduced axonal loss in SOD1^{G93A} mice. This effect was observed both at the pre-symptomatic time point and end-stage. There is currently no evidence to suggest that *jun* plays a direct role in axonal degeneration. The maintenance of axon structure depends

on axonal transport. SOD1^{G93A} mediated disease is characterised by compromised axonal transport (Boillee et al., 2006a). Interventions that reduce the burden on axonal transport, such as genetic removal of neurofilaments (Nguyen et al., 2001), have been associated with decreased neurodegeneration and improved survival in SOD1 mutant mice (Boillee et al., 2006a). There is evidence of a regenerative effort in ALS and SOD1^{G93A} mice, characterised by axonal sprouting (Fischer and Glass, 2007, Gordon et al., 2004, Frey et al., 2000b). Such regenerative efforts would invariably cause a burden on axonal transport. Deletion of *jun* is known to dramatically reduce axonal regeneration following axotomy (Raivich et al., 2004). Thus, it is possible that *jun* deletion may curtail the axonal regenerative efforts mounted by SOD1^{G93A} mice and in this way reduce axonal degeneration and loss. To determine whether *jun* deletion does reduce the axonal regenerative effort in SOD1G93A mice, axonal sprouting could be investigated.

Neuromuscular junction dennervation in c-Jun deficient SOD1^{G93A} mice

Previous studies have suggested that loss of NMJ innervation is the predominant pathological feature driving disease in SOD1^{G93A} mice (Fischer et al., 2004, Gould et al., 2006). In the current study, SOD1^{G93A} mice reached end-stage with the same level of neuromuscular junction innervation independent of *jun* deletion. Here, end-stage was defined as the onset of complete hind limb paralysis. Since motor function depends on NMJ innervation, the fact that there was no difference in NMJ innervation at end-stage between SOD1^{G93A} mice irrespective of *jun* deletion, is unsurprising. It is important to note that SOD^{G93A} mice lacking neural *jun* reached end-stage with a greater numbers of motor neurons and axons compared to *jun* competent mice. This is in line with previous evidence which demonstrates that NMJ denervation precedes or occurs independently of pathological changes at the nerve or motor neuron level (Fischer et al., 2004, Gould et al., 2006, Pun et al., 2006). In fact, when motor neuron death in SOD1^{G93A} mice is completely abolished by genetic deletion of BAX, end-stage is reached with the same level of NMJ innervation irrespective of BAX deletion (Gould et al., 2006). However, the evidence presented in the current studies does not exclude the possibility that *jun* deletion may affect NMJ innervation in wild type mice or in SOD1^{G93A} mice at

earlier time points. Assessment of the effect of *jun* deletion on NMJ innervation in wild type mice and day 70 SOD1^{G93A} mice may shed light on this.

The axotomy response in motor neurons of SOD1^{G93A} mice

The current study demonstrates that neurons in SOD1^{G93A} mice mount a disease mediated axotomy response. This response was characterised by the expression of regeneration-associated proteins in neurons of the ventral horn of the spinal cord, hypoglossal and facial nuclei of SOD1^{G93A} mice. Pro-regenerative efforts have been previous described in human cases of ALS and SOD1^{G93A} mice (Fischer and Glass, 2007, Gordon et al., 2004, Frey et al., 2000b). However, there is little evidence on pro-regenerative changes at the level of motor neuron cell body. Nonetheless, previous neuropathological studies of ALS cases have suggested that motor neurons undergo chromatolysis, which is a morphological feature of the normal axotomy response (Kiernan and Hudson, 1993). In addition, a recent study has demonstrated that motor neurons from the facial nucleus of SOD1^{G93A} mice upregulate mRNA of pro-regenerative genes, namely GAP-43 and βII-Tubulin (Haulcomb et al., 2014). In the current study, neurons were found to be immunoreactive for the pro-regenerative proteins c-Jun, CD44, alpha7 and beta1 integrins. These proteins are expressed the novo or upregulated in motor neurons in response to axotomy and have described functional roles in axonal regeneration (Raivich et al., 2004).

The present study shows that the disease mediated axotomy response observed in SOD1^{G93A} mice is dependent on the expression of *jun.* It has been previously demonstrated that the molecular response of facial motor neurons to axotomy is dramatically reduced in mice when neural *jun* is deleted (Raivich et al., 2004). In line with this, neural deletion of *jun* significantly reduced the expression of axotomy associated proteins in the ventral horn of the spinal cord, hypoglossal nucleus and facial nucleus. The similarity between the molecular responses seen in SOD1^{G93A} mice and following peripheral nerve axotomy in wild type mice, suggests that the upregulation of regeneration associated proteins by neurons in SOD1^{G93A} mice may indeed represent a disease associate axotomy response.

The apparent magnitude of the axotomy response observed in SOD1^{G93A} mice correlates with the level of motor neuron loss in different motor neuron pools. Motor neuron degeneration in ALS cases and SOD1^{G93A} mice has been described to occur at different rates in different neuroanatomical areas (Ravits and La Spada, 2009, Haenggeli and Kato, 2002). In line with previous evidence (Haenggeli and Kato, 2002), the present study demonstrates that the level of motor neuron loss was severe in the spinal cord, moderate in the hypoglossal nucleus and not detectable in the facial motor nucleus of *jun*-competent SOD1^{G93A} mice. Interestingly, the level of axotomy response in these motor neuron pools correlated well with the levels of motor neuron loss. Furthermore, the axotomy response was almost absent at the pre-symptomatic stage were no motor neuron loss was observed. This suggests that the disease mediated axotomy response mounted by surviving motor neurons in SOD1^{G93A} mice, may represent a last ditch attempt at survival prompted by severe motor neuron injury occurring before cell death.

Finally, in line with the observation made in the spinal cord, neural *jun* deletion prevented motor neuron loss and reduced neuroinflammation in the hypoglossal nucleus and in the facial nucleus, of end-stage SOD1^{G93A} mice. This suggests that the role of *jun* in SOD1^{G93A} mediated neurodegeneration observed in the spinal cord is generalizable to different motor neuron pools. Thus, therapeutic intervention targeting *jun* may ameliorate ALS neurodegeneration in the different motor neuron pools which are affected in humans and rodent disease.

Limitations

The effects of neural *jun* deletion on the motor function and weight SOD1^{G93A} mice was not assessed. Rotarod performance could be used to determine whether *jun* deletion affected motor function and weight could be used to determine overall effect on disease progression. These measures may permit determining whether *jun* deletion delayed onset of disease. Data from such assessment would help determine if prolonged survival in *jun* deficient SOD1^{G93A} mice was secondary to an effect on disease onset.

The current study fails to describe the cellular localisation of c-Jun expression in the central nervous system of *jun*-competent or *jun*-deficient SOD1^{G93A} mice.

Double immunofluorescent labelling for c-Jun and markers for neurons, astrocytes, oligodendrocytes, oligodendrocyte precursor cell, microglia or myocytes in *jun* competent or *jun* deficient SOD1^{G93A} mouse tissue could be carried out. Analysis of this would clarify which cell type is responsible for the effects *jun* deletion, driven by the nestin promoter, has on SOD1G93A mediated disease.

Data on the effect of *jun* deletion on neuroinflammatory markers, neuromuscular junction innervation and the expression of axotomy related proteins in mice lacking the SOD1^{G93A} transgene is absent. Obtaining such data would validate the conclusion formed in this study.

The observations made regarding axotomy related proteins requires the identification of the cells expressing these proteins. Double immunofluorescent labelling for the different axotomy related proteins and markers for neurons, astrocytes, oligodendrocytes, oligodendrocyte precursor cell and microglia should be carried out. Analysis would confirm that the expression of axotomy related proteins is indeed neuronal.

Conclusion

Neural deletion of the transcription factor c-Jun prolongs the lifespan of SOD1^{G93A} mice and reduces disease associated motor neuron death, axonal loss and neuroinflammation. Interestingly, in addition to the described neuropathological changes, SOD1^{G93A} mice also demonstrate a disease mediated axotomy response, which is mounted by motor neurons in late stage disease and which depends on c-Jun. In light of the critical role of c-Jun in peripheral nerve regeneration, the observed axotomy response in SOD1^{G93A} mice is likely to be a regenerative effort mounted by motor neurons in a last ditch attempt to recover physiological function.

Chapter 4: Nuclear clearing of ALS associated proteins following peripheral nerve axotomy

Introduction

Familial ALS is associated with mutations in TDP-43, FUS and alsin genes (Andersen and Al-Chalabi, 2011). Histopathological studies of ALS cases have demonstrated changes in the pattern of immunoreactivity for TDP-43 and FUS protein in degenerating motor neurons (Neumann et al., 2006, Neumann et al., 2009). These include the clearing of normally nuclear staining and presence of immunoreactive cytoplasmic inclusions (Neumann et al., 2006, Neumann et al., 2009). The role of such changes in ALS degeneration and the functions of TDP-43, FUS and alsin in motor neurons remain elusive.

Peripheral nerve injury is associated with a molecular response within the injured neuron which can culminate in either axonal regeneration and survival, or degeneration and death (Patodia and Raivich, 2012a). This response is orchestrated by changes in gene expression which involve the up-regulation of cytoskeletal proteins, cell adhesion molecules, and growth and cell death associated genes (Patodia and Raivich, 2012a). Transcription factors play a central role in driving these changes (Patodia and Raivich, 2012b). For example, c-Jun, which is found upregulated in motor neuron cell nuclei following axotomy, plays a crucial role in the response to axonal injury (Raivich et al., 2004, Ruff et al., 2012). Its genetic deletion from neurons results in impaired axonal regeneration and functional recovery but abolishes motor neuron death following facial nerve transection in mice (Raivich et al., 2004, Ruff et al., 2012).

Some of the cellular and molecular changes that occur in ALS resemble those observed following axonal injury (Dadon-Nachum et al., 2011, Fischer et al., 2004). Interestingly, as well as playing a role in ALS, TDP-43, FUS and alsin are all involved in processes related to the neuronal response to axonal injury (Hadjebi et al., 2008, Li et al., 2013a). TDP-43 and FUS are involved in gene transcription and translation (Li et al., 2013a), while alsin has roles in retrograde signalling and growth cone dynamics (Hadjebi et al., 2008). However, the roles of these ALS associated proteins in the response to axonal injury have not yet been characterised.

The aim of this chapter is to describe the pattern of immunoreactivity for TDP-43, FUS and alsin following axonal injury, using the facial nerve axotomy model. It reports the immunoreactivity changes that occur over time following facial nerve transection. It also describes the effects different severities of axonal injury have on the immunoreactivity of TDP-43 and alsin.

Results

Time course of TDP-43 immunoreactivity in regenerating facial motor nucleus

Peripheral nerve transection produces protein changes within axotomised neurons which can be associated with regenerative and degenerative processes (Patodia and Raivich, 2012a). TDP-43 is a ubiquitously expressed nuclear protein involved in gene transcription and mRNA translation (Li et al., 2013a). In ALS, TDP-43 protein is found to leave its nuclear location and form cytoplasmic inclusions within motor neurons (Neumann et al., 2006). To investigate the pattern of TDP-43 immunoreactivity following peripheral nerve axotomy, C57BL/6 mice underwent a unilateral facial nerve transection and were allowed to survive for 0, 1, 4, 7, 14, 21, and 42 days following axotomy (n=4 per time point). Coronal sections through the facial motor nuclei of these animals underwent immunohistochemistry for TDP-43 protein and were analysed using light microscopy.

TDP-43 immunoreactivity in the uninjured facial nucleus was mainly confined to the nuclei of motor neurons and surrounding glial cells (Fig. 1 left column). Some weak immunoreactivity was also observed in the cytoplasm of these two cell types (Fig. 1A). However, transection of the facial nerve resulted in a strong increase in TDP-43 immunoreactivity in the axotomised facial motor nucleus (Fig. 1), starting at day 4 (Fig. 1F) and reaching a peak at day 7 after injury (Fig. 1H). This apparent rise in overall immunoreactivity was associated with clearing of TDP-43 staining from the nuclei of injured motor neurons which started at day 7 (Fig. 1H insert) and was most obvious at day 14 (Fig. 1J insert). Nuclear clearing was accompanied by a marked increase in diffuse cytoplasmic staining which extended to the dendrites of axotomised neurons (Fig. 1H&J inserts). At day 14, TDP-43 immunoreactivity was also found in perineuronal sprouts (Fig. 1J bottom insert). Cytoplasmic and neurite TDP-43 staining diminished from day 21 post facial nerve transection and gave way to increasing nuclear staining

(Fig. 1M insert). By day 42, the pattern of TDP-43 staining returned to that observed in uninjured motor neurons (Fig, 1M and N).

Optical luminosity quantifications (Fig. 2) demonstrated that uninjured facial nuclei had steady levels of TDP-43 immunoreactivity at all time-points studied. In contrast, a peak in TDP-43 immunoreactivity was confirmed at day 7 after axotomy. This was followed by a decline to baseline levels by day 42. TDP-43 optical luminosity levels were higher in the axotomised compared to uninjured facial nuclei from day 2 up to day 21. However, the difference was only statistically significant at day 7 ($p<5\%$; using unpaired Student's T-test between control facial motor nuclei (FMN) and axotomy FMN, at day 7), with TDP-43 immunoreactivity levels that were 57% higher in the axotomised facial nuclei compared to the non-injured facial nucleus.

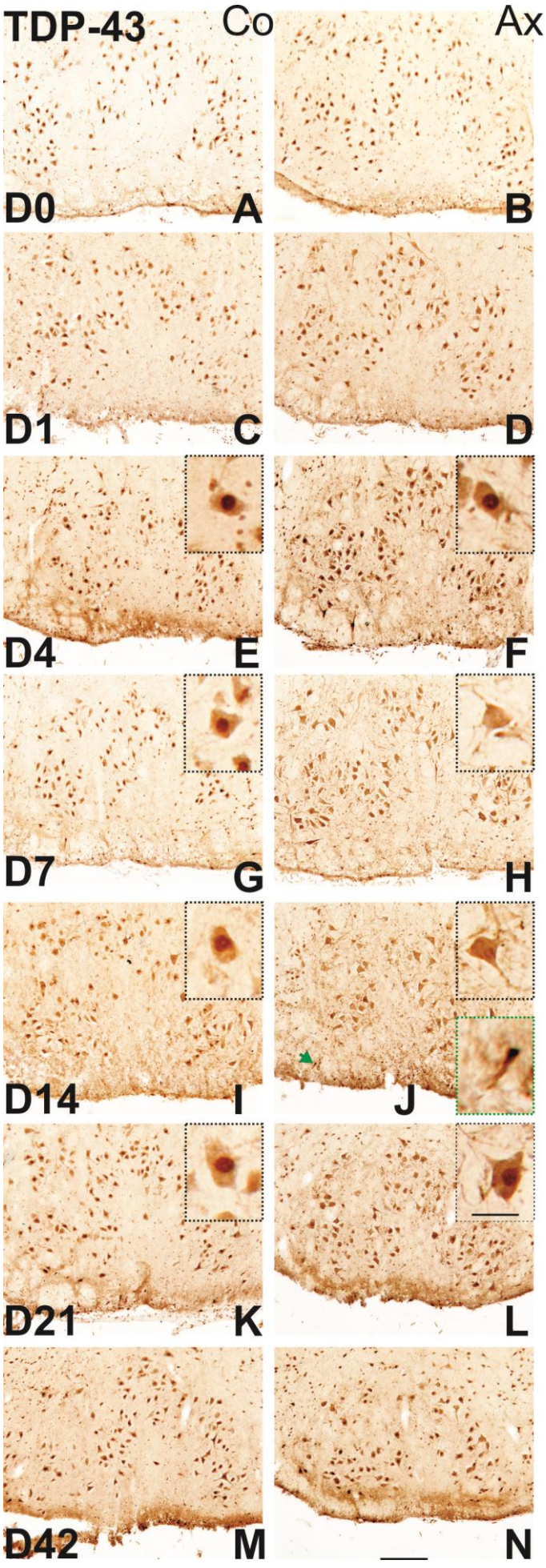

TDP-43
Co
Ax
D0
A
B
D1
C
D
D4
E
F
D7
G
H
D14
I
J
D21
K
L
D42
M
N

Figure 1: TDP-43 immunoreactivity increases in the facial nucleus after facial nerve injury. Coronal sections through the uninjured (Co) and injured (Ax) facial motor nucleus at 0 (A&B), 1 (C&D), 4 (E&F), 7 (G&H), 14 (I&J), 21 (K&L), and 42 days (M&N) after facial nerve transection. Sections were immunostained for TDP-43. From day 4 to day 28, there is more TDP-43 immunostaining in the injured facial motor nucleus compared to the uninjured (Co) facial motor nucleus. Bar scale represent 100um. High magnification inserts show that at 7 (G) and 14 days (I) after facial nerve injury TDP-43 nuclear staining is absent. However, TDP-43 immunostaining returns to the nucleus at 21 days after facial nerve injury (K). At day 14, staining is also evident in neuronal sprouts (green arrow). Neurons of the uninjured facial motor nucleus show strong nucleic staining at all time-points post injury. Bar scale represents 50um.

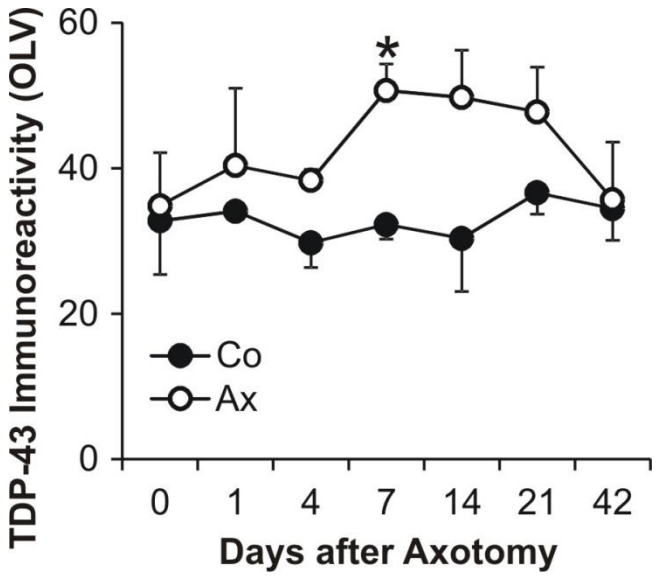


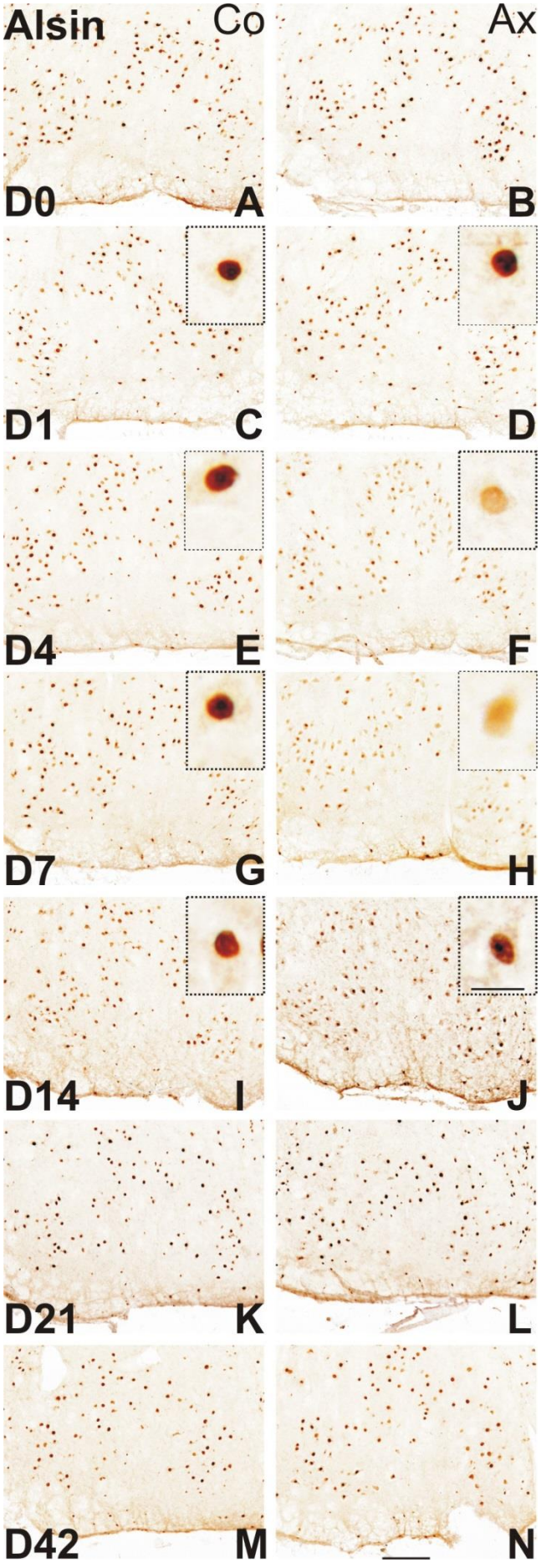
Figure 2. Quantitative analysis of TDP-43 immunoreactivity following peripheral nerve transection. Line graph showing the mean TDP-43 immunoreactivity, quantified using the MEAN-SD algorithm, at 0, 1, 4, 7, 14, 21 and 42 days after facial nerve injury. Black and white circles represent quantifications of uninjured (Co) and axotomised (Ax) facial nucleus, respectively. *p<5% using unpaired Student's T-test between uninjured and injured groups. n=4 for both groups. (OLV: Optical Luminosity Values)

Time-course of Alsin immunoreactivity after facial nerve injury.

Alsin is 1657 amino acid protein encoded by the ALS associated gene ALS2. Its known functions in retrograde signalling and growth cone dynamics suggest a potential role in the response to axonal injury (Tudor et al., 2005, Hadano et al., 2010). To study the pattern of Alsin immunoreactivity following peripheral nerve axotomy, C57BL/6 mice underwent a unilateral facial nerve transection and were allowed to survive for 0, 1, 4, 7, 14, 21, and 42 days following axotomy (n=4 per time point). Coronal sections through the facial motor nuclei of these animals underwent immunohistochemistry for Alsin protein and were analysed using light microscopy.

Alsin immunoreactivity in the uninjured facial nucleus was confined to the nuclei of motor neurons (Fig. 3 left column). Staining was strong in the nucleus but absent from the cytoplasm (Fig. 3C). Transection of the facial nerve resulted in markedly diminished nuclear staining, which started at day 4 (Fig. 3D insert) and was most obvious at day 7 (Fig. 3F insert). Nuclear clearing was not associated with changes in cytoplasmic immunoreactivity (Fig. 3D&F). Nuclear alsin immunoreactivity began to return to normal at day 14 (Fig. 3H insert) after axotomy and reached pre-injury levels at day 21 (Fig. 3K&L).

Quantification of nuclear alsin immunoreactivity (Fig. 4) demonstrated that the uninjured facial nuclei had steady levels at all time-points studied. In contrast, a trough in nuclear immunoreactivity was confirmed at day 7 after axotomy. This was followed by an increase to baseline levels at day 21. Nuclear immunoreactivity levels for alsin were significantly lower in the axotomised compared to uninjured nuclei from day 4 up to day 14 ($p<5\%$; using unpaired Student's T-test between control FMN and axotomy FMN, at day 4 to 14). However, the difference was greatest at day 7, with levels of nuclear immunoreactivity being around 60% lower in the axotomised facial nuclei compared to the non-injured facial nucleus.


Alsin
Co
Ax
D0
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K
L
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M
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Figure 3: Alsin immunoreactivity decreases in the nuclei of neurons of the facial motor nucleus after facial nerve injury. Coronal sections through the uninjured (Co) and injured (Ax) facial motor nucleus at 0 (A&B), 1 (C&D), 4 (E&F), 7 (G&H), 14 (I&J), 21 (K&L), and 42 (M&N) after facial nerve transection. Sections were immunostained for alsin. From day 4 to 14 after facial nerve injury, there is less nuclear alsin immunostaining in the injured facial motor nucleus compared to the uninjured (Co) facial motor nucleus. Bar scale represents 100um. High magnification inserts show that Alsin staining is nucleic in neurons of the injured (D, F, H, J) and uninjured facial motor nucleus (C, E, G, I). Neurons of the uninjured facial motor nucleus show strong nucleic staining at all time-points post injury. Bar scale represents 25um.

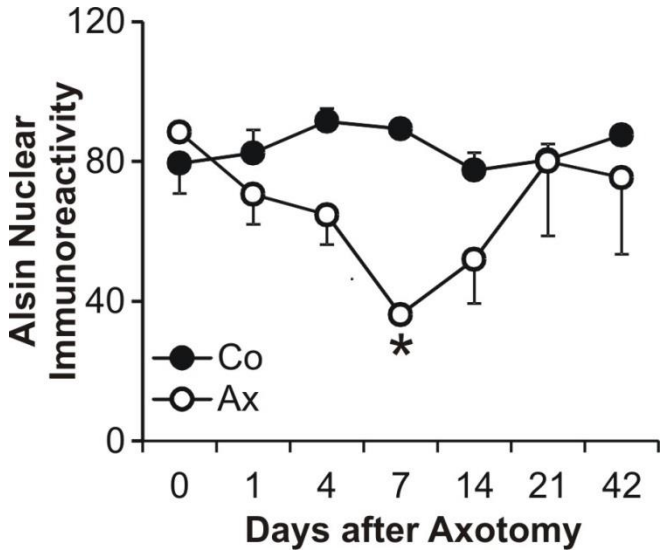

Figure 4. Quantitative analysis of nuclear alsin immunoreactivity following peripheral nerve transection. Line graph showing nuclear alsin immunoreactivity, quantified using a variation of the MEAN-SD algorithm, at 0, 1, 4, 7, 14, 21 and 42 days after facial nerve injury. Black and white circles represent quantifications of uninjured (Co) and axotomised (Ax) facial nucleus, respectively. *p<5% using unpaired Student's T-test between uninjured and injured groups. n=4 for both groups.

Time course of FUS immunoreactivity in regenerating facial motor nucleus

FUS is a 526 amino acid protein which has roles in gene transcription and translation (Li et al., 2013a). It is ubiquitously expressed and normally found in the nucleus, but can shuttle between the nucleus and cytoplasm (Li et al., 2013a). Mutations in the FUS gene are associated with familial ALS (Andersen and Al-Chalabi, 2011). In addition, abnormal patterns of FUS immunoreactivity have been described in motor neurons of ALS cases (Neumann et al., 2009). To study the pattern of FUS immunoreactivity following peripheral nerve axotomy, C57BL/6 mice underwent a unilateral facial nerve transection and were allowed to survive for 0, 1, 4, 7, 14, 21, and 42 days following axotomy (n=4 per time point). Coronal sections through the facial motor nuclei of these animals underwent immunofluorescent labelling for FUS protein and analysis with confocal microscopy.

FUS immunoreactivity in the uninjured facial nucleus was mainly restricted to the nuclei of motor neurons and surrounding glial cells (Fig. 5 left column). Some weak immunoreactivity was also observed in the cytoplasm of both of these cell types (Fig. 5 left column). Transection of the facial nerve resulted in a modest decrease in nuclear FUS immunoreactivity at 14 days after injury (Fig. 5H insert). This reduction was still discernible at day 21 (Fig. 5J insert). However, FUS nuclear staining returned to baseline at day 42 (Fig. 5N).

Quantification demonstrated that nuclear FUS immunofluorescence in the intact facial nuclei was similar at all time-points studied. In contrast, injured motor neurons showed a drop in FUS immunofluorescence, which started at day 14 after injury and was followed by a return to baseline at day 42. Nuclear FUS immunofluorescence levels were lower in the axotomised compared to uninjured nuclei at day 14 and 21. However, the difference was only statistically significant at day 14 ($p<5\%$; using unpaired Student's T-test between control FMN and axotomy FMN, at day 14), with levels of FUS immunofluorescence being around 20% lower in the axotomised facial nuclei compared to the non-injured facial nucleus.

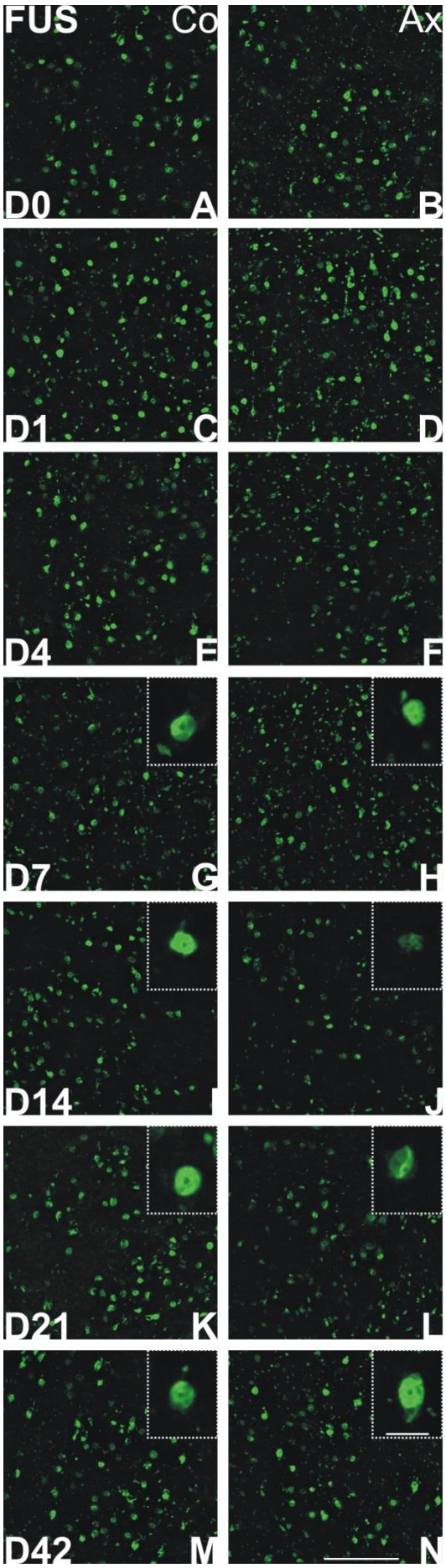
FUS
Co
Ax
D0
A
B
D1
C
D
D4
E
F
D7
G
H
D14
I
J
D21
K
L
D42
M
N

Figure 5: Nuclear FUS immunofluoresence decreases in the nuclei of neurons of the facial motor nucleus after facial nerve injury. Coronal sections through the uninjured (Co) and injured (Ax) facial motor nucleus at 0 (A&B), 1 (C&D), 4 (E&F), 7 (G&H), 14 (I&J), 21 (K&L), and 42 days (M&N) after facial nerve transection. Sections are immunofluorescently labelled for FUS. Labelling was evident in the nuclei of glial and neuronal cells in both injured and uninjured facial motor nucleus at all time-points. Bar scale represents 100um. Inserts (J&L) show that from day 14 to day 21 after facial nerve injury, there is less nuclear FUS labelling in axotomised motor neurons compared to the uninjured ones (I&K). Bar scale represents 25um. Cells of the uninjured facial motor nucleus show strong neuronal nuclei staining at all time-points post injury.

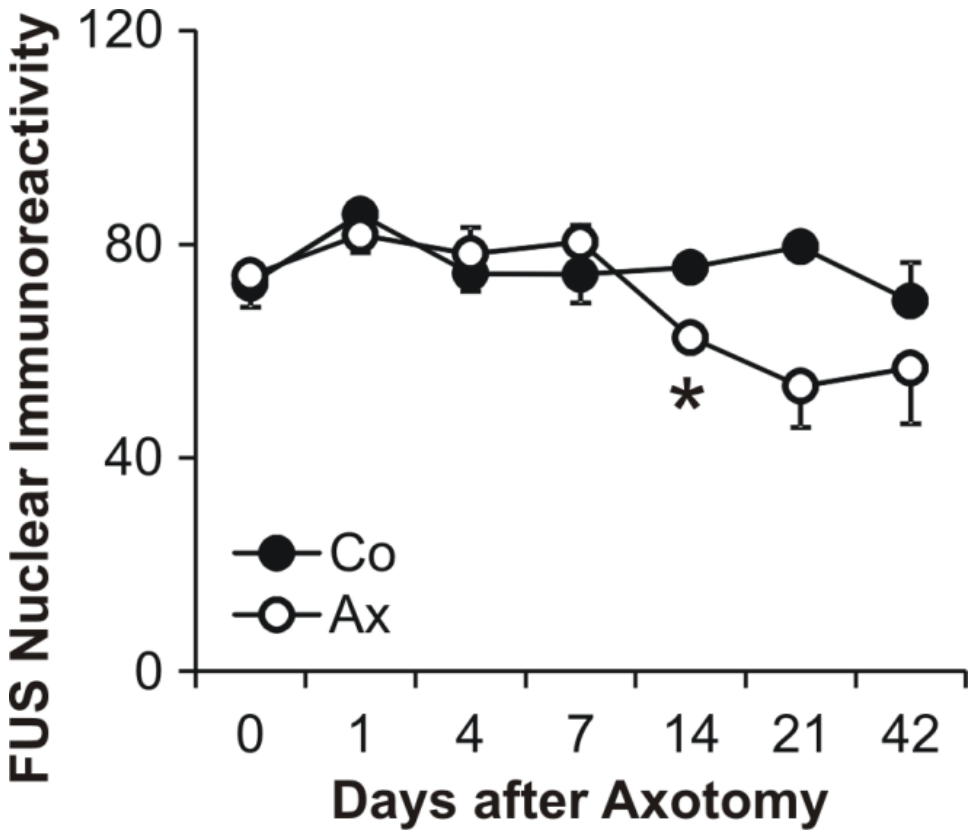

Figure 6. Quantitative analysis of nuclear FUS immunoreactivity following peripheral nerve transection. Line graph showing nuclear FUS immunoreactivity, quantified using a variation of the MEAN-SD algorithm, at 0, 1, 4, 7, 14, 21 and 42 days after facial nerve injury. Black and white circles represent quantifications of uninjured (Co) and axotomised (Ax) facial nucleus, respectively. *p<5% using unpaired Student's T-test between uninjured and injured groups. n=4 for both groups.

Effects of injury severity on TDP-43 and Alsin immunoreactivity

Severity of axonal injury is known to affect the cellular and molecular response to axotomy (Makwana et al., 2010). To investigate whether severity of nerve injury affects axotomy-induced changes in TDP-43 and alsin immunoreactivity, C57BL/6 mice underwent either a unilateral facial nerve transection or crush, and were allowed to survive for 7, 10, and 14 following axotomy (n=3 per time point). Coronal sections through the facial motor nuclei of these animals underwent immunohistochemistry for TDP-43 or alsin protein and were analysed using light microscopy.

TDP-43

Ten days after facial nerve cut (Fig. 7C) or crush (Fig. 7B) injuries, the motor neurons within the facial nucleus demonstrated nuclear clearing of TDP-43 staining, which was associated with pronounced TDP-43 staining in the cytoplasm. Optical luminosity quantifications (Fig. 7A) showed that TDP-43 immunoreactivity in the injured facial nucleus was greater after nerve cut rather than after nerve crush at all time-points after injury. However, the difference between cut and crush injury was only statistically significant at day 10 ($p<5\%$; using unpaired Student's T-test between cut and crush injury, at day 10). At this time point, nerve cut resulted in an increase in TDP-43 immunoreactivity which was about 40% greater than that caused by nerve crush.

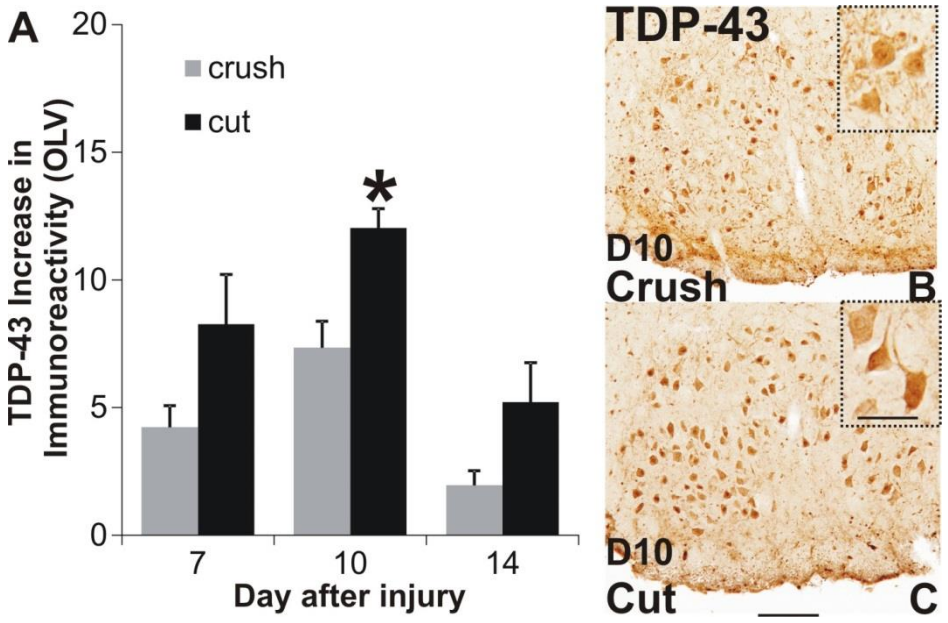

Figure 7. Facial nerve cut produces greater axotomy induced changes in TDP-43 immunoreactivity than crush. (A) Quantitative analysis of TDP-43 immunoreactivity following peripheral nerve injury. Histograms show the difference between TDP-43 immunoreactivity in the facial nuclei ipsilateral to a nerve cut or crush injury, which was quantified using the MEAN-SD algorithm, at 7, 10 and 14 days after facial nerve injury. Grey and black bars represent quantifications of crush and cut, respectively. *p<5% using unpaired Student's T-test between crush and cut groups. n=3 for both groups. (B&C) Coronal sections through the injured facial motor nucleus at day 10 after facial nerve crush (B) or cut (C). Sections were immunostained for TDP-43. TDP-43 immunostaining appears stronger in the cut facial nucleus (C) compared to crush (B) Bar scale represents 100um. High magnification inserts show that nerve cut (C insert) results in more obvious TDP-43 nuclear clearing and increased cytoplasmic staining compared to crush (B insert). Bar scale represents 50um.

Alsin

Ten days after facial nerve cut (Fig. 8C) or crush (Fig. 8B) injuries, the facial motor neurons demonstrated Alsin staining confined to the cellular nucleus rather than the cytoplasm. Nuclear alsin immunoreactivity quantifications (Fig. 8A) revealed that the levels of alsin immunoreactivity was similar at 7, 14 or 21 days after a cut or crush injuries. At all studied time-points nuclear alsin immunoreactivity was greater after cut rather than after nerve crush injury. However, the difference between cut and crush injury was only statistically significant at day 10 ($p<5\%$; using unpaired Student's T-test between cut and crush injury, at day 10). At this time point, nerve cut resulted in clearing of nuclear alsin immunoreactivity which was around 80% greater than that caused by nerve crush.

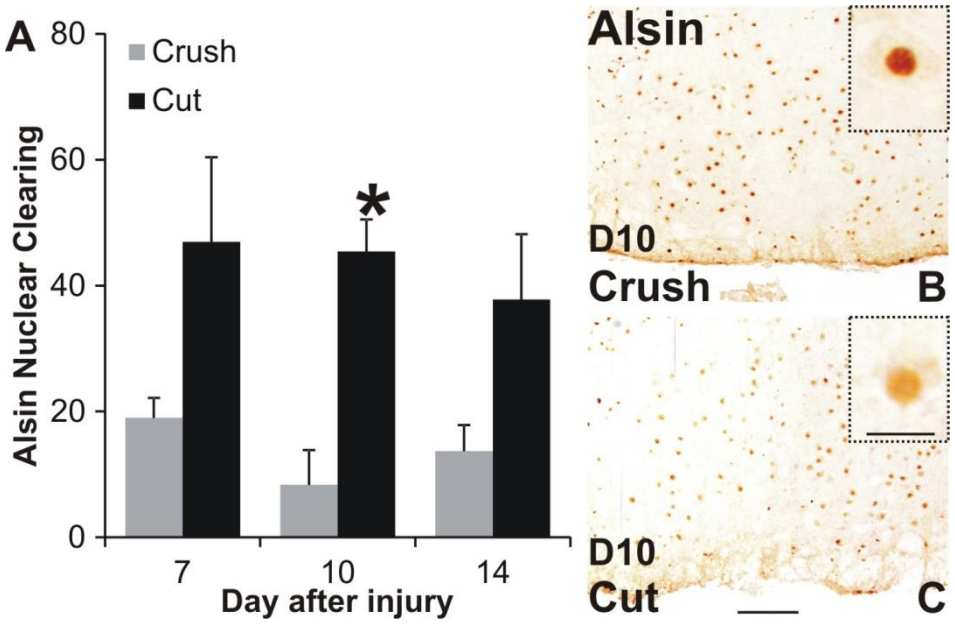

Figure 8. Facial nerve cut produces greater axotomy induced changes in alsin immunoreactivity than crush. (A) Quantitative analysis of alsin immunoreactivity following peripheral nerve transection. Histograms show the difference between the immunoreactivity of alsin in the nucleus of facial motor neurons that have undergone a facial nerve cut or crush injury, which was quantified using a modified MEAN-SD algorithm, at 7, 10 and 14 days after facial nerve injury. Grey and black bars represent quantifications of crush and cut, respectively.

*p<5% using unpaired Student's T-test between crush and cut groups. n=3 for both groups. (B&C) Coronal sections through the injured facial motor nucleus at 10 days after facial nerve crush (B) or cut (C). Sections were immunostained for alsin. Alsin immunostaining appears weaker in the cut facial nucleus (C) compared to crush (B) Bar scale represents 100um. High magnification inserts show that there is less alsin staining in the neuronal nucleus after facial nerve injury (C) than after nerve crush (B insert). Bar scale represents 50um.

Discussion

ALS is associated with cellular and molecular changes which resemble those observed following peripheral nerve injury (Dadon-Nachum et al., 2011). Axotomy causes changes in the immunoreactivity of a number of growth and death associated proteins within neurons (Patodia and Raivich, 2012a). TDP-43, FUS and ALS2 are genes which cause familial ALS (Andersen and Al-Chalabi, 2011) and have functions which may implicate them in the neuronal response to axotomy (Hadjebi et al., 2008, Li et al., 2013a). This study investigated whether facial nerve axotomy produces changes in the normal immunoreactivity pattern of TDP-43, FUS and Alsin protein within injured neurons. It also investigated whether these changes depend on the severity of nerve injury. It demonstrates that neuronal TDP-43, FUS and Alsin immunoreactivity changes following axotomy, in a time-dependent manner after injury. It also shows that changes in TDP-43 and Alsin immunoreactivity are affected by the severity of axonal injury.

TDP-43 immunoreactivity in the facial motor nucleus following facial nerve axotomy

The current study demonstrates that TDP-43 immunoreactivity in the facial motor nucleus changes following facial nerve axotomy in a time-dependent manner. In line with previous studies, TDP-43 immunohistochemical staining was localised to the cellular nucleus of neurons and glia in the uninjured facial nucleus (Moisse et al., 2009b, Sato et al., 2009). Following facial nerve cut, TDP-43 staining moved out from the neuronal nucleus into the cytoplasm, increasing the overall level of immunoreactivity in the injured facial nucleus. These changes were transient, returning to normal by around the time when peripheral target reinnervation occurs in the facial nerve axotomy model (Makwana et al., 2010). The observations reported here are consistent with

previous studies which have described similar changes in TDP-43 immunoreactivity in the sciatic nerve motor pool following peripheral nerve cut and in the hypoglossal nucleus after nerve ligation (Moisse et al., 2009b, Sato et al., 2009).

It is unclear whether the increase in overall TDP-43 immunoreactivity following axotomy represents an increase in TDP-43 expression at the protein level or a redistribution of the protein. Previous studies have suggested that TDP-43 is upregulated in injured neurons at both the mRNA and protein level following sciatic nerve cut and hypoglossal nerve ligation (Moisse et al., 2009b, Sato et al., 2009). However, whether this is the case in facial motor neurons following axotomy remains to be determined.

This study also describes for the first time the localisation of TDP-43 in putative regenerative perineuronal sprouts following axotomy. TDP-43 immunoreactive structures, morphologically characterised by a bulb and tail (Makwana et al., 2010), were found in the perineuronal white matter in the injured facial nucleus. Previous in-vitro studies have reported that TDP-43 translocates to axons and dendrites following axonal injury and repetitive depolarisation (Wang et al., 2008a, Sato et al., 2009), but none have described TDP-43 in regenerating neurites in vivo. Perineuronal sprouting in the facial nucleus has been suggested to correlate with the regenerative potential of neurons after injury (Makwana et al., 2010, Makwana et al., 2009). Previous studies have implicated TDP-43 in mRNA transport and translation at the synapse, and axonal growth (Fallini et al., 2012, Liu-Yesucevitz et al., 2011, Tripathi et al., 2014). Thus, TDP-43 may be functionally involved in the process of axonal regeneration. This hypothesis is tested in vivo with the use of TDP-43 transgenic mice and the facial nerve axotomy model in the next chapter of this thesis.

Alsin immunoreactivity in the facial motor nucleus following facial nerve axotomy

The current study describes, for the first time, alsin immunoreactivity in the nucleus of motor neurons. This is in contrast to previous immunohistochemical studies which show normal alsin localisation to be cytoplasmic (Tudor et al., 2005, Otomo et al., 2003, Devon et al., 2005) in line with its known functions in

endosomal trafficking and neurite outgrowth (Hadano et al., 2010, Tudor et al., 2005).

It is possible that the motor neuron nuclear staining obtained in the current study was not specific for alsin. The commercially available antibody used here was raised against a purified synthetic peptide sequence within the c-terminal of the alsin protein (LKACYYQIQREKLN) (Everest Biotech, 2014). The specificity of antibodies raised against peptides can be affected by contamination from other proteins during antibody production and by cross reactivity with proteins which contain similar sequences in vivo (Ramos-Vara, 2005). No previous studies have used this antibody to detect alsin. In order to determine whether the nuclear staining described in this study is due to alsin, the specificity of the antibody must be validated. Future work should use western blotting to determine if the molecular weight of the protein detected by the antibody matches that of alsin, and immunohistochemistry on a negative control tissue, such as alsin deficient mouse brain, to demonstrate that nuclear staining from the antibody is absent when alsin protein is not present.

Although not previously described, endogenous alsin protein may indeed be localised to the cellular nucleus. Alsin is a large protein with six RCC1 like domains (RLDs) in its N-terminus (Hadjebi et al., 2008). The RCC1 protein, from which the term originates, is made up of 7 homologous amino acid repeats which fold into a seven-bladed β-propeller domain (Hadjebi et al., 2008). Loops within the RCC1 β-propeller domain interact with the histone component and DNA component of the nucleosome core particle in the cell nucleus (Makde et al., 2010). Thus, immunohistochemistry for RCC1 protein demonstrates nuclear immunoreactivity (Hadjebi et al., 2008). The RLDs in alsin are thought to take a similar β-propeller structure in vivo (Hadjebi et al., 2008) and thus may potentially bind to the nucleosome and be localised in the nucleus, as described in the current study.

Gros-Louis et al. (2008) produced two antibodies against alsin with differential immunohistochemical staining patterns. One, raised against a short sequence in the N-terminal of alsin, resulted in cytoplasmic labelling within neurons of the central nervous system. The other, which was raised against a peptide

sequence in the C-terminal, showed both cytoplasmic and nuclear labelling within neurons (Gros-Louis et al., 2008). The immunoreactivity obtained with the anti C-terminal alsin antibody was reported as absent in lysates from ALS2 knock out mice brains, suggesting that the antibody was specific (Gros-Louis et al., 2008). The antibody used in the current study was also raised against a peptide sequence in the c-terminal. Thus, alsin may reside in the nucleus of neurons and may be detectable using antibodies against the c-terminal of the protein.

The majority of previous descriptions of the subcellular localisation of alsin used antibodies which have not been raised against the C-terminal of the protein (Tudor et al., 2005, Otomo et al., 2003, Devon et al., 2005, Yamanaka et al., 2003). The majority have been raised against the N-terminal and middle portion of alsin and have described cytoplasmic immunoreactivity within neurons (Tudor et al., 2005, Otomo et al., 2003, Yamanaka et al., 2003). Nonetheless, some studies using antibodies raised against the c-terminal have failed to report nuclear staining (Topp et al., 2004). However, when exogenous alsin tagged with eGFP is overexpressed by cells in vitro, it localises to the nucleus as well as the cytoplasm of cells (Topp et al., 2004). Importantly, when truncated sections of eGFP-alsin protein are expressed by cells in vitro, those which contain the RCC1 domain localise to the nucleus and those without it do not (Topp et al., 2004).

Overall, it is possible that antibodies raised against the N-terminal and middle portions of alsin protein may not detect nuclear alsin because the antigenic portions of the protein could be interacting with nuclear structures via the RCC1 like domains. The antibody used in this study may avoid this antigen masking by binding with a part of the protein which does not interact with nuclear structures. Similarly, the lack of cytoplasmic alsin immunoreactivty, found using the antibody in the current study, may be explained by antigen masking. The antibody was raised against a sequence within the c-terminal of alsin protein. The cellular functions mediated by alsin in the cytoplasm depend on domains within its c-teminus. In order to carrying out these functions the target amino

acids may be engaged with other proteins via its antigenic epitopes in its c-terminus, thus the antibody may be prevented from binding to cytoplasmic alsin.

Axotomy produces transient clearing of nuclear alsin immunoreactivity. It is uncertain whether this is the result of down regulation, degradation or redistribution of alsin protein. Quantitative RT-PCR and western blotting with subcellular fractionation for alsin would help elucidate the mechanism of nuclear clearing. It is interesting to note that RCC1 leaves the nucleus during cellular stress (Kelley and Paschal, 2007). The cellular stress caused by axotomy may release alsin from its putative RLD-mediated nuclear localisation. This would allow it to carry out its known cytoplasmic functions which appear to involve it in axonal regeneration (Hadano et al., 2010, Tudor et al., 2005). Therefore, potentially axotomy induce nuclear clearing may represent the redistribution of alsin from the nucleus to the cytoplasm to promote axonal regeneration.

FUS immunoreactivity in the facial motor nucleus following facial nerve axotomy

The current study describes clearing of FUS immunofluorescence from the cell nucleus of motor neurons after axotomy. Prior to injury, labelling for FUS was nuclear in neurons and glial cells of the facial nucleus. Facial nerve transection resulted in a late and transient clearing of nuclear immunofluorescence. Previous studies have described clearing of FUS immunoreactivity from the nucleus of degenerating motor neurons in cases and animal models of ALS (Huang et al., 2011, Neumann et al., 2009). In addition, FUS has been described as part of stress granules (SG), structures which are involved in managing RNA homeostasis during cellular stress (Li et al., 2013a). It has been previously reported that FUS shuttles from the nucleus to the cytoplasm upon cellular stress induction (Li et al., 2013a). Once in the cytoplasm it rapidly associated with SGs. When the instigated stress resolves, SGs resolve and FUS returns to the nucleus (Li et al., 2013a). Thus, while FUS nuclear clearing occurs following cellular stress has been reported in the past, the current study is the first to described clearing of FUS from the nucleus of motor neurons following peripheral nerve injury.

The redistribution of nuclear FUS may have functional effects in either the cytoplasm or nucleus. Previous studies have demonstrated that activation of

mGluR5 neuron receptors results in the recruitment of FUS to dendrites where it mediates a rise in local RNA content (Fujii et al., 2005). FUS appears to have functional effects in the dendrite, since neurons lacking FUS demonstrate abnormal dendritic spine morphology and density, in vitro (Fujii et al., 2005). In addition, FUS has roles in nucleus, including mRNA splicing, RNA stability, and transcriptional regulation (Lagier-Tourenne et al., 2012). Depletion of FUS in vivo has been associated with increase Jun and CD44 expression (Lagier-Tourenne et al., 2012), which are both genes important in nerve regeneration. Thus, nuclear clearing of FUS protein in the nucleus following peripheral nerve axotomy may mediate processed involved in axonal regeneration.

Effect of injury severity on axotomy induced changes in TDP-43 and alsin immunoreactivity

The current study demonstrates that severity of nerve injury determines the magnitude of axotomy induced changes in TDP-43 and alsin immunoreactivity in motor neurons. Facial nerve transection resulted in more pronounced changes in immunoreactivity for both proteins compared to nerve crush. These observations are consistent with previous studies which have shown that the neuronal response to axotomy dependens on the severity of nerve injury (Moran et al., 2001, Matteoli et al., 1986, Lieberman, 1971, Saika et al., 1991a, Saika et al., 1991b, Saika et al., 1993, Makwana et al., 2010). Nerve cut is more severe than crush because as well as severing the perineurum and axons; it also injures the epineurium of a nerve. Compared to crush, nerve transection is associated with longer and more robust electrophysiological, morphological and molecular changes in the injured neuronal cell body (Moran et al., 2001, Matteoli et al., 1986, Lieberman, 1971, Saika et al., 1991a, Saika et al., 1991b, Saika et al., 1993, Makwana et al., 2010). However, reinnervation is known to occur earlier, more promptly, and with less error after nerve crush than after cut (Nguyen et al., 2002, Witzel et al., 2005). Thus the greater changes in TDP-43 and alsin immunoreactivity observed following nerve transection, compared to crush, may be explained by greater injury signals from the initial injury and by delayed trophic support from reinnervation of targets.

Nuclear clearing of ALS associated proteins

Histopathological studies of ALS cases describe the clearing of normal nuclear TDP-43 or FUS immunoreactivity in affected motor neurons (Neumann et al., 2006, Neumann et al., 2009). The nuclear clearing of TDP-43 and FUS described in the current study mirror the changes that occur in motor neurons affected by ALS. This suggests that nuclear clearing of these proteins in ALS could be a normal physiological response to disease and not necessarily a change that drives it. Nuclear clearing of TDP-43 or FUS is often accompanied by cytoplasmic inclusions of either protein within ALS affected motor neurons (Neumann et al., 2006, Neumann et al., 2009). In the current study, FUS or TDP-43 cytoplasmic inclusions were not identified within axotomised motor neurons. This suggests that nuclear clearing of TDP-43 or FUS is not sufficient to drive the formation of cytoplasmic inclusions in normal motor neurons.

Nuclear-cytoplasmic shuttling of TDP-43 and FUS has also been described during RNA-protein (RNP) formation (Li et al., 2013a). During cellular stress, both proteins leave the nucleus bind to non-translating mRNAs forming SGs. This process is physiological and reversible but has been implicated in the formation of cytoplasmic inclusions in ALS (Li et al., 2013a). Although not previously described, it is possible that the cellular stress caused by axotomy may result in the formation of SGs within motor neurons. However, the current study did not look for TDP-43 or FUS positive SGs. Future work should aim to determine whether axotomy induces SG formation and whether TDP-43 and FUS located to these.

Timing of changes and how they marry with what happens in facial nerve axotomy

Axotomy induced the clearing of alsin, TDP-43 and FUS immunostaining from the nucleus of motor neurons. All of these proteins have cytoplasmic functions which may be involved in driving the regenerative process following axonal injury (Hadjebi et al., 2008, Li et al., 2013a). TDP-43 nuclear clearing was associated with increased cytoplasmic staining. However, increase in cytoplasmic staining was not observed after alsin and FUS nuclear clearing. Nonetheless, this does not mean that alsin and FUS could not have been

redistributed to cytoplasm after axotomy. This is because conformational changes and protein binding on nuclear-cytoplasmic shuttling can hide target epitopes which would be normally recognised by the antibodies used (Ramos-Vara, 2005). Thus, it is possible that alsin, TDP-43 and FUS nuclear clearing after axotomy is associated with the redistribution of all those proteins to the cytoplasm.

Cytoplasmic redistribution following axotomy may promote alsin, TDP-43 and FUS to carry out the functions which may be important in axonal regeneration. Interestingly, the timing of nuclear clearing matches well the known function of each of the proteins. Alsin nuclear clearing is earliest, beginning at day 4 after injury. Its known function in retrograde signalling (Tudor et al., 2005, Hadano et al., 2010), and the importance of this process in the early stages following axonal injury (Patodia and Raivich, 2012a), may make its redistribution to the cytoplasm at this time-point appropriate. TDP-43 nuclear clearing and cytoplasmic redistribution begins at day 7. At this time-point the process of axonal elongation and sprouting takes central stage (Makwana et al., 2010). TDP-43 has been implicated previously in these processes (Fallini et al., 2012, Liu-Yesucevitz et al., 2011, Tripathi et al., 2014). Finally, FUS clearing begins at day 14, a time point which the process of reinnervation and NMJ maturation begins (Makwana et al., 2010). The known function of FUS in synapse stabilisation make its putative redistribution to the cytoplasm at this time point appropriate (Fujii et al., 2005). These hypotheses regarding the potential functional roles of alsin, TDP-43 and FUS could be tested using the facial nerve axotomy on mutant mice which lack or overexpress the respective genes.

Limitations

In the current study, the changes of protein expression after facial nerve injury of three candidate proteins, TDP-43, alsin and FUS, was chosen for analysis because of their known biological functions and their relation to motor neuron disease. However, the expression of other proteins was not analysed in the current study. Thus it is possible that the changes observed in TDP-43, alsin and FUS protein expression may be stereotypic to all protein. However, previous studies have demonstrated that protein expression changes in a

variety of ways depending on the protein analysed (Patodia and Raivich, 2012a, Patodia and Raivich, 2012b, Moran and Graeber, 2004). In addition, in Chapter 3 the expression of c-Jun, CD44, alpha7, Beta1 and galanin proteins are described as raised in the motor neurons of facial nucleus 14 days following facial nerve transection. Time course of the expression of these proteins and others following facial nerve axotomy have been previously published (Raivich et al., 1992, Raivich et al., 1995, Moller et al., 1996, Jones et al., 1997, Werner et al., 1998, Bohatschek et al., 1999, Jones et al., 2000, Werner et al., 2000, Vogelezang et al., 2001, Raivich et al., 2004). Nonetheless, expression of such proteins should be analysed in the tissues collected for the current study to ensure that the findings described for TDP-43, alsin and FUS are not stereotypic for all proteins.

In this study, commercially available antibodies were used to detect TDP-43, alsin and FUS. The antibodies for TDP-43 and FUS have been used in previous published reports (Moisse et al., 2009b, Neumann et al., 2009). In addition, the immunoreactivity pattern observed for TDP-43 and FUS in the current study match previous descriptions. However, the specificity of the antibodies used in the current study needs to be validated using western blotting and immunohistochemistry in positive and negative control tissue (such as normal and knock CNS tissue). This is particularly important for to confirm the observations made regarding alsin immmunoreactivity. This is because the antibody for alsin, has never been used in a published study, and the subcellular localisation of alsin immunoreactivity found with this antibody has never been described in the past.

The description of changes in immunoreactivity in the current study has only focused on motor neurons. However, previous post mortem studies of ALS cases have demonstrated similar changes to FUS and TDP-43 immunoreactivity in glial cells as those observed in motor neurons (Neumann et al., 2009, Neumann et al., 2006). The current study did not examine patterns of immunoreactivity in glial cells. Future work could make use of double immunolabelling for glia and TDP-43, alsin or FUS to determine if changes in immunoreactivity for this ALS associated occur in glial cells after axotomy.

In the current study, the changes observed in immunoreactivity for TDP-43 were quantified using the MEAN-SD algorithm (Moller et al., 1996). The changes in nuclear immunoreactivity of alsin and FUS were measured using a modified version of the algorithm, which quantified the percentage of nuclei with immunoreactivity above a certain threshold. The MEAN-SD algorithm has been used to measure changes in immunoreactivity in a number of published reports (Moller et al., 1996, Ruff et al., 2012, Raivich et al., 2004, Makwana et al., 2009). However, the accuracy of this method in measuring changes in protein expression depends on the quality and specificity of immunohistochemical labelling, which can vary from slide to slide (Moller et al., 1996). Western blotting is a more robust method of determining changes in protein expression, which could be used to validate the results in the current study. This method coupled with subcellular fractionation may provide a more definitive description to the observations made here. In addition, changes in mRNA levels of TDP-43, alsin and FUS following axotomy should be measured using RT-PCR, to determine whether changes in mRNA mirror those at the protein level.

Conclusion

The current study has demonstrated that peripheral nerve axotomy results in the clearing of TDP-43 and FUS from the nucleus of injured motor neurons. In addition, after axotomy TDP-43 immunoreactivity is increased to the cytoplasm and localises to proregenerative perineuronal sprouts. Although the observation of nuclear clearing for TDP-43 and FUS resemble those in ALS affected motor neurons, the effects of axotomy on the proteins are reversible and physiological. In addition, this study describes for the first time alsin to be subcellularly localised to the nucleus of motor neurons. Similar to FUS and TDP-43, alsin is also cleared from nucleus following axotomy. Finally, the magnitude of axotomy mediated effects on TDP-43 and alsin appear to be dependent on the severity of nerve injury. Overall this study suggests that TDP-43, FUS and alsin may have a functional role in the neuronal response to axotomy.

Chapter 5: The effect of ALS related mutant TDP-43 on peripheral nerve regeneration

Introduction

Epidemiological studies have linked trauma resulting in injury to the nervous system with the development of ALS (Chen et al., 2007). Injury to the central and nervous system of $SOD1^{G93A}$ mice results in a defective cellular and molecular response (Malaspina et al., 2010, Yip and Malaspina, 2012, Mesnard et al., 2011a, Haulcomb et al., 2014, Mesnard-Hoaglin et al., 2014). However, the response to peripheral nerve injury in TDP-43 mutant mice has not been assessed.

Peripheral nerve injury induces a number of molecular and cellular changes within the injured neuron and neighbouring cells (Navarro et al., 2007). At the cellular level, motor neurons undergo chromatolysis in the cell body and mount perineuronal axonal sprouting (Makwana et al., 2010). In addition, astrocytes and microglia become activated and there is T-lymphocytes infiltration around axotomised neurons (Raivich et al., 1999). At the molecular level, there are dramatic changes in gene expression, orchestrated by transcription factors, such as c-Jun (Patodia and Raivich, 2012b). There is increased expression of regeneration related proteins such as adhesion molecules (CD44) and neuropeptides (CGRP and galanin) (Patodia and Raivich, 2012a). These changes normally culminate on axonal regeneration, target reinnervation and functional recovery in mice (Raivich et al., 2004). However, following axotomy a number of injured neurons die, most likely due to failed target reinnervation and loss of neurotrophic support (Sendtner et al., 1996).

TDP-43 is a ubiquitously expressed 414-amino acid protein with DNA, RNA, and protein binding motifs (Buratti and Baralle, 2001). TDP-43 is involved in gene transcription, mRNA transport and translation (Buratti and Baralle, 2008, Fallini et al., 2012). It is a major component of the proteinaceous inclusions within motor neurons which are characteristic of ALS (Neumann et al., 2006). Mutations in TDP-43 have been found in families suffering from inherited ALS and in sporadic cases of the disease (Pesiridis et al., 2009, Sreedharan et al., 2008). Transgenic mice overexpressing human TDP-43 carrying ALS

associated mutations have been used to model the disease (Lee et al., 2012). One of these models is the A315T TDP-43 mutant mouse which demonstrates ALS-like changes including axonal degeneration and motor neuron loss (Wegorzewska et al., 2009).As described in the previous chapter, the normally nuclear immunoreactivity of TDP-43 protein changes after peripheral nerve injury (Moisse et al., 2009b), suggesting a possible functional role in the response to axonal injury. To begin to elucidate such role, the current study investigates the effect of A315T mutant TDP-43 overexpression on the normal physiological response to peripheral nerve injury.

The following chapter on reports the motor function assessment of whisker movements and histological and immunohistochemical analysis of the facial nucleus following facial nerve transection in A315T mutant TDP-43 overexpressing mice and their littermate wild-type controls.

Results

Mutant TDP-43 reduces functional recovery after facial nerve transection without affecting target reinnervation

Facial nerve transection in mice results in a transient paralysis of the muscles which control whisker movement. Functional recovery is gradual following injury, by four weeks, whisker movement is back to almost normal levels in wild type mice (Raivich et al., 2004).

To find out whether A315T mutant TDP-43 affects the normal recovery of function observed following peripheral nerve injury in mice, transgenic TDP-43 mice were used. These expressed a human TDP-43 construct containing the A315T mutation, seen in patients with familial ALS, under the control of the mouse prion protein promoter (Wegorzewska et al., 2009).

TDP-43 mutant mice (n=5) and their non-transgenic wild-type littermates (n=8) underwent a unilateral facial nerve transection (Moran and Graeber, 2004). Whisker hair motor performance on the ipsilateral side to injury was scored from 0 (no movement) to 3 (strong, normal movement as on the uninjured side) by two observers, unaware of the genotypes, from 7 to 28 days after injury (Raivich et al., 2004).

Recovery of whisker function occurred in both mutant (Fig. 1A- red bars) and non-transgenic control mice (Fig. 1A- blue bars). Motor scores increased gradually over time reaching a measured peak at day 28 in both groups. However, the level of functional recovery was similar for TDP-43 mutant mice and their non-transgenic littermate controls up to day 11 (Mean score ± SEM: 0.5 ± 0.1 for both groups). By day 14, functional recovery in the TDP-43 mutant mice began to lag behind; with average scores for control mice being 0.8 ± 0.1 compared to 0.6 ± 0.1 for mutants ($p<5\%$ in an unpaired Student T-test). This lag continued until day 25 were the difference between motor scores for controls (2.0 ± 0.2) and mutants (1.2 ± 0.3) was greatest ($p<5\%$ using unpaired Student T-test). By day 28, there was only a trend for poorer motor function in the TDP-43 mutant mice (control: 2.2 ± 0.1 vs mutant: 1.4 ± 0.3, $p=7\%$ in an unpaired Student T-test).

To obtain an average motor score independent of time, a functional recovery index (FRI) for each animal was determined by calculating the area under the curve for motor scores of mutant and control mice at 7 days to 28 days after facial nerve injury. Overall, control animals showed an average motor score of 1.8 ± 0.1, which was significantly less than 1.2 ± 0.2 motor score in TDP-43 mutant mice ($p <5\%$ using unpaired Student T-test).

To determine if the defective whisker movement in TDP-43 mutant mice was due to differences in target reinnervation, the fluorescent tracer Fluorogold (FG) was applied into the injured and uninjured whisker pads 28 days after facial nerve injury (Raivich et al., 2004). The retrograde tracer was allowed to act for 2 days and coronal sections through the facial nucleus were then collected. FG labelled neurons in the control and axotomised facial nuclei were counted using fluorescent microscopy at low power using a x20 objective lens.

There was no significant difference between the total number of FG labelled motor neurons in the uninjured facial nucleus of mutant TDP-43 (154 ± 25, n=5) and control mice (173 ± 12, n=8). The overall ratio of labelled neurons on the axotomised versus the contralateral side was calculated (Fig. 1C). TDP-43 mutant mice had 22% ± 8% motor neurons labelled which was not significantly

different to 18% ± 5% in control mice. This showed that there was no difference in target reinnervation between the studied groups at day 28.

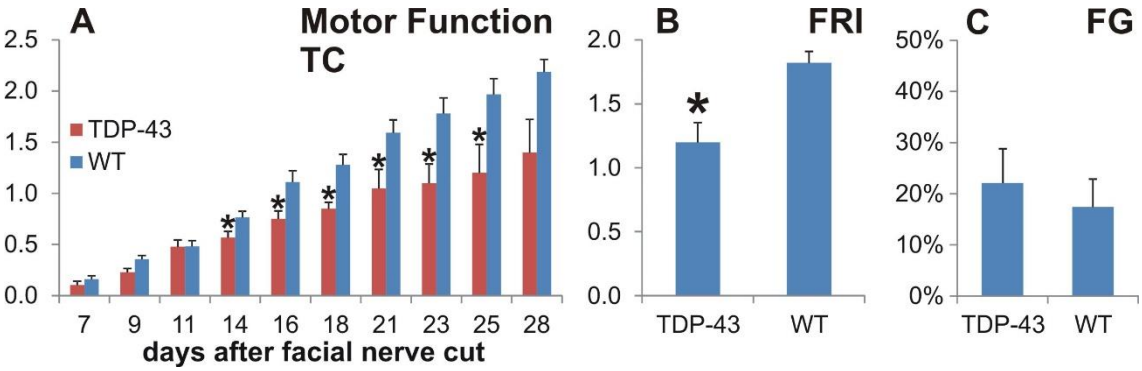

Figure 1. Overexpression of ALS associated A315T mutant TDP-43 delays recovery of motor function but does not affect target reinnervation following facial nerve axotomy. (A) Time course of whisker hair motor function. Functional recovery was measured at the indicated time points after axotomy, on a scale of 0 (no movement) to 3 (normal, strong movement as on the unoperated side); n=5 for A315T mutant TDP-43 mice and n=8 for WT littermate control mice. *p<5% between TDP-43 and WT groups using unpaired Student's T-test. (B) Functional recovery index (FRI), calculated by integrating the motor function score of each studied time point across the time course (A). *p=1.1% using unpaired Student's T-test. (C) Quantification of retrograde labelling of facial motor neurons with fluorogold, performed 28 days after facial nerve cut. The overall ratio of labelled neurons in the operated/unoperated side is shown (n=5 for TDP-43 and n=8 WT).

ALS mutant TDP-43 does not affect axotomy induced cell death

Facial nerve transection in mice is associated with the loss of a proportion of motor neurons in the facial nucleus (Raivich et al., 2004). ALS is also characterised by motor neuron death (Wegorzewska et al., 2009) and mutant TDP-43 has been shown to be neurotoxic in vitro (Suzuki et al., 2011).

To characterise the effect of ALS mutant TDP-43 on axotomy induced neuronal cell death, coronal sections through the facial nuclei were obtained 30 days following facial nerve cut. Serial section underwent cresyl violet staining and subsequent motor neuron quantification by light microscopy using a x20 objective.

The number of motor neurons in the uninjured facial nucleus of TDP-43 mutants was 1167 ± 27 (n=5), which was not significantly different from 1175 ± 41(n=8) motor neurons counted in the wild-type mice (Fig. 2A-blue bars). 30 days following facial nerve injury the number of neurons within the facial motor nucleus ipsilateral to the side of axotomy was significantly lower than the control side in both the mutant and the control groups (Fig. 2A-red bars). To determine the overall level of neuronal cell death, a ratio between motor neuron counts in axotomised versus control side was calculated (Fig. 2B). TDP-43 mutant mice had 42% ± 4% neuronal death, which was not significantly different when compared to 38% ± 5% neuronal death found in their wild-type littermate controls (Fig. 2B) (p>5% using unpaired Student's T-test).

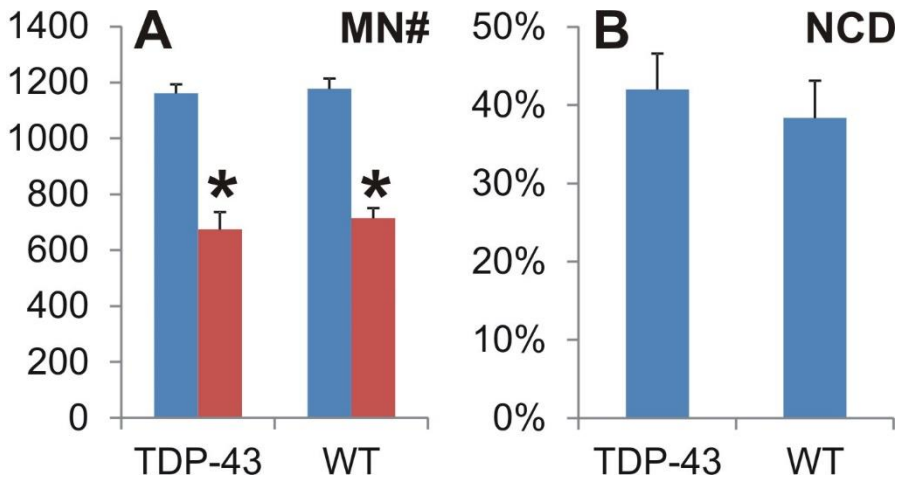

Figure 2. Overexpression of ALS associated mutant TDP-43 does not result in motor neuron loss in the injured facial nucleus and does not affect axotomy induced motor neuron cell death. (A) Quantification of motor neuron number in the facial nucleus in mutant (TDP-43; n=5) and wild-type littermate (WT; n=8) mice on the control (blue bars) and operated side (red bars, 30 days after facial nerve cut (*p<5% between control and operated side using unpaired Student's T-test) (B) Motor neuron cell death (NCD) in the facial nucleus of TDP-43 (n=5) and WT mice (n=8), calculated as the overall ratio of motor neurons in the operated/unoperated side, 30 days after facial nerve cut.

Mutant TDP-43 promotes microglia activation and phagocytosis following facial nerve axotomy

Facial nerve transection results in a glial and inflammatory response in the injured facial nucleus. Perineuronal microglia and astrocytes become activated,

and there is formation of microglia phagocytic clusters as well as infiltration of T-lymphocytes (Raivich et al., 1999). Neuroinflammation is also observed around degenerating motor neurons in ALS (Alexianu et al., 2001).

To determine the effect of ALS related mutant TDP-43 overexpression on axotomy induced neuroinflammation and astogliosis, TDP-43 mutant mice (n=5) and their littermate wild-type controls (n=5) received facial nerve transections. Coronal sections through the facial nuclei were taken 14 days after injury and underwent immunohistochemistry to visualise microglia in early (αM) and late stage (B7.2) activation, activated astrocytes (GFAP), and T-lymphocytes (CD3).

Facial nerve transection induced microglial activation in the injured facial motor nucleus of both control (Fig.3B) and mutant mice (Fig.3D), which was characterised by greater αM staining. Quantification of αM immunohistochemical staining in the injured or uninjured facial nuclei were carried out using the MEAN-SD algorithm (Moller et al., 1996). The level of αM immunoreactivity in the uninjured facial motor nuclei of TDP-43 and wild-type mice was not significantly different (Fig. 4A-blue bars: $p>5\%$ using unpaired Student T-test). However, αM immunoreactivity was 18% greater in the injured facial motor nucleus of TDP-43 mice compared to control mice, 14 days after facial nerve injury (Fig. 4A-red bars: $p <5\%$ using unpaired Student T-test).

αM-positive microglial clusters, defined as 3 or more tightly aggregated phagocytes, were counted in the injured or uninjured facial motor nuclei. Clusters were almost absent in the uninjured nuclei of both TDP-43 mutants and their littermate controls (Fig. 4B-blue bars). However, 14 days following facial nerve transection, αM-positive microglial clusters were observed in the injured facial nuclei of mutant and control animals. TDP-43 mice had 19 ± 2 αM-positive microglial clusters in the injured facial nucleus, which was significantly more than 12 ± 3 found in controls (Fig. 4B-red bars: $p <5\%$ using unpaired Student T-test).

Phagocytic cluster formation was further evaluated by staining for the phagocytosis related protein B7.2 that is expressed by microglia in middle and late stages of activation (Raivich et al., 1999). There were no B7.2-positive

clusters in the uninjured facial motor nucleus of both mutant (Fig.3G) and wild-type mice (Fig.3E; Fig. 4C-blue bars). However, B7.2-positive clusters were found in the injured facial nuclei of both TDP-43 (Fig.3H) and control mice (Fig.3F), 14 days after facial nerve transection. In keeping with the αM results above, the number of B7.2-positive clusters found in the injured facial nucleus of TDP-43 overexpressing mice (10 ± 1) was 2-fold greater than those found in their littermate control mice (5 ± 1; Fig. 4C-red bars: $p < 5\%$ using unpaired Student T-test).

Astrocyte activation, characterised by GFAP-immunoreactivity, was apparent in the injured facial nucleus of wild-type (Fig. 3J) and mutant mice (Fig. 3L), 14 days after facial nerve injury. Quantification of GFAP immunolabelling, using optical luminosity values, demonstrated that GFAP immunoreactivity in the injured facial nucleus was similar for both control and mutant mice (Fig. 4D-red bars; $p > 5\%$ using unpaired Student T-test). However, there was 11% greater GFAP-immunolabelling in the uninjured motor nucleus of TDP-43 mutant mice compared to control mice (Fig. 4D-blue bars; $p < 5\%$ using unpaired Student T-test).

Finally, axotomy induced the influx of CD3-positive T-lymphocytes into the injured facial motor nucleus of TDP-43 (Fig. 3P) and control mice (Fig. 3N). Quantification of CD3-positive T-lymphocytes in the injured facial motor nucleus, demonstrated similar numbers of lymphocytes recruited in both groups (Fig. 4E-red bars; $p > 5\%$ using unpaired Student T-test). Interestingly, TDP-43 mutant mice (Fig. 3O) had more T-lymphocytes in the uninjured facial motor nucleus than control mice (Fig. 3M). However, this difference was minimal with only 1 ± 1 T-lymphocyte being present in mutant TDP-43 uninjured facial nuclei compared no none in wild-type littermate mice (Fig. 4E-blue bars; $p < 5\%$ using unpaired Student T-test).

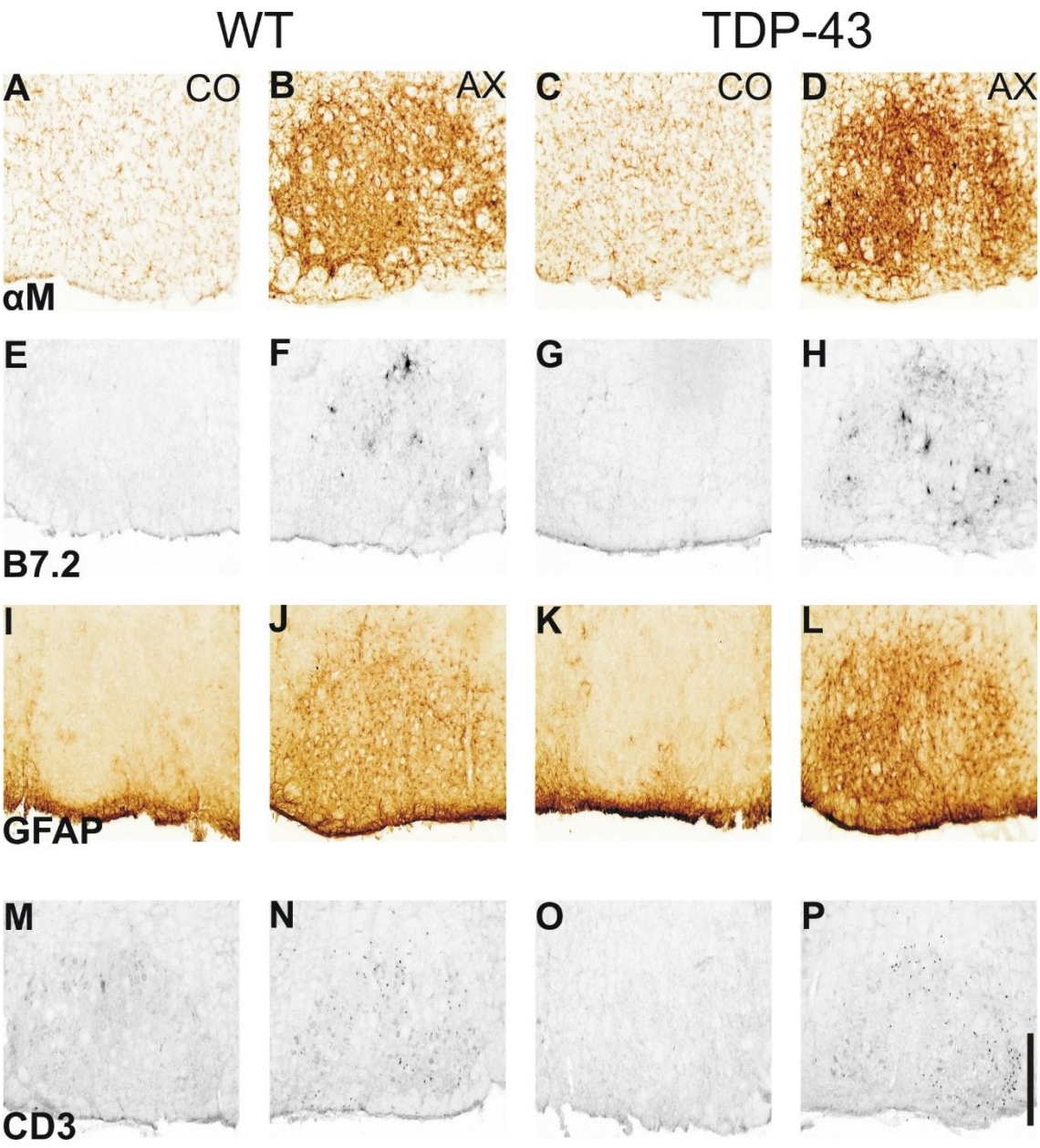

Figure 3. Overexpression of mutant TDP-43 increases microglial response to peripheral nerve axotomy and causes minor neuroinflammatory changes around uninjured motor neurons. Coronal sections through the uninjured (Co) and injured (Ax) facial nuclei of mutant (TDP-43) and wild type littermates (WT), 14 days after facial nerve cut, immunohistochemically stained for: (A-D) early microglial activation marker αM, (E-H) phagocyte marker B7.2, (I-L) activated astrocyte marker GFAP, and (M-P) T-lymphocyte maker CD3. Scale bar represents 400µm.

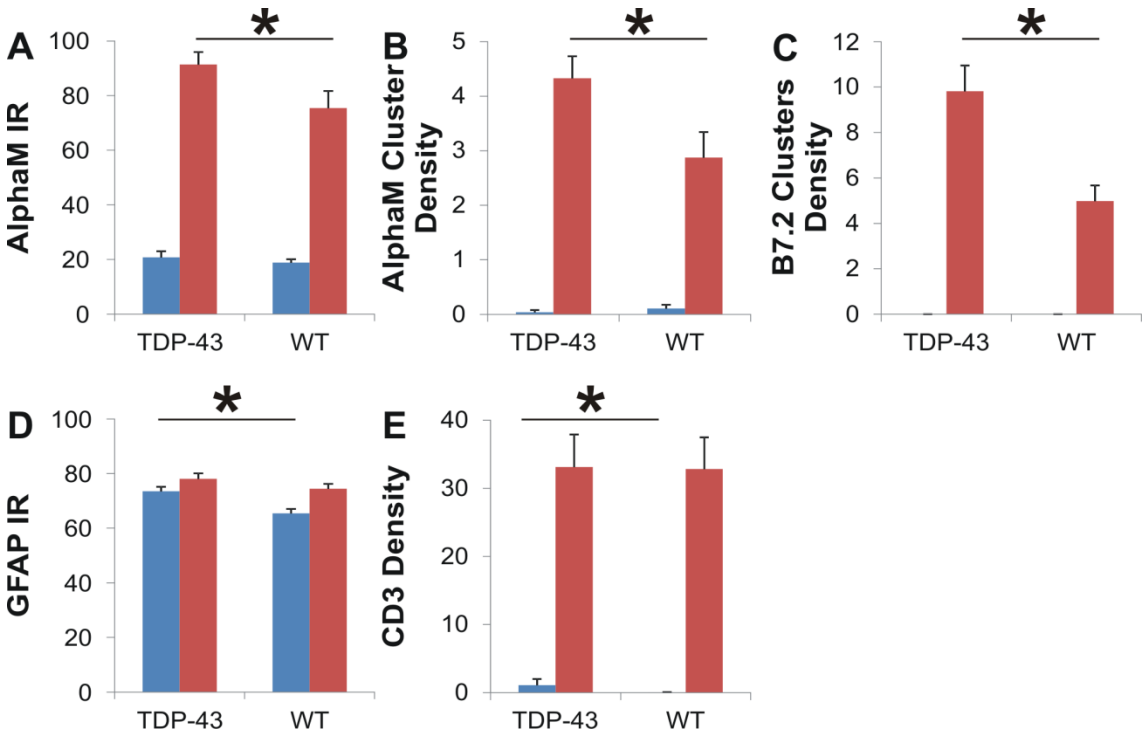

Figure 4. Quantification of non-neuronal response to axotomy in mutant TDP-43 and wild-type mice. Histograms representing: immunoreactivity (IR) for αM (A) and GFAP (D) quantified using the MEAN-SD algorithm (Moller et al., Glia, 1996); the number of αM- (B) and B7.2- (C) positive microglial clusters and CD3-positve lymphocytes (E). Blue and red bars represent quantifications of uninjured and axotomised facial nucleus, respectively. *p<5% using unpaired Student's T-test between mutant (TDP-43) and wild-type littermates (WT) groups. n=5 for both groups (A-E).

Mutant TDP-43 overexpression promotes motor neuronal CD44-expression but supresses perineuronal sprouting after facial nerve transection

Following axonal injury, neurons of the peripheral nervous system upregulate regeneration associated molecules such as c-Jun, CD44, Galanin and CGRP (Raivich et al., 2004). Axonal injury also induces neurite sprouting in close proximity to the injured neuronal cell bodies (Makwana et al., 2010). In addition, as demonstrated in Chapter 5, TDP-43 protein clears from the nucleus of neurons following peripheral nerve injury and can be observed within perineuronal sprouts. To investigate whether over expression TDP-43 affects the neuronal response to injury, coronal section through the facial motor nucleus of mutant TDP-43 (n=5) and wild-type mice (n=5), with unilateral facial nerve injury, were stained for, TDP-43 (Fig.5A-D), c-Jun (Fig.5E-H), CD44 (Fig.5I-L), Galanin (Fig.5M-P) or CGRP (Fig.5Q-T).

Mice overexpressing ALS associated A315T TDP-43 transgene demonstrated stronger TDP-43 immunohistochemical labelling in both injured and uninjured facial nuclei compared to their wild-type littermate mice (Fig.5A-D). Quantification of nuclear TDP-43 immunoreactivity (NIR) in motor neurons, using the modified MEAN-SD method described in chapter 5, demonstrated 25% greater level of TDP-43 immunohistochemical labelling in TDP-43 transgenic mice compared to wild-type littermate controls (Fig.6A). At the cellular level, uninjured neurons demonstrated mainly nuclear TDP-43 immunoreactivity with some cytoplasmic staining. Fourteen days after facial nerve transection there was evidence of some clearing of nuclear TDP-43 immunoreactivity from axotomised neurons in both TDP-43 mutant and wild-type littermate mice (Fig.5A-D). Quantification demonstrated a 50% drop in the level of nuclear TDP-43 immunoreactivity in the injured (Fig.6A-red bars) compared to the uninjured facial nucleus of both mutant and wild-type mice (Fig.6A-blue bars).

Immunohistochemistry for c-Jun showed staining of neuronal nuclei in the facial motor pool of both mutant TDP-43 (Fig.5G&H) and wild-type mice (Fig.5E&F). Fourteen days following facial nerve transection, there was an increase of c-Jun nuclear immunolabelling in injured neurons of both mutant TDP-43 (Fig.5H) and wild-type mice (Fig.5F). Quantification showed that TDP-43 mutant mice had a trend for 15% greater nuclear c-Jun immunoreactivity in the injured facial nucleus compared to wild-type controls (Fig. 6B-red bars; p=6% using unpaired Student T-test). However, there was no significant difference in the level of c-Jun nuclear immunoreactivity in the uninjured facial nuclei of mutant (Fig.5E) and wild-type (Fig.5F & 6A-blue bars; p>5% using unpaired Student T-test).

Immunoreactivity for the adhesion molecule CD44 demonstrated labelling of the perinuclear white matter, but no staining in the uninjured facial nuclei of both TDP-43 mutant (Fig.5I&J) and wild-type mice (Fig.5K&L) (Fig. 6C&D-blue bars; p >5% using unpaired Student T-test). However, CD44 immunolabelling was found in the injured facial nucleus of both studied groups at 14 days after axotomy (Fig.5J&L). Optical luminosity quantifications showed that there was no difference in the levels of CD44 in the injured facial nucleus of mutant and

control mice (Fig. 6C-red bars; p>5% using unpaired Student T-test). However, following manual counts of CD44-positive motor neurons (MN), TDP-43 mice showed significantly more highly CD44-immunoreactive neuronal cell bodies (7 ± 2) in the injured facial nucleus than wild-type mice (3 ± 1) (Fig. 6D-red bars; p<5% using unpaired Student T-test).

Immunohistochemistry for the neuropeptides galanin (Fig.5M&O) and CGRP (Fig.5Q&S) showed staining of motor neuronal cell bodies in the uninjured facial nucleus of both mutant TDP-43 and control mice. Staining for both neuropeptides increased in the injured facial nucleus in both studied groups, 14 days following axotomy (Fig.5N&P; R&T). There was also evidence of de novo appearance of galanin- and CGRP-positive perineuronal sprouts (neurites with a large terminal bulb) in and around the injured facial nucleus in both animal groups. To evaluate the effect of TDP-43 on axonal sprouting, galanin- or CGRP-positive perineuronal sprouts were manually counted. In comparison to control mice (Fig. 5N), which had 50 ± 9 galanin-positive sprouts in the injured facial nucleus, there were significantly fewer sprouts in the facial nucleus of TDP-43 mutant mice (Fig. 5P), with 28 ± 6 sprouts counted in the injured facial nucleus (Fig. 6E-red bars; p <5% using unpaired Student T-test). In addition, a trend towards fewer numbers of CGRP-positive sprouts were found in the injured facial nucleus of TDP-43 mice (Fig.5T) when compared to those counted in the facial nucleus of control mice (Fig.5R) (Fig. 6F-red bars; p= 6% using unpaired Student T-test).

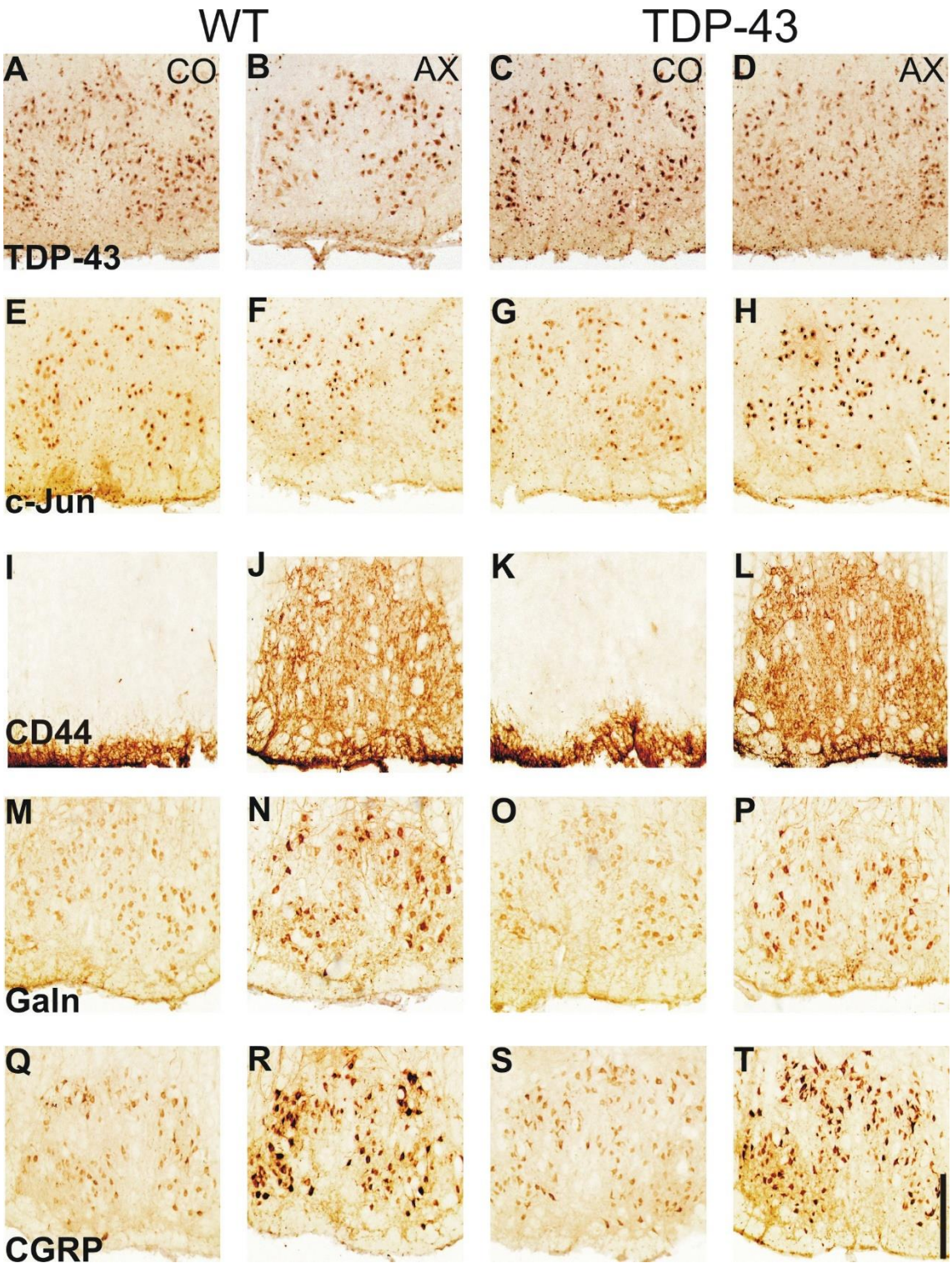
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Figure 5. Overexpression of mutant TDP-43 increases the axotomy induced upregulation of CD44 by injured motor neurons and decreases normal perineuronal sprouting. Coronal sections through the uninjured (Co) and injured (Ax) facial nuclei of mutant (TDP-43) and wild type littermates (WT), 14 days after facial nerve cut, immunohistochemically stained for: (A-D) TDP-43, (E-H) transcription factor c-Jun, (I-L) adhesion molecule CD44, neuropeptides (M-P) galanin (Galn) and (Q-T) CGRP. Scale bar represents 400µm.

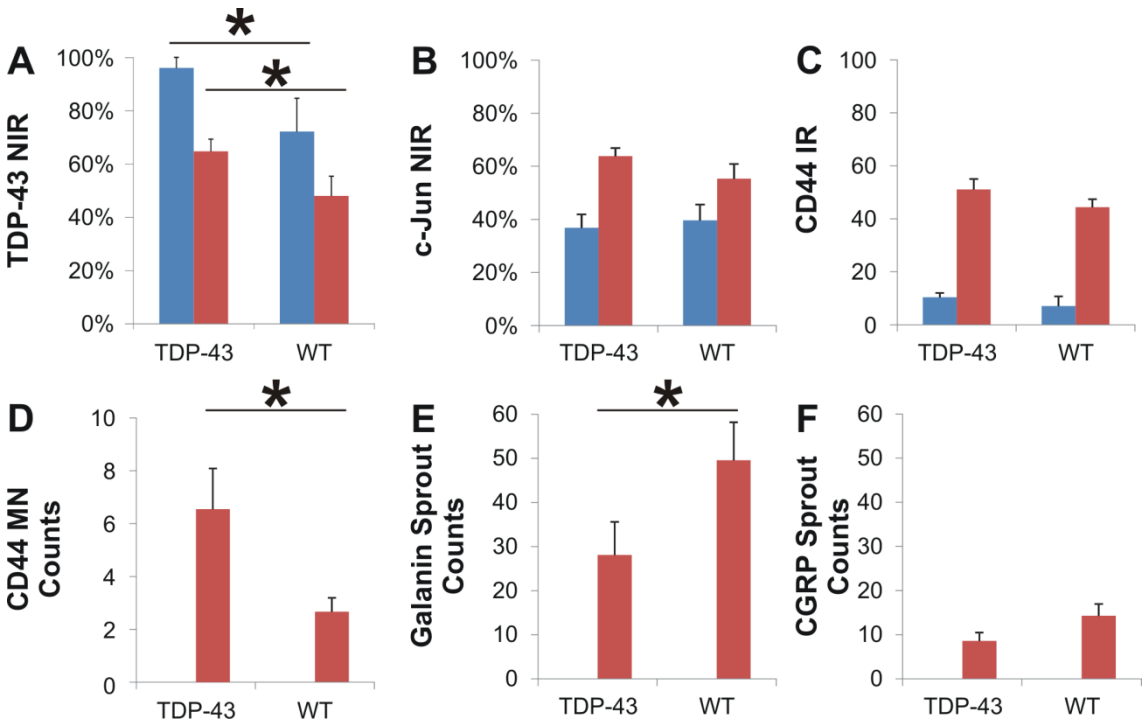

Figure 6. Quantification of motor neuron response to axotomy in mutant TDP-43 and wild-type mice. Histograms representing: nuclear immunoreactivity (NIR) for TDP-43 (A) and c-Jun (B) quantified using a variation of the MEAN-SD algorithm (Moller et al., Glia, 1996), immunoreactivity (IR) for CD44 (C) manual counts of highly immunoreactive CD44 motor neurons (D), galanin- (E) and CGRP-positive (F) sprouts. Blue and red bars represent quantifications of uninjured and axotomised facial nucleus, respectively. *p<5% using unpaired Student's T-test between mutant (TDP-43) and wild-type littermates (WT) groups. n=5 for both groups (A-F).

Discussion

TDP-43 is a major component of the protein inclusions characteristic of ALS. Mutations in the TDP-43 gene have been found to cause some forms of familial ALS. Transgenic mice overexpressing TDP-43 with such mutations develop pathology similar to that seen in the disease. The cellular localisation of TDP-43 protein changes following peripheral nerve injury, suggesting a role in the response to axotomy. Here, it was investigated whether the overexpression of human TDP-43 carrying the ALS associated mutation A315T affected the physiological response to facial nerve axotomy. The current study demonstrates that overexpression of mutant TDP-43 results in a reduced functional recovery that is associated with increased perineuronal inflammation and reduced perineuronal sprouting after facial nerve axotomy.

Overexpression of mutant A315T TDP-43 reduces functional recovery after facial nerve transection

Peripheral nerve injury in mice is normally associated with nerve regeneration, target reinnervation and functional recovery over time (Raivich et al., 2004). Overexpression of mutant A315T TDP-43 delays functional recovery after facial nerve transection. This effect might be secondary to a delay in reinnervation of the whiskerpad, similar to those previously described in α7 integrin knock out mice (Werner et al., 2000). In these mice, target reinnervation was poor at day 9 after facial nerve axotomy but reached normal levels at day 21 (Werner et al., 2000). In this study, levels of target reinnervation were similar in both mutants and controls at day 30 after axotomy. However, retrograde labelling at earlier time points might demonstrate poorer target reinnervation in mutant TDP-43 mice compared to controls.

Motor function also depends on accurate NMJ innervation (Bendella et al., 2011, Zhang et al., 2013, Magill et al., 2007) and appropriate synaptic function (Brown et al., 1981). TDP-43 appears to play important physiological roles in both of these processes (Godena et al., 2011, Wang et al., 2008a, Polymenidou et al., 2011, Medina et al., 2014). In addition, there is electromyographic (Wegorzewska et al., 2009) and anatomical evidence that mutant A315T TDP-43 causes NMJ degeneration in mice (Herdewyn et al., 2014). Overexpression

of mutant A315T TDP-43 may have delayed motor function recovery after facial nerve injury through effects at the NMJ. Future experiments should look at NMJ electrophysiology and anatomical reinnervation after facial nerve injury in TDP-43 mutant and control mice.

The delay in functional recovery may also represent effects of mutant TDP-43 on upper motor neurons which regulate the function of the facial nucleus. Intact A315T mutant TDP-43 mice demonstrate progressive motor dysfunction which appears to be caused more by a disruption of descending motor pathways in the spinal cord rather than by the disturbance of peripheral motor nerves (Esmaeili et al., 2013, Wegorzewska et al., 2009). In A315T mutant TDP-43 mice, there is only around 16% axonal loss in the femoral motor nerve compared to around 30-50% axonal loss in the dorsal and lateral corticospinal tracts (Wegorzewska et al., 2009). After facial nerve transection, there is a molecular and cellular response by the neurons that control the injured facial nucleus (Hundeshagen et al., 2013, Peeva et al., 2006). This response includes the reorganization of synaptic inputs on axotomised facial motor neurons (Hundeshagen et al., 2013, Peeva et al., 2006). A315T mutant TDP-43, which preferentially affect upper motor neurons in mice, might provoke poor functional recovery by affecting this response.

The interpretation of whisker assessment data following facial nerve axotomy is limited in the current study because measures of baseline score of whisker activity were formally taken prior to injury or in the uninjured side after injury. However, whisker activity on the injured side was compared to the uninjured side and a score was given in comparison to that side.

Overexpression of mutant A315T TDP-43 does not affect motor neuron death following facial nerve transection

Peripheral nerve injury is often associated with some axotomy induced neuronal cell death (Sendtner et al., 1996). Overexpression of ALS associated mutant A315T TDP-43 in mice did not produce spontaneous loss of uninjured facial motor neurons and did not affect axotomy induced motor neuron death after facial nerve transection. Animal models of ALS should encompass the features of the disease, which include the loss of lower motor neurons (Van den Bosch,

2011). Previous studies have disagreed in the level of lower motor neuron death in A315T TDP-43 mouse model of ALS (Esmaeili et al., 2013, Wegorzewska et al., 2009, Herdewyn et al., 2014). The initial description of these mice claimed a 20% loss in spinal motor neurons (Wegorzewska et al., 2009). However, later studies did not find evidence of motor neuron loss at the spinal cord (Herdewyn et al., 2014, Esmaeili et al., 2013). There has been no description of motor neuron viability in the intact facial nucleus of these mice. However, as seen in Chapter 3 of this thesis and in previous studies (Haenggeli and Kato, 2002), in the SOD1 mouse model of ALS there is only minor motor neuron loss at the facial nucleus even at the end-stage of the disease, when motor neuron loss in the spinal cord is high. Thus, the absence of spontaneous loss of facial motor neurons in the A315T TDP-43 mutant mice fits well with previous literature and suggests that facial motor neurons have reduced vulnerability to ALS disease in mice (Haenggeli and Kato, 2002).

Overexpression of mutant A315T TDP-43 has been demonstrated to provoke loss of cortical and mesenteric neurons (Esmaeili et al., 2013, Wegorzewska et al., 2009, Herdewyn et al., 2014). In the current study, the prodegenerative effect of mutant TDP-43 overexpression did not exacerbate axotomy induced motor neuron loss. In contrast, previous studies have demonstrated that ALS mutant SOD1 mice have greater motor neuron loss than littermate controls 28 days after facial nerve axotomy (Mariotti et al., 2002, Ikeda et al., 2005, Haulcomb et al., 2014, Mesnard et al., 2011b). Interestingly, it has been shown that motor neuron loss in control wild type mice reaches levels comparable to those in mutant SOD1 mice at day 56 after facial axotomy injury (Haulcomb et al., 2014). Suggesting that mutant SOD1 expedites cell death of motor neurons that were destined to die as a result of axotomy, but it does not make other injured motor neurons more vulnerable to axotomy (Haulcomb et al., 2014). Since determining effects on motor neuron loss depends on the time point studied, it would be interesting to measure motor neuron number at earlier and later time points in mutant TDP-43 mice after facial nerve axotomy.

Overexpression of mutant TDP-43 causes gliosis and T-cell recruitment in the uninjured facial nucleus and increases axotomy induced microglial activation and cluster formation

Axotomy produces a glial and inflammatory response at the level of the injured neuron (Raivich et al., 1999). In this study, mice overexpressing mutant A315T TDP-43 demonstrated spontaneous astrogliosis and T-cell recruitment in their uninjured facial nucleus and had greater levels of axotomy-induced microglial activation and cluster formation than wild-type controls after facial nerve transection.

Astrocyte activation has been previously described in layer 5 of the motor cortex and in the ventral horn of the spinal cord in mice overexpressing mutant A315T TDP-43 (Wegorzewska et al., 2009, Herdewyn et al., 2014). In the current study, the description of spontaneous astrogliosis has been extended to the uninjured facial motor nucleus of these mice. Astrocytes may be central in the pathogenesis of mutant TDP-43 mediated disease (Tong et al., 2013). Astrocyte activation has been described to precede other disease changes in mice overexpressing mutant A315T TDP-43 fragments (Swarup et al., 2011). Expression of mutant TDP-43 by astrocytes is associated with the production of substances that cause neuronal cell death in vitro (Bi et al., 2013, Rojas et al., 2014, Huang et al., 2014). In addition, rats overexpressing human mutant M337V TDP-43 in astrocytes, through an astrocyte specific promoter, develop motor neuron degeneration (Tong et al., 2013).

T-cell recruitment into areas undergoing neurodegeneration has been described in human ALS and the SOD1 mouse model of the disease (Alexianu et al., 2001). However, T-cells recruitment has not been previously studied in mice overexpressing mutant A315T TDP-43. The current study demonstrates modest but significant T-cell infiltration into the uninjured facial nucleus in these mice. In the SOD1 mouse model of ALS, T-cell infiltration is an early event that precedes the onset of motor dysfunction (Chiu et al., 2008). Genetic ablation of T-cells in mutant SOD1 mice results in a delayed onset of disease (Tada et al., 2011) but accelerated disease progression (Chiu et al., 2008). Thus, the presence of T-cell recruitment in the uninjured facial nucleus of mutant A315T TDP-43 mice may be a feature of the early stages of disease in these mice.

Microglia activation accompanies motor neuron degeneration in ALS (Alexianu et al., 2001) and has been described in the motor cortex of mice overexpressing mutant A315T TDP-43 (Wegorzewska et al., 2009). However, it has not yet been assessed in the lower motor neuron pools of these mice. Nonetheless, microglia activation has been described in the ventral horn of mice overexpressing fragments of mutant A315T TDP-43 (Swarup et al., 2011). In the current study, there was no evidence of spontaneous microglia activation in the uninjured facial nucleus of mutant A315T TDP-43 mice, despite the presence of astrogliosis and T-cell recruitment. Microglia activation has been described to occur after astrocyte activation in the spinal cord of ALS mutant SOD1 mice (Fischer et al., 2004). Similarly, increase in microglia number occurs after T-cell recruitment in mutant SOD1 mice (Chiu et al., 2008).The results in the current study do not exclude mutant TDP-43 mediated microglia activation in the uninjured facial nucleus, as this may occur at a later time point, after spontaneous astrogliosis and T-cell recruitment in mutant A315T TDP-43 mice.

Facial nerve axotomy results in the activation of astrocytes and microglia as well as the infiltration of T-cells in the injured facial nucleus (Raivich et al., 1999). The effect of overexpression of mutant A315T TDP-43 in axotomy induced neuroinfammation has not been previously described. In the current study, microglia activation and cluster formation after facial nerve transection was found to be greater in mutant A315T TDP-43 mice than wild-type littermate controls. However, mutant A315T TDP-43 had no effect on post-axotomy astrocyte activation or T-cell recruitment. In contrast, previous studies in ALS mutant SOD1 mice have demonstrated that peripheral nerve axotomy causes less microglia and astrocyte activation, as well as less T-cell recruitment around injured neurons in ALS mutant SOD1 mice than wild type controls (Kawamura et al., 2012, Haulcomb et al., 2014).

TDP-43 has been shown to control the expression of a number of genes involved in the inflammatory response by neurons (Polymenidou et al., 2011). Thus mutant A315T TDP-43 may enhance axotomy-induced microglia activation and cluster formation via affecting the levels of expression of pro-

inflammatory cytokines. This effect may be secondary to a defect in the gene regulatory function of TDP-43 (Polymenidou et al., 2011), or the cellular stress caused by the expression of mutant TDP-43 protein (Aggad et al., 2014). Mutant A315T TDP-43 protein expressed in *C. elegans* motor neurons is susceptible to misfolding, aggregate formation (Vaccaro et al., 2012a), activation of the endoplasmic reticulum unfolded protein response (Vaccaro et al., 2012b), and cellular toxicity by the dysregulation of intracellular calcium (Aggad et al., 2014). This type of cellular stress has been associated with the activation of the immunoproteasome which is involved in the prevention of protein aggregates (Bendotti et al., 2012). The immunoproteasome protects the cell during viral infection by generating antigenic peptides and presenting them to immune cells via cell surface MHCI molecules (Yewdell and Haeryfar, 2005). Interestingly, motor neurons increase the expression of MHCI after axotomy, suggesting that the immunoproteasome may be also activated in this condition (Bendotti et al., 2012). Thus, increased microglia activation and cluster formation in A315T TDP-43 mice may be due to the combined effect of proteasome activation by mutant TDP-43 protein and the axotomy induced stress.

Overexpression of mutant TDP-43 enhances axotomy-induced CD44 expression by motor neuron but diminishes perineuronal sprouting

Axotomy induces a molecular response in the injured cell body which includes the upregulation of transcription factors, neuropeptides and adhesion molecules (Patodia and Raivich, 2012a). In the current study, it was demonstrated that injured and uninjured facial motor neurons in mutant TDP-43 mice have greater levels of TDP-43 immunoreactivity in their cellular nucleus compared to their littermate controls. The mouse model of ALS used in this study overexpresses human A315T mutant TDP-43 under the control of the prion protein promoter (Wegorzewska et al., 2009). TDP-43 protein has been described to be present at levels approximately 3-fold higher than endogenous protein (Wegorzewska et al., 2009). Thus, the greater level of nuclear TDP-43 immunostaining found in the mutant mice in this study is consistent with previous observations.

After axotomy, both TDP-43 mutant and control littermate mice showed a decrease in nuclear TDP-43 immunoreactivity. This is consistent with the data presented in Chapter 5 of this thesis and previous studies (Moisse et al., 2009b) which show clearing of TDP-43 from the nucleus of motor neurons after axonal injury. The level of nuclear TDP-43 clearing was not different between the mutant TDP-43 mice and littermate controls. Demonstrating that overexpression of A315T TDP-43 does not affect the axotomy-induced nuclear clearing of TDP-43 immunoreactivity. Spontaneous nuclear clearing has been described in neurons of the motor cortex of A315T TDP-43 mutant mice (Wegorzewska et al., 2009). However, this observation was uncommon, suggesting that the A315T mutation does not particularly affect the nuclear localisation of TDP-43 as do other mutations in the TDP-43 gene (Cohen et al., 2011).

In this study the level of nuclear c-Jun immunoreactivity in uninjured facial motor neurons was not different between TDP-43 mutant mice and littermate controls. After axotomy, nuclear c-Jun immunoreactivity increased in facial motor neurons of both TDP-43 mutant mice littermate controls. There was a trend for marginally higher levels (15%) of nuclear c-Jun immunoreactivity in TDP-43 mutant mice than controls. There are no previous studies analysing c-Jun expression or phosphorylation in A315T TDP-43 mutant mice. However, a previous study, using the mouse motor neuron like NSC34 cell line, has shown that TDP-43 overexpression leads to neuronal death via the JNK/c-Jun signalling pathway (Suzuki and Matsuoka, 2013). In this model, expression of dominant negative c-Jun resulted in reduced cell death (Suzuki and Matsuoka, 2013). Thus, neurodegeneration caused by mutant A315T TDP-43 overexpression may depend on c-Jun. The lack of effect of mutant TDP-43 overexpression on c-Jun immunoreactivity in this study may be explained by a low level of neurodegeneration in the facial motor neurons at the time point of analysis. Perhaps, as disease progresses and facial motor neurons degenerate a change in c-Jun reactivity may ensue.

In this study, it was found that overexpression of mutant TDP-43 was associated with higher levels of axotomy induced CD44 immunoreactivity. This finding is in contrast to a previous study, which demonstrates that CD44 mRNA

expression increases when TDP-43 is depleted (Polymenidou et al., 2011). In that study, endogenous TDP-43 mRNA and protein was reduced in the mouse striatum using antisense oligonucleotides (Polymenidou et al., 2011). However, here a human mutant form of TDP-43 is overexpressed within injured motor neurons. The increased expression of CD44 may be because mutant TDP-43 was acting in a dominant negative fashion inhibiting the function of endogenous TDP-43 and thus reducing its normally inhibitory effect on CD44 expression. A previous study on A315T TDP-43 transgenic mice, has indeed shown that human mutant TDP-43 overexpression reduces endogenous TDP-43 expression (Herdewyn et al., 2014). Thus, increased immunoreactivity of CD44 after axotomy may be due to the reduction in endogenous TDP-43 function caused by the overexpression of mutant TDP-43. The increased CD44 immunoreactivity in the axotomised motor neurons of A315T TDP-43 transgenic mice may also be explained by the higher levels of inflammation observed in these mice after axotomy. Compared to indirect or mild injury, severe neural injury or inflammation causes more CD44 expression by neurons, astrocytes, T-cells and microglia (Jones et al., 2000, Makwana et al., 2007). Thus, the additional cellular stress caused by mutant TDP-43, and the associated inflammation may promote the expression of CD44 after axotomy.

Peripheral nerve axotomy is associated with neurite sprouting at the level of the injured motor neuron (Makwana et al., 2010). Overexpression of mutant A315T TDP-43 reduced axotomy induced perineuronal sprouting after facial nerve axotomy. TDP-43 appears to be involved in the formation and maintenance of synapses at the level of the neuromuscular junction (Godena et al., 2011) and in the central nervous system (Wang et al., 2008a, Medina et al., 2014). There is accumulating evidence that TDP-43 has important roles in the transport of mRNA along axons and its translation at the synapse (Liu-Yesucevitz et al., 2011, Fallini et al., 2012). In vitro, TDP-43 is actively transported in motor neuron axons in response to BDNF, it interacts with axonal mRNA binding proteins and its overexpression causes less axonal growth and branching (Fallini et al., 2012). In vivo, TDP-43 overexpression results in truncation of chick embryo motor axon growth to peripheral targets and the perturbation of their cytoskeletal components (Tripathi et al., 2014). Similarly, in drosophilia

depletion of the TDP-43 homolog TBPH resulted in structural defects at the neuromuscular junctions and alterations in the organization of synaptic microtubules (Godena et al., 2011). Thus, overexpression of mutant TDP-43 may affect perineuronal sprouting by interfering with axonal mRNA transport and local translation.

Perineuronal sprouting was analysed using immunohistochemistry for the neuropeptides galanin and CGRP. The expression of these peptides are upregulated following axotomy and appear to be under the transcriptional control of c-Jun (Raivich et al., 2004). Since TDP-43 has an important role in gene expression, the reduction in perineuronal sprouting might be secondary to an effect of overexpression of mutant TDP-43 on the transcriptional regulation or translation of these two peptides. However, previous microarray and splicing-sensitive junction array analysis have failed to demonstrate that CGRP or galanin expression is under the regulation of TDP-43 (Polymenidou et al., 2011). In the current study, the overall level of CGRP and galanin immunoreactivity in the facial motor nucleus was not measured. Measuring this may elucidate a difference in peptide levels between the studied groups, and thus may demonstrate that the observed reduction in sprouting is due to sprouts not being visible, due to decreased sprout marker expression, rather than an actual effect of mutant TDP-43 on the sprouting process.

Conclusion

This study has demonstrated that the neuronal overexpression of ALS associated mutant TDP-43 reduces functional recovery, increases perineuronal inflammation and reduces perineuronal axonal sprouting after peripheral nerve injury. It suggests that mutations in TDP-43 associated with ALS may promote defects in the normal physiological regenerative response that motor neurons mount following injury. Such deficits might be part of the progressive degenerative nature of ALS disease.

Chapter 6: General discussion

Summary of Results

The molecular pathways mediating motor neuron degeneration in Amyotrophic lateral sclerosis and axonal regeneration following peripheral nerve injury remain elusive.

The transcription factor c-Jun, an important orchestrator of axonal regeneration, has been found upregulated in the spinal cord of ALS cases and the SOD1G93A mouse model of the disease. However the functional role of c-Jun in ALS degeneration has not been defined. The current thesis demonstrates that genetic deletion of c-Jun from neurons and neuroglia prolonged the lifespan of SOD1G93A mice. Associated with this effect on survival, motor neuron loss, axonal loss and neuroinflammation were attenuated in c-Jun deficient SOD1G93A mice. Interestingly, in addition to the neurodegenerative changes, motor neuron pools affected by ALS disease in SOD1G93A mice demonstrated a disease-mediated axotomy response, which was characterised by the expression of regeneration associated proteins and was dependent on the expression of c-Jun.

Since the expression of regeneration-associated proteins changes in ALS, it was next investigated whether the expression of ALS-associated proteins changes during axonal regeneration. This thesis demonstrates that the pattern of expression of ALS associated proteins; TDP-43, FUS and alsin, does indeed change within motor neurons following facial nerve axotomy, in wild type mice. In addition, it describes for the first time that alsin protein may localise to nucleus in uninjured motor neurons. Interestingly, axotomy resulted in a transient clearing of the normal nuclear immunoreactivity for all three studied proteins. The size of this effect was dependent on the severity of nerve injury for TDP-43 and alsin.

Finally, to begin to investigate the functional role of ALS associated genes in axonal regeneration the A315T TDP-43 model of ALS was assessed following facial nerve transection. Genetic overexpression of ALS associated A315T mutant TDP-43 resulted in delayed motor function recovery following facial nerve axotomy in mice. This effect was associated with increased perineuronal

inflammation and increased motor neuron expression of the adhesion molecule CD44 but reduced perineuronal sprouting. However, overexpression of A315T mutant TDP-43 did not affect anatomical target reinnervation, motor neurons loss or the pattern of expression of other regeneration associate proteins.

The role of c-Jun in ALS

Targeting c-Jun/JNK pathway for the treatment of ALS

The transcription factor c-Jun has been found upregulated in the astrocytes and motor neurons of the spinal cord in ALS cases and mutant SOD1 mice (Vlug et al., 2005, Jaarsma et al., 1996). However, the functional role of c-Jun in ALS degeneration has not been defined. In this thesis, genetic deletion of c-Jun from neurons and glia in SOD1G93A mice produced an increase in lifespan of 10 days compared to controls. This effect may appear modest but it represents an increase of 10% to the lifespan of SOD1G93A mice. The size of this effect on survival seems clinically significant as the only approved medical therapy for ALS, riluzole, prolongs survival by around 2 months in ALS patients which on average have an age of 50 years (increase 0.3% of total lifespan) (Scott et al., 2008). Thus, pharmacological inhibition of c-Jun activation or expression may represent a novel therapeutic avenue to be explored in the treatment of ALS. In this vein, a number of JNK inhibitors have been developed and tested in neurodegenerative disorders such as Parkinson's Disease (Chambers et al., 2011); in fact, an orally active JNK inhibitor (CC-930) has now reached stage II clinical trials for the treatment of interstitial pulmonary fibrosis (Krenitsky et al., 2012). Thus, future experiments may aim to determine whether pharmacological inhibition of JNKs modifies disease in SOD1G93A mice.

Elucidating the mechanism of c-Jun ALS neurodegeneration

The genetic deletion of c-Jun from neurons and glia attenuated a number of neuropathological changes in SOD1G93A mice, demonstrating that c-Jun plays a functional role in ALS associated neurodegeneration. However, the mechanism through which c-Jun does this was not elucidated in this thesis. The transcription factor c-Jun has described roles in cell death, neuroinflammation, and axonal regeneration by controlling the expression of a number of gene targets (Raivich et al., 2004). Determining what effects neural deletion of c-Jun has on gene expression of SOD1G93A mice would help identify the mechanism

through which it promotes neuroprotection in this model. Using laser guided dissection, microarrays analysis of gene expression specifically within motor neurons and in the surrounding neuropil may indicate whether c-Jun deletion affects processes involved in ALS disease, such as proteostasis, mitochondrial function, cell death pathways or cytokine gene expression. In addition, it would be interesting to find out if the neuroprotective effect of c-Jun deletion is secondary to its absence from the degenerating motor neurons or from surrounding glia. Following axotomy, mice with neuronal specific deletion of c-Jun, under the control synapsin promoter, show an identical phenotype as those mice with neural specific deletion c-Jun, under the control of the nestin promoter (Ruff et al., 2012). This suggests that the effects of c-Jun on motor neuron death and neuroinflammation in SOD1G93A mice may be due to the lack of c-Jun in neurons. However, future experiments may aim to determine whether this is in fact the case.

The role of a disease mediated axotomy response in ALS

This thesis describes the presence of a disease mediated axotomy response mounted in degenerating motor neuron pools in SOD1G93A mice. This response was characterised by the expression of regeneration associated proteins and was dependent on the expression of c-Jun. The functional role of this response and the mechanism that triggers it have not been characterised in the current study. Intuitively, a regenerative response mounted by degenerating motor neurons can only represent an attempt at maintaining function and structure. This view is supported by evidence that regenerative terminal sprouting at the NMJ compensates for neurodegenerative muscle denervation in ALS (Tsujihata et al., 1984, Atsumi, 1981, Hegedus et al., 2008, Hegedus et al., 2007). In addition, enhancing this regenerative effort via pharmacological or genetic interventions has been shown to attenuate disease in the SOD1 mouse model of ALS (Tonges et al., 2014, Gunther et al., 2014). In contrast, genetic inhibition of this regenerative effort exacerbates neurodegeneration in mutant SOD1 mice (Williams et al., 2009).

However, this thesis demonstrates that neuronal and glial c-Jun deletion, which in wild-type mice results in a dramatic deficit in axonal regeneration following axotomy (Raivich et al., 2004), did not worsen disease in SOD1G93A mice but

instead attenuated it. In addition, the disease mediated pro-regenerative axotomy response mounted by neurons in SOD1G93A mice was absent if c-Jun was genetically deleted from neurons and neuroglia. This suggests that the axotomy response mounted by motor neurons in mutant SOD1 mice may not necessarily represent a beneficial phenomenon, but may be a part of pro-degenerative process that is slowed down by the genetic deletion of c-Jun. In support of this, it has been reported that sciatic nerve crush, which promotes peripheral nerve regeneration, results in an acceleration in disease progression and enhanced motor neuron loss in SOD1G93A mice (Sharp et al., 2005). However, a different group has demonstrated that sciatic nerve ligation followed by transection improves axonal survival in SOD1 G93A mice (Kong and Xu, 1999). The difference in the results from these two studies has been explained by the fact that the crush injury permits axonal regeneration while nerve ligation prevents it (Sharp et al., 2005). It is suggested that axonal regeneration may play a pro-degenerative role in ALS by increasing the burden on what is already compromised axonal transport. Thus deletion of c-Jun may delay degeneration in SOD1G93A mice by inhibiting the axonal regeneration programme initiated in these mice by the disease.

Alternatively, the failure to exacerbated disease despite attenuating a regenerative effort at the level of the motor neuron cell body in SOD1G93A mice may be interpreted as a disassociation between the regenerative efforts that occur at the neuromuscular junction and those that occur at the cell body. There is evidence that sprouting at NMJ can be mediated by local protein synthesis independent of protein production at the neuronal cell body or axonal transport (Alvarez et al., 2000). Thus, c-Jun deletion may not affect sprouting and reinnervation at NMJ despite the fact that it affects gene expression at the cell body that promotes axonal regeneration following peripheral nerve axotomy. However, in this thesis the effect of c-Jun deletion on NMJ innervation was only studied at end stage of disease when levels of denervation were similar between c-Jun deficient or competent SOD1 mice. NMJ innervation, terminal and collateral sprouting should be studied at earlier time points in the disease to determine whether c-Jun has any effect on this type of regenerative effort.

The pattern of expression of ALS associated proteins changes after peripheral nerve injury

The ALS associated proteins TDP-43, FUS and alsin have previously described functions in the maintenance of neurite structure and growth (Fallini et al., 2012, Liu-Yesucevitz et al., 2011, Tripathi et al., 2014, Fujii et al., 2005, Tudor et al., 2005). This thesis describes the changes in the pattern of expression of these proteins within motor neurons following axonal injury, in vivo. Transient clearing of the normal nuclear immunoreactivity for all of the three studied proteins was observed following axotomy. This suggests that all these proteins may be involved in axonal regeneration following peripheral nerve injury, in vivo.

Interestingly, nuclear clearing of TDP-43 and FUS immunoreactivity has been described in the motor neurons of ALS cases, and has been implicated in the pathogenesis of the disease by aberrantly altering gene expression (Lagier-Tourenne et al., 2012). However, as observed here the transient clearing of these proteins from the nucleus represents a physiological response to injury and this could also be the case in ALS (Deng et al., 2010). Nonetheless, ALS is characterised by the presence of protein inclusions which may sequester TDP-43 and FUS from the nucleus permanently and thus dysregulate gene expression (Lagier-Tourenne et al., 2012). Interestingly, absence of TDP-43 and FUS from the motor neuron nucleus may promote a regenerative gene expression programme, as suggested by this thesis, which as discussed above may promote neurodegeneration in ALS.

In addition, in this thesis, alsin, immunoreactivity, which has been described as a cytoplasmic protein associated with endosomes (Tudor et al., 2005, Otomo et al., 2003, Devon et al., 2005), is for the first time described as nuclear in uninjured motor neurons. Alsin is a large protein with a number of functional motifs, some of which may localise it to the nucleus (Yang et al., 2001). This suggests that, just like TDP-43 and FUS, alsin may have, up to now undescribed, functional roles in the nucleus. The nuclear clearing of alsin following axotomy further suggest that it may play a role in axonal regeneration in vivo. However, the observations made in this thesis need to be coupled with molecular studies which provide evidence that the immunoreactivity observed with the anti-alsin antibody used here is indeed specific for alsin.

Future work should focus in determining whether the proteins studied in this thesis have functional roles in axonal regeneration in vivo. This may be carried out using the facial axotomy model on the available TDP-43, FUS and alsin transgenic and knock out mice. In addition, other ALS associated proteins should be assessed to determine whether they are also involved in the neuronal response to axotomy and axonal regeneration.

ALS associated human mutant TDP-43 disrupts peripheral nerve regeneration in vivo

The change in the pattern of TDP-43 immunoreactivity following axotomy suggests that TDP-43 may play a functional role in the response to peripheral nerve injury. This thesis demonstrates that the overexpression of ALS associated human mutant A315T TDP-43 delayed motor function recovery in mice following facial nerve transection. This effect was associated with enhanced neuroinflammation around axotomised motor neurons and decreased perineuronal sprouting following axotomy. However, the overexpression of mutant TDP-43 did not affect axotomy induced motor neuron loss or the anatomical reinnervation of target muscle. Because TDP-43 has roles in the regulation of gene expression, the effect observed here may be explained by mutant TDP-43 mediated dysregulation of gene expression (Polymenidou et al., 2011, Raivich et al., 2004). In support, the adhesion molecule CD44, a target of TDP-43 (Polymenidou et al., 2011), was found aberrantly upregulated in the cell body of injured motor neurons. However, the expression of other regeneration associated proteins was not dysregulated in injured motor neurons of mutant TDP-43 mice. Thus, it is possible that A315T TDP-43 may not mediate its effects on regeneration by affecting gene expression at the level of the cell body. Since TDP-43 has roles in transport of mRNA along axons and its translation at the synapse (Liu-Yesucevitz et al., 2011, Fallini et al., 2012), it is possible that mutant TDP-43 may affect axonal regeneration through effects on local protein translation. To elucidate the mechanism by which A315T TDP-43 causes delayed functional recovery, the rate of axonal elongation and effects on axonal sprouting at the neuromuscular junction should be assessed. In addition, to the determine the molecular mechanisms, analysis of gene and protein expression may be carried out at the motor neuron cell body and axon terminal of mutant TDP-43 and control mice following facial nerve axotomy.

The defect in axonal regeneration observed in the TDP-43 mouse model of ALS may suggest that there is an underlying defect in axonal regeneration in ALS. However, facial nerve transection in mutant SOD1 mice reveals that the molecular response to axotomy is phenotypically regenerative and indistinguishable from that of wild type mice (Mesnard et al., 2011a). Interestingly, axotomy induced motor neuron death is exacerbated in mutant SOD1 mice (Haulcomb et al., 2014). In contrast, such exacerbation was not observed here in mutant TDP-43 mice. This may suggests that SOD1 and TDP-43 associated ALS may represent two different mechanisms of neurodegeneration. Indeed, there are many clinical and pathological differences between SOD1 and TDP-43 associated ALS in mice (Gurney et al., 1994, Wegorzewska et al., 2009) and in humans (Phukan et al., 2012, Wicks et al., 2009). Further work may aim to characterise the regenerative responses found in different forms human ALS. This would help elucidate the common or specific mechanism of degeneration involved in the different forms of the disease.

General Conclusion

This thesis presents evidence that ALS degeneration involves cellular and molecular changes that resemble the regenerative response to acute axonal injury. It demonstrates that genetic deletion of c-Jun, an important orchestrator of axonal regeneration, attenuates SOD1 associated ALS disease in mice, while abolishing a disease-associated regenerative response in degenerating motor neurons. In addition, this thesis demonstrates that the expression of ALS associated proteins, TDP-43, FUS and alsin, changes in motor neurons after axonal injury in mice. Finally, it demonstrates that ALS associated mutant TDP-43 disrupts axonal regeneration following peripheral nerve injury in mice. Thus, the molecular changes in ALS-associated motor neurodegeneration and peripheral nerve injury-associated regeneration overlap. This suggests that insights obtained from studying one of these processes may be applied to further understand the other.

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Appendix