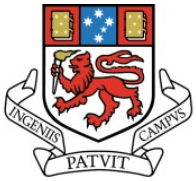

UNIVERSITY
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Distal axon and neuromuscular junction degeneration in amyotrophic lateral sclerosis

by

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BBiotech (Hons)

Submitted in fulfillment of the requirements for the
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KS performed experiments, data analysis and co-authored the manuscript with AK. AK and CB provided assistance with some experiments. TD participated in conceptualisation, design, coordination and manuscript editing.

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KS performed experiements, data analysis and wrote the manuscript. AK and CB provided assistance with some experiments and manuscript editing. GM and AK provided technical assistance. TD participated in conceptalisation, design, coordination and manuscript editing.

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SUMMARY

The cause of axon degeneration in ALS remains poorly understood. In recent years it has become clear that the onset of symptoms in ALS occurs after a potentially lengthy pre-symptomatic period in which key processes become dysfunctional, notably axonal transport, regulation of calcium and communication between motor neurons and non-neuronal cells. This thesis aims to investigate the relationship between excitotoxicity, non-neuronal cells and degeneration of the motor neuron axon.

Substantial evidence implicates glutamate excitotoxicity in the pathogenesis of ALS. However, the mechanism by which excitotoxicity results in axon degeneration is not well understood. This thesis has utilised primary cell culture techniques and immunocytochemistry to investigate the effect of targeted excitotoxin exposure to cortical neurons. Excitotoxicity in the somatodendritic compartment resulted in degeneration of the untreated distal axon and extensive degeneration of neuronal structures in the treated compartment. However, targeted excitotoxicity to the distal axon also resulted in degeneration of the axon, in the absence of degenerative changes to the untreated somatodendritic compartment. Immunocytochemical and western blot analysis indicated distally mediated excitotoxicity likely occurred via the AMPA receptor. In addition, distally triggered degeneration occurred in a caspase-dependent manner.

Degeneration of the neuromuscular junction occurs early in the development of ALS, occurring in conjunction with die-back of the motor neuron distal axon. This thesis has examined a wide range of neuromuscular junction-associated proteins in the mSOD1 G93A mouse model of ALS. Analysis of key structural proteins at the gastrocnemius muscle indicated very early differences in the structure of the mSOD1 G93A neuromuscular junction relative to the wild-type controls. For some structural

components (rapsyn and nestin), the differences were most apparent at the onset of physical symptoms (12 weeks), however for nerve terminals and Schwann cells, significant differences were apparent as early as 8 weeks of age. Most previous research has focused on loss of the presynapse from the post-synaptic acetylcholine receptors (AChR) as a marker for loss of the neuromuscular junction, however this thesis indicates that many key structural proteins are affected in the early stage of ALS prior to AChR changes. Targeting such proteins may provide a novel therapeutic target for treatment in ALS.

In this thesis, primary cell culture techniques have been used to develop a novel *in vitro* model for motor neurons, incorporating glial cells, motor neurons and skeletal muscle with the spatial organisation as occurs *in vivo*. Spinal motor neurons co-cultured with either glial cells or skeletal muscle under standard culture conditions developed different morphological characteristics, with the cell feeder layer affecting the development of neurites, axonal extension and survival during early development. Incorporation of spatially organised cells resulted in improved survival of motor neurons, whilst promoting robust axonal extension. The formation of rudimentary neuromuscular junctions within the distal compartment indicated maturation of the circuit. These cultures are the first to replicate the spatial organisation of the lower motor neuron/neuromuscular junction circuit within an *in vitro* model. Additionally, such preparations are achieved without the addition of extraneous growth factors, known to affect later maturation of the motor neuron. Targeted excitotoxicity to the somatodendritic compartment of mature cultures resulted in significant axon degeneration and loss of proximal structures (soma, dendrites and axons), however targeted excitotoxicity to the distal axon did not result in distal axon degeneration.

These results indicate a number of novel findings: Firstly, the distal axon of cortical neurons is capable of mediating excitotoxicity in a caspase-dependent manner. Secondly, degeneration of the neuromuscular junction *in vivo* is preceded by changes to the underlying structures. Thirdly, appropriate growth of motor neurons *in vitro* requires both the presence of glial and muscle cells, and relevant spatial organisation of these cells. Targeted excitotoxicity to motor neurons yielded a different response to that from compartmented cortical cells. In conclusion, this thesis demonstrates the importance of often under-recognised components of the lower-motor neuron - neuromuscular junction circuit in the pathogenesis of ALS, culminating in the development of a novel cell culture preparation for the investigation of spinal motor neurons *in vitro*. In addition, this thesis has uncovered two novel sites for potential therapeutic intervention in neurodegenerative disease; the cortical neuron axon and structural changes at the neuromuscular junction.

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TABLE OF CONTENTS

ABBREVIATIONS

α-BTx	α-Bungarotoxin
ACh	Acetylcholine
AChR	Acetylcholine receptor
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPA-R	AMPA receptor
AMPK	AMP-activated protein kinase
BDNF	Brain-derived neurotrophic factor
C9ORF72	Chromosome 9 open reading frame 72
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CSF	Cerebrospinal fluid
DGC	Dystrophin glycoprotein complex
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DPR	Di-peptide repeat proteins
E14.5	Embryonic day 14.5
EAAT/GLT1	Excitatory amino acid transporter/Glutamate transporter 1
EDTA	Ethylenediaminetetraacetic acid
Eph4	Ephrin receptor
ER	Endoplasmic reticulum
fALS	Familial ALS
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobe degeneration
FUS/TLS	Fused in sarcoma/Translated in liposarcoma
GABA	γ-Aminobutyric acid
GDNF	Glial-derived neurotrophic factor
GluR2	Calcium-permeable AMPA-R subunit
GluR5-7	Kainate receptors
HBSS	Hanks buffered salt-solution

IGF-1	Insulin growth factor-1
LMN	Lower motor neuron
LRP4	Lipoprotein receptor 4
M1	Activated microglia
MASC	Myotube-Associated Specificity Component
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein K
MAPT	Microtubule-associated protein tau
MCT1	Monocarboxylate transporter 1
mGluR	Metabotropic glutamate receptor
MK801	(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
MND	Motor neuron disease
mSOD1 G93A	Mutant SOD1 with glycine to alanine substitution at amino acid 93
MuSK	Muscle-specific kinase
NMDA	*N*-methyl-D-aspartate
NMDA-R	NMDA receptor
NMJ	Neuromuscular junction
NRG-1	Neuregulin
NT-3	Neurotrophin 3
P30	Post-natal day 30
$P75^{NTR}$	Neurotrophin receptor
PNS	Peripheral nervous system
RNA	Ribonucleic acid
S100	Calcium-binding protein/Schwann cell marker
sALS	Sporadic ALS
SOD1	Superoxide dismutase 1
TDP-43	tar-DNA binding protein 43
TNF-α	Tumour-necrosis factor α
UMN	Upper motor neuron
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system

VAPB	Vesicle-associated membrane protein
Wld^{s}	Slowed Wallerian degeneration

1 LITERATURE REVIEW

1.1 AMYOTROPHIC LATERAL SCLEROSIS

The mammalian motor system is complex and highly integrated, linking the motor cortex of the brain to skeletal muscle fibres in the periphery. Disruption to components of this circuit can result in loss of voluntary control and onset of paralysis. The motor system contains a number of sites which are particularly vulnerable to damage, notably the motor neuron itself, the skeletal muscle fibre and the synapse which connects the two; the neuromuscular junction (NMJ). Motor neuron disease (MND), initially reported in the 1840s by Duchenne and Aran, relates primarily to disturbed functioning of the motor system. Subsequent histological and pathological studies of MND indicated selective degeneration of the corticospinal tract motor neurons and the spinal cord (Charcot and Joffrey, 1869; Cleveland, 1999). The term motor neuron disease now collectively refers to progressive motor neuron degeneration involving either upper or lower motor neuron populations or both (Turner *et al.*, 2013).

MND is the third most frequent cause of neurodegenerative disease, behind Alzheimer's disease and Parkinson's disease (Forman *et al.*, 2004). In 1869 the French physician Jean-Martin Charcot described a specific form of MND, termed amyotrophic lateral sclerosis (ALS) (Charcot and Joffrey, 1869). ALS is recognised as the most common form of adult-onset MND, affecting 1-2 individuals per 100,000 (Chio *et al.*, 2013; Mehal *et al.*, 2013). ALS progresses rapidly from onset and death occurs usually 2-5 years from diagnosis primarily from respiratory failure (Gruis and Lechtzin, 2012) or cardiac conduction abnormalities (Scherer and Bedlack, 2012). Clinical diagnostic criteria are derived from the El Escorial and modified Airlie House criteria (Brooks *et al.*, 2000; Traynor *et al.*, 2000), which requires evidence of both upper and lower motor neuron

degeneration (Figure 1.1). Patients who present with symptoms indicating only lower motor neuron (LMN) involvement are classified as progressive muscular atrophy (PMA), whilst patients demonstrating only upper motor neuron (UMN) involvement are diagnosed with primary lateral sclerosis (PLS) (reveiwed in Van Damme and Robberecht, 2013). Furthermore, a diagnosis of ALS requires the absence of neuropathological features of other diseases, including neurological disturbances to sensation, autonomic and visual systems (Ince *et al.*, 1998b; Turner *et al.*, 2013). Initial clinical presentation in ALS varies widely but typically manifests as focal asymmetric weakness in one or more limbs (limb onset) in 80% of patients, or slurring of speech indicating bulbar onset in 20% of cases (Leigh and Ray-Chaudhuri, 1994; Jackson and Bryan, 1998; Ravits and La Spada, 2009; Turner *et al.*, 2013). Subsequent symptoms segregate according to the involvement of specific populations of motor neurons. Initial involvement of LMNs causes weakness, muscular atrophy and fasciculations, whereas predominantly UMN involvement causes weakness, spasticity and hyperreflexia (Rothstein, 2009; Turner *et al.*, 2013). Dysphagia and dysarthria may present regardless of specific motor neuron population involvement. Whilst most neurologists identify ALS as a single disease based on the characteristic nature of progressive motor neuropathy, strong evidence points to a potentially wider aetiopathogenic spectrum (Turner et al., 2013).

Neurons in the prefrontal and temporal cortex appear affected in ALS to varying degrees (Ringholz *et al.*, 2005). Frontotemporal lobar degeneration (FTLD) affects 3-4 individuals per 100,000, with the resulting language and behavioural abnormalities termed frontotemporal dementia (FTD). FTD is the second-most common form of dementia in adults under 65 years of age after Alzheimer's disease (Snowden *et al.*, 2007; Ratnavalli, 2010) and fourth-most common cause of dementia after 65 following Alzheimer's disease, dementia with Lewy bodies and vascular dementia (Barker *et al.*,

2002; Arvanitakis, 2010; Ratnavalli, 2010). FTLD is estimated to affect up to 15% of ALS patients (Ringholz *et al.*, 2005; Lillo and Hodges, 2009). A further 50% of ALS patients have evidence of subtle deficits in cognition or behaviour (Ringholz *et al.*, 2005). Similarly, 40% of patients with FTLD show evidence of motor involvement (Lomen-Hoerth *et al.*, 2002; Burrell *et al.*, 2011). Several histological findings are common to both ALS and FTLD, notably mislocalisation of the DNA binding protein tar-DNA binding protein 43 (TDP-43) (Neumann *et al.*, 2006). Additionally, chromosome 9 open reading frame 72 (*C9ORF72*) mutations are common to both ALS and FTLD (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). These links between FTLD and ALS suggest the two disorders form part of a continuum of neurological diseases (Neumann *et al.*, 2006; Lillo and Hodges, 2009; Robberecht and Philips, 2013).

Current therapeutic strategies for ALS are largely limited to palliative care and the administration of a single drug, riluzole (Rilutek) (Bensimon *et al.*, 1994). Riluzole has a modest effect in ALS, increasing patient life-expectancy by 2-3 months, however there is no demonstrable effect on clinical measures such as muscle strength (Miller *et al.*, 2012). A benzothiazole derivative, riluzole is proposed to act via a number of neuroprotective mechanisms including modulation of glutamatergic neurotransmission to reduce excitotoxicity via modulation of glutamate release, blockade of Ca^{2+} and Na^{+} channels and activity on the GABAergic system (Cheah *et al.*, 2010). Cell culture investigations indicate riluzole can prevent glutamate and *N*-methyl-D-aspartate (NMDA) induced degeneration of cultured cortical and motor neurons (Malgouris *et al.*, 1994; Estevez *et al.*, 1995; Koh *et al.*, 1999). In addition, riluzole acts on non-neuronal cells, by increasing glutamate uptake on cultured astrocytes (Dall'igna *et al.*, 2013) and increasing glutamate transport in cultured muscle and neuronal cells via activation of the AMP-activated protein kinase (AMPK) pathway (Daniel *et al.*, 2013). To date, discovery of new drugs

for the treatment of ALS has been unsuccessful. Dexpramipexole, a similar benzothiazole derivative, recently held some clinical promise (Cudkowicz *et al.*, 2011; Rudnicki *et al.*, 2013), however phase III clinical trials were discontinued in early 2013 due to failure to demonstrate efficacy in function or survival.

1.1.1 Causes of ALS

About 10% of ALS cases demonstrate a clear genetic link, however the cause remains unknown for 90% of cases, termed sporadic ALS (sALS) (Turner *et al.*, 2013). Despite this, clinical and pathological similarities between familial and sporadic onset ALS suggests a common pathogenesis, implicating a wide array of disease modifying processes irrespective of specific mutant activity (Bruijn *et al.*, 2004; Robberecht and Philips, 2013). The ALS brain and spinal cord is characterised by extensive activation of glial cells (reviewed in Philips and Robberecht, 2011). Altered communication between motor neurons and glial cells has been linked with many of the pathological signs of ALS motor neurons (Julien, 2007; Haidet-Phillips *et al.*, 2011). Furthermore, the toxicity of mutations, particularly those in superoxide dismutase 1 (SOD1) may be mediated via glial cells rather than directly within motor neurons (Clement *et al.*, 2003; Di Giorgio *et al.*, 2007; Nagai *et al.*, 2007; Kang *et al.*, 2013). The resulting non-cell autonomous theory of ALS highlights the widespread nature of pathology; ALS is not limited to motor neurons (Clement *et al.*, 2003; Boillée *et al.*, 2006a,b). Current hypotheses propose that motor neurons represent a 'weak link' in many motor disorders in which rescuing the mutant phenotype in motor neurons alone does not ablate the disease (Roselli and Caroni, 2012). Onset of disease and degeneration of motor neurons may occur following the

cumulative effect of multiple sub-threshold insults resulting in motor neuron dysfunction, degeneration and ultimately neuronal death (Adalbert and Coleman, 2012).

At present, the causative factors for ALS remain unknown, with age and sex the only proven risk factors, with risk increasing with age (peak incidence between 60-70 years of age) and increased susceptibility in males (ratio of 1.5 to 1) (McCombe and Henderson, 2010). Non-proven risk factors include smoking (Alonso *et al.*, 2010), environmental risk factors (Vinceti *et al.*, 2012) including toxin exposure (Bradley *et al.*, 2013) and some pesticides (Kamel *et al.*, 2012). It has even been suggested that previous exposure to poliomyelitis or other viruses may result in ALS via retrograde axonal transport of pathogens (Chou and Norris, 1993; Alfahad and Nath, 2013). ALS is also putatively associated with a history of musculoskeletal trauma and concussion with particular regard to sporting injuries (Lehman *et al.*, 2012), heavy manual activities and electric shock (Sirdofsky *et al.*, 1991; Abhinav *et al.*, 2007). Increased incidence of ALS in retired Italian soccer players (Chio *et al.*, 2005) and American NFL players (Lehman *et al.*, 2012) raises concerns over safety within these sports and toxin exposure via playing grounds, although these associations remain unconfirmed (Vanacore *et al.*, 2013). The incidence of ALS is more common amongst individuals with a high level of fitness and lower than average body mass index prior to onset of disease, suggesting a possible premorbid phenotype (Scarmeas *et al.*, 2002; Veldink *et al.*, 2005). The variance in disease onset relative to risk exposure suggests early and repeated damage to motor neurons may predispose them to degeneration, however disease is likely to occur from a combination of genetic and environmental factors.

1.1.2 Genetics of ALS

Familial ALS (fALS) or ALS cases with a clear inherited link account for 10% of patients and involves mutations to a large number of genes, including SOD1, RNA processing proteins, and those that regulate cellular homeostasis and axonal transport (Table 1.1).

Mutations to SOD1 (mSOD1), accounting for 20% of fALS cases, were discovered over 20 years ago following identification of a disease-relevant locus on chromosome 9 (Siddique *et al.*, 1991). SOD1 mutations have since been well characterised, with over 100 mutations spread throughout the encoding sequence of the gene (Rosen *et al.*, 1993; Robberecht and Philips, 2013). SOD1 is a 32kDa cytosolic homodimeric metalloenzyme that binds Cu^{2+} within the active site for dismutase activity and a regulatory Zn^{2+} (Fridovich, 1978; Banci *et al.*, 2008). SOD1 undergoes a number of post-translational modifications required for dimerisation of the subunits, formation of disulphide bonds and acquisition of the catalytic metal ions (Arnesano *et al.*, 2004; Banci *et al.*, 2008).

SOD1 mutations present with varying onset and disease progression, however with few exceptions, mutations are inherited in an autosomal dominant manner (Clement *et al.*, 2003). Within North American populations the alanine to valine substitution at position 4 (A4V) is the most common mutation, occurring in approximately 50% of cases and is typified by aggressive onset and progression (Boillée *et al.*, 2006b), with minimal involvement of the corticospinal tract (Cudkowicz *et al.*, 1998). Toxicity of mutant SOD1 in fALS was initially attributed to loss of dismutase activity (Rosen *et al.*, 1993), however ablation of SOD1 or overexpressed wild-type SOD1 in mutant mice did not alter disease (Bruijn *et al.*, 1998). Toxicity of mSOD1 is generally attributed to a novel gain of function (Tsuda *et al.*, 1994), however recent investigations into $mSOD1^{-/-}$ mice indicates some pathology may still be attributed to loss of dismutase activity (Flood *et al.*, 1999;

Fischer *et al.*, 2011). mSOD1 misfolds, undergoes ubiquitination, oligomerises and forms insoluble aggregates (Basso *et al.*, 2006), placing stress on both the proteasomal and autophagy protein degradation pathways (Bendotti *et al.*, 2012; Chen *et al.*, 2012b). Both human ALS tissue and animal models contain increased phagosomes, indicative of perturbed protein degradation (Morimoto *et al.*, 2007; Sasaki, 2011). This process induces unfolded protein stress on the cell, resulting in eventual microglial activation (Saxena *et al.*, 2009; Saxena and Caroni, 2011). The mechanisms by which misfolded mSOD1 escapes degradation are not known.

Other known toxic effects of mSOD1 include accumulation of neurofilaments (Rouleau *et al.*, 1996) and damage to mitochondria (Liu *et al.*, 2004; Vijayvergiya *et al.*, 2005; Deng *et al.*, 2006). The role of biochemically altered SOD1 in sALS remains highly speculative (Rothstein, 2009), however oxidised wild-type SOD1 is also able to misfold and aggregate, with demonstrable toxic effects on motor neurons (Rakhit *et al.*, 2002; Ezzi *et al.*, 2007; Bosco *et al.*, 2010). Clinical similarities between sporadic and inherited forms of ALS suggest that elucidating the mechanisms underlying fALS may provide insight into both forms of the disease (Bruijn *et al.*, 2004; Robberecht and Philips, 2013).

In 1994, a mSOD1 mouse line was developed via the overexpression of a single-amino acid substitution of glycine to alanine at position 93 in human SOD1 (Gurney *et al.*, 1994) and has since become one of the most widely used models of ALS. The G93A model recapitulates many aspects of human ALS, including axonal and mitochondrial dysfunction, progressive neuromuscular dysfunction, gliosis and loss of motor neurons (Gurney *et al.*, 1994; Ripps *et al.*, 1995; Bruijn *et al.*, 1997; Dal Canto and Gurney, 1997; Nagai *et al.*, 2001). A number of other mSOD1 rodent models have also been developed, notably the G85R strain that is characterised by a slower disease trajectory (Bruijn *et al.*, 1997). The development of ALS-like symptoms in these models is dependent on the

specific mutation, the transgene expression level, gender, and genetic background (Heiman-Patterson *et al.*, 2005). Both G93A and G85R mutations are associated with the toxic gain-of function attributed to mSOD1 toxicity, however the two mutations differ in dismutase activity which is present in the G85R and lacking in G93A (Borchelt *et al.*, 1994; Bruijn and Cleveland, 1996). It has been suggested that the G85R mutation results in a much greater propensity for the protein to aggregate thus resulting in toxicity (Bruijn *et al.*, 1997; Wang *et al.*, 2009). The average life-expectancy of mSOD1 mouse models is greatly affected by the genetic background, with a mixed background generally conferring a longer lifespan (Heiman-Patterson *et al.*, 2005; Heiman-Patterson *et al.*, 2011). mSOD1 G93A mice develop an obvious disease phenotype at 111 days, termed 'onset' (Dobrowolny *et al.*, 2005), characterised by hind-limb tremors (Wooley *et al.*, 2005).

Cellular abnormalities are present even during embryonic development, notably abnormal neuronal architecture (Amendola *et al.*, 2004) and neuronal hyperexcitability (van Zundert *et al.*, 2008). mSOD1 positive aggregates are present in the ventral horn from P30 (Gould *et al.*, 2006). A number of pathologies are present just before symptom onset, including abnormal muscle fibre distribution (Baloh *et al.*, 2007), abnormal action potentials (Geracitano *et al.*, 2003) increased expression of UPS (ubiquitin-proteasome system) target genes (Kieran *et al.*, 2007), presence of neurofilament positive spheroids in the anterior and lateral horns of the spinal cord (Tu *et al.*, 1996), gliosis (Kieran *et al.*, 2007) and loss of motor neurons (Lambrechts *et al.*, 2003). Muscle atrophy is apparent as the disease progresses (Dobrowolny *et al.*, 2005).

A number of other genes have become implicated in ALS, with the notable discovery of mutations to RNA/DNA binding proteins within the last 5 years. Mutations to TDP-43 were described in 2008 and account for 5% of fALS cases (Kabashi *et al.*, 2008;

Sreedharan *et al.*, 2008; Da Cruz and Cleveland, 2011). This was perhaps not surprising following the discovery that aggregated wild-type TDP-43 forms a key component of the large ubiquitinated protein aggregations that are also characteristic of sALS cases (Neumann *et al.*, 2006). TDP-43 pathology is not exclusive to ALS and forms a key component of pathology in FTLD. Toxicity of TDP-43 occurs via two proposed mechanisms; loss of nuclear activity and presence in the cytoplasm, with mutant TDP-43 demonstrating a higher tendency for cytoplasmic mislocalisation and aggregation (Johnson *et al.*, 2009; Barmada *et al.*, 2010). TDP-43 is involved in the regulation of over half of human and mouse brain transcripts (Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011; Lanson and Pandey, 2012), in particular, neuronal development proteins (Fujii *et al.*, 2005; Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011). TDP-43 also auto-regulates its own expression (Ayala *et al.*, 2011; Avendano-Vazquez *et al.*, 2012). A number of genes are potentially affected by mutant TDP-43 misregulation in ALS including *ATXN-2* (Elden *et al.*, 2010), survival motor neuron (*SMN*) and apolipoprotein A-II (Bose *et al.*, 2008), neurofilament light chain (*NFL)* (Strong *et al.*, 2007) and histone deacetylase 6 (*HDAC-6*) (Kim *et al.*, 2010). *NFL* and *HDAC-6* are both reduced in the motor neurons of ALS patients (Kabashi *et al.*, 2008). In addition, proteins involved in synaptic activity are affected by loss of nuclear TDP-43, including an NMDA receptor, an ionotropic glutamate receptor and neurexins 1 and 3 (Da Cruz and Cleveland, 2011; Polymenidou *et al.*, 2011; Lanson and Pandey, 2012). Recently, TDP-43 levels have also been correlated with regulation of fat deposition and glucose homeostasis in mice (Stallings *et al.*, 2013).

Mutations in the gene encoding fused-in-sarcoma and translocated in sarcoma (FUS/TLS), another RNA binding protein, were discovered the year after TDP-43 (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). FUS/TLS mutations are proposed to account for 4% of fALS (Da Cruz and Cleveland, 2011; Lanson and Pandey, 2012). FUS-

linked fALS appears clinically similar to TDP-43 fALS, with the notable absence of TDP-43 positive inclusions, rather, the characteristic cytoplasmic aggregates are immunoreactive for FUS (Sun *et al.*, 2011; Lagier-Tourenne *et al.*, 2012). FUS mutations are relatively specific for ALS, with the exception of ALS-linked FTLD (Lagier-Tourenne *et al.*, 2010). FUS is not associated with other neurodegenerative disorders, including 'pure' FTLD. FUS is a member of the 'spliceome', forming a component of mRNA splicing machinery (Andersen and Al-Chalabi, 2011; Ishigaki *et al.*, 2012), consequently, FUS/TLS is implicated in alternative splicing of a large number of mRNA transcripts (Rogelj *et al.*, 2012; Orozco and Edbauer, 2013). Many of the binding partners to FUS have not yet been identified, however FUS/TLS has been proposed to play an indirect role in regulation of initiation of transcription (Sephton *et al.*, 2011). TLS may be involved in regulation of the actin stabilising protein Ndl-L required for stabilisation of dendritic spines (Fujii and Takumi, 2005). A number of FUS targets overlap with TDP-43 binding, including *HDAC-6* and microtubule-associated protein tau (*MAPT*) (Kim *et al.*, 2010; Orozco *et al.*, 2012).

Most recently, a large hexanucleotide repeat in the non-coding region of the *C9ORF72* promoter was discovered (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). Mutated C9ORF72 accounts for approximately 40% of fALS patients, and is also associated with around 40% of inherited FTLD cases (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). C9ORF72 mutations are not exclusive to ALS and FTLD, with mutations also present in Alzheimer's disease (Majounie *et al.*, 2010), however C9ORF72 mutations are not associated with movement disorders such as Parkinson's disease (Majounie *et al.*, 2012a; Harms *et al.*, 2013). C9ORF72 cases have a characteristic phenotype with common bulbar onset, frequent frontotemporal involvement and may form a subset of ALS patients (Byrne *et al.*, 2012; Chio *et al.*, 2012). Some C9ORF72 patients may

develop psychosis (Snowden *et al.*, 2012). The hexanucleotide (GGGGCC) repeat acts in a dose-dependent manner, with <20 repeats considered normal and >30 repeats correlated with disease, with many patients having 100-1000 repeats (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). Susceptibility to the pathogenic expansion is age-dependent, with under 35 year olds considered non-penetrant and those over 80 years of age considered fully penetrant (Majounie *et al.*, 2012b). One of the mechanisms of C9ORF72 toxicity has been proposed to occur via the production and aggregation of short di-peptide repeat proteins (DPR) (Ash *et al.*, 2013; Mori *et al.*, 2013). DPR proteins are produced via alternative repeat-associated non-ATG-initiated (RAN) translation and may interfere with protein expression or gene translation (Zu *et al.*, 2011). The discovery of C9ORF72 in combination with TDP-43 and FUS strongly supports a potential role for altered RNA translation in the pathogenesis of ALS. This is supported by the finding of TFP-43 and FUS in rare sALS cases (Kabashi *et al.*, 2008; Mackenzie *et al.*, 2010; Lanson and Pandey, 2012), and that C9ORF72 may account for up to 10% of sALS (Debray *et al.*, 2013).

Additional mutations are involved in familial ALS across a diverse range of genes and cellular processes. Mutated alsin (Yang *et al.*, 2001) and senataxin (Chen *et al.*, 2004b) result in defective DNA processing and repair. Mutations to optineurin (Maruyama *et al.*, 2010) and valosin-containing protein (Johnson *et al.*, 2010) result in formation of intracellular aggregates that colocalise with TDP-43. Defects in the proteasome system occur with mutations to ubiquilin 2 resulting in impaired proteins degradation (Deng *et al.*, 2011). Mutations within hypoxia-inducible genes including vascular endothelial growth factor (Oosthuyse *et al.*, 2001) and the related angiogenin (Greenway *et al.*, 2004; Greenway *et al.*, 2006) result in hypoxic-related cell stress. Mutations to the cytoskeletal proteins include neurofilaments (Al-Chalabi *et al.*, 1999), peripherin (Gros-Louis *et al.*,

2004) and profilin-1, essential for conversion of monomeric actin to filamentous actin (Wu *et al.*, 2012a), result in cytoskeletal abnormalities and associated deficits in cellular transport. Additionally, deficits in axonal transport are caused by mutations to vesicle-associated membrane protein (VAPB) (Nishimura *et al.*, 2004). Mutations to ataxin-2 demonstrate a dose-dependent toxicity, with 22-23 poly-Q repeats present in normal individuals, 23-24 repeats present in a number of ALS patients, and >34 repeats associated with spinocerebellar ataxia (Elden *et al.*, 2010). The longer ataxin poly-Q repeat results in formation of stress granules and TDP-43 positive aggregations (Hart and Gitler, 2012). Mutations to the tyrosine kinase ephrin receptor (Eph4) modify disease progression, with missing Eph4 slowing disease in animal models and human patients (Van Hoecke *et al.*, 2012). A similar disease modifying locus 1p34.1 has been recently described to modulate age of onset (Ahmeti *et al.*, 2013).

1.2 PATHOGENESIS OF ALS

ALS is characterised by widespread loss of motor neurons throughout the corticospinal system (Sasaki and Iwata, 1999; Zhang *et al.*, 2011), including the primary motor cortex, brainstem cranial nerves and the anterior spinal cord (Kiernan and Hudson, 1991; Nihei *et al.*, 1993; Milonas, 1998; Tsuchiya *et al.*, 2002). The cause of this degeneration is poorly understood. The complex biology of ALS indicates many concurrent disease processes, including axon degeneration, slowed axon transport, neuronal death, glial activation and glutamate excitotoxicity (reviewed in Robberecht and Philips, 2013) (Figure 1.2). However, it is difficult to ascertain which of these processes are pathogenic and which are homeostatic adaptive responses (Quinlan, 2011). Additionally, pathology is not restricted to motor neurons, with inhibitory interneurons lost throughout the

corticospinal system (Nihei *et al.*, 1993; Petri *et al.*, 2003; Maekawa *et al.*, 2004). These same affected regions are characterised by extensive glial activation in humans (Kushner *et al.*, 1991; Kawamata *et al.*, 1992; Nagy *et al.*, 1994; Schiffer *et al.*, 1996; Turner *et al.*, 2004; Philips and Robberecht, 2011).

The key pathological finding in ALS is the presence of inclusion bodies within motor neurons (Hirano *et al.*, 1967). These inclusions develop prior to neuronal loss and include the ALS-specific Bunina bodies, comprising predominantly tubular and vesicle structures (Wood *et al.*, 2003; Okamoto *et al.*, 2008), perikaryal accumulations of phosphorylated neurofilaments and ubiquitinated proteins (Cluskey and Ramsden, 2001) as well as widespread ubiquitinated proteinaceous inclusions (Leigh *et al.*, 1991; Ince *et al.*, 1998a). The main protein constituent of the latter inclusion type was discovered to be TDP-43 in most sALS and non-SOD1 or FUS fALS (Neumann *et al.*, 2006; Tan *et al.*, 2007; Liscic *et al.*, 2008). Somal phosphorylated eosinophilic inclusions comprising organelles, aggregated misfolded proteins, ubiquitin, neurofilaments, proteasome components and other sequestered proteins are also common in motor neurons throughout the spinal cord and primary motor cortex (Murayama *et al.*, 1989; Wood *et al.*, 2003).

Recent investigations indicate that contrary to what was previously thought, proteinaceous inclusions are not restricted to motor neurons, with cytoplasmic ubiquitin-positive inclusions present in the hippocampus, cerebellum and neocortex of some ALS patients, particularly those with C9ORF72 repeat expansions (Ikemoto *et al.*, 2000; Mahoney *et al.*, 2012). Similar pathology is also present in interneurons (Martin and Chang, 2012) and glial cells (Stieber *et al.*, 2000; Haidet-Phillips *et al.*, 2011). The formation of astrocytic hyaline inclusions (Kato *et al.*, 1999; Miller *et al.*, 2004; Yamanaka *et al.*, 2008b) may precede motor neuron pathology in mouse models (Barbeito *et al.*, 2004). The toxicity of these inclusions is not well understood, however

their presence highlights an important role for glial cells in the pathogenesis of ALS (Barbeito *et al.*, 2004; Estes *et al.*, 2013).

Non-neuronal cells are being increasingly appreciated in the pathogenesis of ALS. Glial cells have been shown to mediate the effects of mSOD1 in chimeric animal models (Clement *et al.*, 2003) as well as in cell culture models utilising human embryonic stem cell derived motor neurons (Hedlund and Isacson, 2008). The now commonly used 'non-cell autonomous' theory of ALS pathogenesis, or the notion that motor neurons are not affected alone, came to the forefront of research through experiments performed with chimeric animals and culture systems demonstrating the critical role of astrocytes in the disease (Boillée *et al.*, 2006a). Survival of cultured motor neurons is drastically reduced when co-cultured with mSOD1 expressing astrocytes, or following exposure of motor neurons to conditioned medium from mSOD1 expressing astrocytes (Nagai *et al.*, 2007; Hedlund and Isacson, 2008). Several studies indicate that mSOD1 expression must occur in both motor neurons and glial cells to induce toxicity (Gong *et al.*, 2000; Pramatarova *et al.*, 2001; Boillée *et al.*, 2006b). Conversely, mSOD1 expressing motor neurons remain protected when surrounded by glial cells expressing wild-type SOD1 (Clement *et al.*, 2003).

Activated astrocytes and microglia are widespread throughout the primary motor cortex, lateral descending and corticospinal tracts and the spinal cord in human ALS and mouse models (Barbeito *et al.*, 2004; Miquel *et al.*, 2012). Gliosis is a complex response, resulting in the release of a diverse range of factors including growth factors, cytokines, cell surface and matrix proteins, nitric oxide, excitotoxins, proteases and proinflammatory molecules (Hanisch, 2002; Kuhle *et al.*, 2009). Astrocytes and microglia are implicated in disease progression (Boillée *et al.*, 2006b; Henkel *et al.*, 2009; Appel *et al.*, 2011) and dysfunction of motor neurons prior to neuronal death (Barbeito *et al.*, 2004; Van Den

Bosch and Robberecht, 2008). Furthermore, mSOD1 expression in astrocytes exacerbates loss of the motor neurons (Di Giorgio *et al.*, 2007). Astrocyte mediated damage may occur via altered glucose metabolism between astrocytes and motor neurons (Cassina *et al.*, 2008; Ferraiuolo *et al.*, 2011). Abnormal proliferation of astrocytes occurs in ALS and has been likened to that of cancerous cells (Miquel *et al.*, 2012), however ablation of astrocyte proliferation does not alter disease outcome in ALS mouse models (Lepore *et al.*, 2008).

In mSOD1 models, early disease is characterised by increased levels of proinflammatory mediators indicating chronic inflammation (Almer *et al.*, 2001; Ilzecka *et al.*, 2001; Yasojima *et al.*, 2001; Almer *et al.*, 2002; Kuhle *et al.*, 2009), similar findings are present in human CSF (Simpson *et al.*, 2004; Goldknopf *et al.*, 2006). Extensive gliosis is also present within the spinal cord (Hall *et al.*, 1998; Levine *et al.*, 1999; Alexianu *et al.*, 2001; Gowing *et al.*, 2008) and sciatic nerve of ALS mice (Chiu *et al.*, 2009). However whilst anti-inflammatory drugs such as minocycline have proven effective in mSOD1 mouse models (Kriz *et al.*, 2002; Van Den Bosch *et al.*, 2002; Zhu *et al.*, 2002), none have translated into human therapy (Fondell *et al.*, 2012). A critical outcome of a pro-inflammatory state is down-regulation of glial cell trophin expression (Hanisch and Kettenmann, 2007). The cause of this inflammatory state is attributed to increasing numbers of activated (M1) microglia (Chiu *et al.*, 2013), with M1 microglial numbers correlating with disease severity (Turner *et al.*, 2004; Moisse and Strong, 2006). Studies into the role of M1 microglia in ALS indicates that they may play a role in determining progression of disease potentially via further propagation of the inflammatory response (Hanisch, 2002; Zhao *et al.*, 2006; Ransohoff and Perry, 2009).

The role of oligodendrocytes in ALS is being increasingly recognised. Oligodendrocytes are proposed to play two key roles in ALS, notably myelination and energy transfer with

axons needed for axonal health (Lee *et al.*, 2012). The latter process of lactate shunting is critical for axonal function (Funfschilling *et al.*, 2012). Perturbations to the oligodendrocyte-axon lactate shunt via downregulation of the monocarboxylate transporter 1 (MCT1) in mice results in axon degeneration and neuronal loss (Lee *et al.*, 2012). Furthermore, MCT1 is reduced in the motor cortex of ALS patients (Lee *et al.*, 2012). Interestingly a similar lactate-dependent mechanism may be mediated via astrocytes (Cassina *et al.*, 2008; Ferraiuolo *et al.*, 2011). Oligodendrocytes are progressively lost in ALS (Kang *et al.*, 2013), however their role in ALS remain uncertain, in particular, their role in determining disease onset (Yamanaka *et al.*, 2008a; Kang *et al.*, 2013). Of particular interest to this thesis, is that oligodendrocytes are vulnerable to glutamate excitotoxicity, a known pathogenic process in ALS (refer to section 1.3). So-termed white-matter excitotoxicity, oligodendrocyte vulnerability to glutamate is likely to perturb protective interactions between oligodendrocytes and axons (both cortical and motor). Furthermore, evidence indicates that glutamate receptor subunits are present along myelinated axons (internodal regions). Whilst the function of these receptors is unknown, their presence could mean that excitotoxicity may be mediated directly via the axon (Matute *et al.*, 2007; Matute and Ransom, 2012). These findings raise interesting questions about how excitotoxicity affects the neurons within the cortex and spinal cord, with relevance to ALS, FTLD and other neurodegenerative diseases.

1.2.1 Upper motor neurons

Much of the information regarding cortical pathology in ALS has been gathered from human tissue at autopsy, and in particular loss of the Betz cells and pyramidal neurons in

the motor cortex is one of the defining features of the disease (Nihei *et al.*, 1993). Additional evidence for UMN involvement in ALS comes from cognitive impairment in some patients, strengthening the links between ALS and FTLD. Cortical neurons in FLTD contain ALS-like ubiquitinated intracellular aggregates (Ikemoto *et al.*, 2000; Mahoney *et al.*, 2012). Gliosis is also prevalent throughout the cortex in ALS and FTLD (Mahoney *et al.*, 2012). Additional investigations indicate that the integrity of the blood-brain barrier is compromised in ALS raising questions over vascular integrity in ALS (Winkler *et al.*, 2013).

It was initially thought that mSOD1 mouse models, whilst recapitulating distal motor neuron degeneration, had limited or no cortical involvement (Wong *et al.*, 2002; Ralph *et al.*, 2005; Niessen *et al.*, 2006). However recent advances in experimental technology have enabled cortical investigation in mSOD1 mice, with results indicating the cortical involvement is present (Ozdinler *et al.*, 2011). UMNs (corticospinal and corticobulbar motor neurons) are lost at post-natal day 30 (P30) in mSOD1 G93A mice (Ozdinler *et al.*, 2011), with notable dendritic pathology at P60 (Yasvoina *et al.*, 2013). In addition, cortical involvement in mSOD1 G93A mice is not restricted to the motor cortex, with neuronal loss throughout the neocortex. This was accompanied by gliosis within these brain regions (Ozdinler *et al.*, 2011).

1.2.2 Lower motor neurons

Large swellings are frequent in the proximal portion of the ALS motor neuron axon (Delisle and Carpenter, 1984). These swellings contain aggregated neurofilaments, ubiquitinated proteins and mitochondria (Bergeron et al., 1994). The ALS LMN axon is characterised by a slowing of bidirectional axonal transport (Rothstein *et al.*, 1992;

Fischer-Hayes *et al.*, 2013; Millecamps and Julien, 2013), particularly with regard to mitochondria transport (Sheng and Cai, 2012). Consequently, motor neurons have slowed mitochondrial turnover (Fischer-Hayes *et al.*, 2013; Millecamps and Julien, 2013) with increasing prevalence of abnormal mitochondria at the distal axon terminals, and within distal axonal varicosities as a result of slowed anterograde transport (Dadon-Nachum *et al.*, 2011; Garcia *et al.*, 2013). Selective damage to dynein, a retrograde transport molecule results in a severe motor phenotype in mice, attributed to dysfunctional transport of target-derived factors to the soma (LaMonte *et al.*, 2002).

Slowed axon transport in mSOD1 G93A mice is dependent on activation of axonal p38 mitogen-activated protein kinases (MAPKs) and phosphorylation of the bidirectional motor protein kinesin, reducing mobility (Morfini *et al.*, 2013). The combination of these processes results in axonal-specific pathology and has been proposed as a potential mechanism for gain-of-function in SOD1 mutants (Morfini *et al.*, 2013).

Accumulated autophagosomes in spinal motor neurons are a common feature of sALS (Li *et al.*, 2008; Sasaki, 2011), and are associated with sequestered cytoplasmic organelles including mitochondria (Sasaki, 2011). Autophagosome accumulation is linked to reduced activity of the retrograde motor protein dynein/dynactin (Ikenaka *et al.*, 2013). *In situ* hybridisation analysis indicates dynein/dynactin levels are reduced in the spinal motor neurons of ALS patients (Jiang *et al.*, 2007). Normal functioning of molecular motor proteins is dependent on binding to microtubules (Reed *et al.*, 2006; Dompierre *et al.*, 2007), however reduced microtubule acetylation perturbs binding (Dompierre *et al.*, 2007; d'Ydewalle *et al.*, 2011). Inactivation of HDAC-6, a key α-tubulin deacetylase and putative splice target of FUS/TLS and TDP-43, is neuroprotective in neurodegenerative disease models, including Huntington's disease, Parkinson's disease (Outeiro *et al.*, 2007; Pallos *et al.*, 2008) and recently in mSOD1 G93A mice (Taes *et al.*, 2013). Additionally,

HDAC-6 regulates transport of mitochondria in hippocampal neurons (Chen *et al.*, 2010a), highlighting the importance of maintaining axonal transport systems.

Mouse models have been used extensively to demonstrate that ALS is primarily a distal axonopathy, whereby extensive distal axon degeneration occurs prior to symptom onset, preceding loss of motor neurons (Frey *et al.*, 2000; Fischer *et al.*, 2004; Dadon-Nachum *et al.*, 2011). A similar finding was discovered following analysis of an ALS patient who died unexpectedly, demonstrating severe distal degeneration without measurable changes in the number of spinal motor neurons (Fischer *et al.*, 2004). Moreover, in both mSOD1 G93A and G85R models, a significant decrease in the number of spinal motor neurons is only present after symptom onset, by which time approximately two thirds of ventral root motor axons had degenerated (Fischer *et al.*, 2004; Schaefer *et al.*, 2005; Gould *et al.*, 2006; Pun *et al.*, 2006). Analysis of MNs indicates deficits in axon transport well prior to degeneration in mSOD1 mice (Warita *et al.*, 1999; Williamson and Cleveland, 1999) and in human ALS (Bradley *et al.*, 1983; Sasaki and Iwata, 1996). These similarities, along with the cortical findings mentioned above, strengthen the notion that mSOD1 mice are valid models of human ALS and early degenerative changes in the mSOD1 mouse may similarly reflect early changes in many human cases.

1.2.2.1 Axon degeneration

Axon degeneration is proposed to be a fundamental process in the development of clinical symptoms in ALS. Axon degeneration in ALS is suggested to occur via a 'dying back' pathology, where degeneration begins with the distal axon and progresses proximally (Fischer *et al.*, 2004; Fischer and Glass, 2007; Adalbert and Coleman, 2012) (Figure 1.3). This is supported by the finding of extensive loss of motor neuron axons in

ALS patients and mouse models without the corresponding loss of cell bodies (Fischer et al., 2004; Gould et al., 2006; King et al., 2012). The mechanism by which this degeneration occurs is not well understood, with hypotheses extending from degeneration and dysfunction within the motor neuron (Rothstein, 2009; Barrett *et al.*, 2011) to a potential pathological effect exerted by the skeletal muscle (Dobrowolny *et al.*, 2008; Wong and Martin, 2010; Da Cruz *et al.*, 2012). In addition, it is not clear how glutamate excitotoxicity, a known pathological process in ALS, results in degeneration of the motor neuron distal axon.

Wallerian degeneration, a form of axon degeneration that occurs following injury, shares many of the features of axon degeneration in ALS (Beirowski *et al.*, 2010). During Wallerian degeneration, the distal portion of the axon below the injury forms 'beads' which is then followed by fragmentation (Adalbert and Coleman, 2012). Much of the information regarding Wallerian degeneration has come from investigating a spontaneous mouse mutant with dramatically slowed Wallerian degeneration (Lunn *et al.*, 1989; Coleman and Freeman, 2010). Damaged axons of this *Wld*^{S} mouse survive for longer than wild-type axons (Mack *et al.*, 2001; Coleman and Freeman, 2010). Transected axons are capable of conducting action potentials for up to 3 weeks following injury (Gillingwater and Ribchester, 2001) and neuromuscular transmission persists at some NMJs for up to 14 days in *Wld*^{S} mice in contrast to 12-24 hours in axotomised wild-type NMJs (Ribchester *et al.*, 1995). Similarly, neuropathy induced by topical neurotoxin application shows delayed degeneration in *Wld*^{S} mice (Wang *et al.*, 2001).

When crossed with the mSOD1 G93A mouse, *Wld*^{S} only resulted in a very modest increase in survival, with no discernible change in the number of motor neurons (Fischer *et al*., 2005). A number of factors may underlie this lack of protection. *Wld*^{S} protection requires normal mitochondrial function (Avery *et al.*, 2012; Fang *et al.*, 2012) which is

known to be perturbed in ALS (Garcia *et al.*, 2013). Additionally, as mentioned above, ALS is increasingly considered to occur from motor neuron die-back which may begin at the NMJ (Frey *et al.*, 2000; Fischer *et al.*, 2004). There is no evidence at present that *Wld*^{S} actively protects the NMJ, instead NMJ preservation is likely to occur secondary to prolonged survival of the distal axon segment.

The prolonged survival of *Wld*^{S} axons is attributed to the formation of a novel fusion protein between the NAD^+ synthesising enzyme Nmnat1 with the ubiquitination factor Ube4b (Mack *et al.*, 2001; Schoenmann *et al.*, 2010). The mechanism by which the fusion protein promotes axonal survival is currently not known, with some evidence suggesting a role in mitochondrial function, however the fusion protein may alternatively act via a cytoplasmic or golgi locus (Coleman and Freeman, 2010; Avery *et al.*, 2012; Wang and Barres, 2012). A similar effect is achieved with knock-out of dSarm/Sarm1 in Drosophila, preventing distal axon degeneration following axotomy (Osterloh *et al.*, 2012). These results support the hypothesis that axon degeneration may be mediated by local energy demand rather than the soma (Zhu and Sheng, 2011). Indeed, before the mechanisms underlying the delayed degeneration characteristic of the *Wld*^{S} mouse were discovered, injury-induced axon degeneration was proposed not to occur via lack of nutrients, instead injured axons were proposed to self-destruct through an active and regulated process (Buckmaster *et al*., 1995; Raff *et al*., 2002) as distinct from apoptosis (Deckwerth and Johnson, 1994; Burne *et al*., 1996; Finn *et al*., 2000). The initial selectivity for degeneration of fast-fatiguable motor neurons in ALS supports a theory of local energy demand versus function (Saxena *et al.*, 2009).

1.2.3 The neuromuscular junction

The NMJ is a highly specialised synapse between the skeletal muscle fibre and spinal motor axon terminal. Degeneration of the neuromuscular junction (NMJ) is considered a hallmark feature of ALS pathology, resulting in muscle weakness and progressive paralysis (reviewed in Rocha *et al.*, 2013). The process of NMJ degeneration in ALS is similar to that which occurs in developmental remodelling and also following axotomy (Gillingwater and Ribchester, 2003). Interestingly, loss of the NMJ occurs before symptom onset in mouse models (Frey *et al.*, 2000; Fischer *et al.*, 2004; Pun *et al.*, 2006). Loss of synaptic connectivity is preceded by weakened synaptic transmission (Gillingwater *et al.*, 2002; Rocha *et al.*, 2013) and progressive displacement of synaptic vesicles (Gillingwater *et al.*, 2003). It is interesting to note that neurodegenerative mechanisms appear to be compartmented in neurons and that the synapse represents a distinctive neuronal compartment (Mattson *et al.*, 1998; Gillingwater and Ribchester, 2001; Raff *et al.*, 2002). Loss of the NMJ in ALS is often considered to occur in conjunction with distal die-back of the motor neuron (Da Cruz *et al.*, 2012; Johri and Beal, 2012; Krakora *et al.*, 2012).

Loss of NMJs in ALS is not uniform across fibre types: Fast-twitch muscle fibres which are innervated by fast-firing phasic motor neurons are preferentially vulnerable in G93A disease in human and mouse models (Frey *et al.*, 2000; Atkin *et al.*, 2005; Pun *et al.*, 2006). Small-caliber type I axons persist later into disease in both human patients and mSOD1 rodent models (Kawamura *et al.*, 1981; Bruijn *et al.*, 1997). This preferential vulnerability of type II fast motor neurons is exacerbated by transient ischaemic injury in mSOD1 G93A mice (David *et al.*, 2007). Sprouting of the disease resistant motor neurons and subsequent reinnervation of fibres maintains muscle function through the early stages of disease (Hegedus *et al.*, 2008; Gordon *et al.*, 2010; Johnson and Mitchell,

2013). This is accompanied by the presence of unusually large motor units in ALS, comprising predominantly type I motor neurons, suggesting compensatory plasticity (McComas *et al.*, 1971; Schaefer *et al.*, 2005). In healthy systems, this reinnervation will trigger a switch in muscle fibre type following reinnervation by a different motor terminal (Pette, 2001), however this does not occur in symptomatic ALS, with the muscle fibre retaining the original fibre characteristics (Baloh *et al.*, 2007). It remains unclear whether the lack of fibre-type switching occurs due to dysfunction within the muscle fibre or originates from the motor terminal.

The mature NMJ is a tripartite synapse, with a terminal (or junctional) Schwann cell enveloping the synapse, much in the same way as an astrocyte does around a CNS synapse (Feng and Ko, 2008). An additional population of peri-synaptic cells at the NMJ, the extralaminar capping cells, has been proposed (Court *et al.*, 2008), however both the identity of the cells and their role in ALS remains poorly understood. Much of the information regarding the key signals at the NMJ has been gathered from developmental studies, utilising genetically modified models (Wu *et al.*, 2010). Consequently, the role of many of the proteins involved in the NMJ development, maintenance and repair are not known in ALS.

Development of the NMJ requires precise and coordinated signalling between the motor neuron terminal and skeletal muscle fibre (Ferraro *et al.*, 2011) (Figure 1.4). Skeletal muscle fibres prepare for innervation by expression and prepatterning of AChRs along the myofibre surface at low density (1,000μm^{-2}) (Hartzell and Fambrough, 1973; Cohen and Fischbach, 1977). In contrast, the density of AChRs at the mature synapse is >10,000 μm^{-2} directly adjacent to the motor terminal, and less than <10 μm^{-2} on the extrasynaptic membrane (Hartzell and Fambrough, 1973; Cohen and Fischbach, 1977; Salpeter and Loring, 1985; Salpeter *et al.*, 1988). The location of prepatterned AChR clusters does not

influence the site of NMJ formation (Anderson and Cohen, 1977; Frank and Fischbach, 1979; Ferraro *et al.*, 2011).

Release of nerve-derived factors rapidly localises AChRs to the motor axon terminal (Godfrey *et al.*, 1988). Agrin, a 400kDa proteoglycan (Tsen *et al.*, 1995; Denzer *et al.*, 1997) induces aggregation of AChRs (Nitkin *et al.*, 1987; Magill-Solc and McMahan, 1988; McMahan, 1990) via interactions with MuSK (muscle-specific kinase), rapsyn and Dok7 (Apel *et al.*, 1997; Bruneau *et al.*, 2005; Kim and Burden, 2008; Inoue *et al.*, 2009). MuSK was initially proposed as the post-synaptic target of agrin (DeChiara *et al.*, 1996), however the two molecules do not directly interact, implicating involvement of an intermediate protein/complex (MASC) (Glass *et al.*, 1996). MASC was recently identified as LRP4, a member of the low-density lipoprotein lipase receptor family (Wu *et al.*, 2012b). MuSK-mediated aggregation of AChRs can occur in the absence of agrin, such as during pre-patterning of AChRs via MuSK autophosphorylation, however localisation of AChRs along the myofibre surface is widespread and uneven (DeChiara *et al.*, 1996; Mittaud *et al.*, 2004; Wu *et al.*, 2010). In the presence of agrin, MuSK-mediated AChR aggregation is firmly localised to the motor terminal.

Expression of AChRs is maintained by synaptic nuclei via nerve-derived neuregulin (NRG1) interacting with ErbB proteins (Altiok *et al.*, 1995; Chu *et al.*, 1995; Jo *et al.*, 1995; Moscoso *et al.*, 1995; Zhu *et al.*, 1995). Additionally, loss of NRG-1/ErbB signalling results in destabilisation of AChR via dephosphorylation of a-dystrobrevin-1 (Schmidt *et al.*, 2011). NRG-1 bound ErbBs activate ERK (Bevan and Steinbach, 1977; Altiok *et al.*, 1997), and JNK (Si *et al.*, 1996; Si *et al.*, 1999) to activate GABP-α (LaMarco *et al.*, 1991; Thompson *et al.*, 1991). Activated GABP-α dimerises with GABP-β within synaptic nuclei to promote AChR transcription (LaMarco *et al.*, 1991; Thompson *et al.*, 1991). Additional targets of GABP-α/β transcription include utrophin

and acetylcholinesterase (AChE) (Fromm and Burden, 1998; Sapru *et al.*, 1998; Schaeffer *et al.*, 1998).

Simultaneously, non-synaptic nuclear expression of AChRs is downregulated in a voltage-dependent manner (Borodinsky and Spitzer, 2006) via Cdk-5 activation/inhibition (Fu *et al.*, 2005; Lin *et al.*, 2005). Release of ACh at the developing junction depolarises the muscle fibre, raising intracellular Ca^{2+}, activating CamKII, protein kinase C and calmodulin (Li *et al.*, 1992; Tang *et al.*, 2001; Fu *et al.*, 2005), subsequently deactivating myogenin transcription of AChRs (Sanes and Lichtman, 2001) via phosphorylation of myogenin (Mendelzon *et al.*, 1994; Macpherson *et al.*, 2002). Myogenin is upregulated following denervation via HDAC-4 to reduce DACH2 inhibition of myogenin to increase AChR expression (Eftimie *et al.*, 1991; Mejat *et al.*, 2005; Tang and Goldman, 2006; Cohen *et al.*, 2007; Tang *et al.*, 2009).

Maturation of the NMJ occurs during the first month postnatally in mice, as determined by the presence of synapsin I (Lu *et al.*, 1996) and clustering of mitochondria in the perijunctional area of the muscle fibre (Lee and Peng, 2005). NMJs mature from an initial 'plaque' organisation to take on the characteristic "pretzel" shape of the mature synapse comprising a central fold with branching secondary folds (Marques *et al.*, 2000). Motor units are established as extraneous polysynaptic connections are systematically pruned from muscle fibres (Brown *et al.*, 1976; Walsh and Lichtman, 2003; Wu *et al.*, 2010). Motor neurons that fail to make synaptic connections with skeletal muscle undergo programmed cell death via $p75^{NTR}$ dependent caspase activation, potentially reflecting successful acquisition of limited skeletal muscle-derived trophins (Taylor *et al.*, 2007).

The role of Schwann cells at the neuromuscular junction is being increasingly recognised. The non-myelinating Schwann cells that encapsulate the junction, termed terminal or

perisynaptic Schwann cells play an active role during development of the neuromuscular synapse and regulate long term synaptic changes (Araque *et al.*, 1999; Feng and Ko, 2008). Whilst not essential for appropriate motor axon pathfinding, Schwann cells are crucial for full development of the *in vivo* synapse (Riethmacher *et al.*, 1997; Lin *et al.*, 2000). Schwann cells migrate alongside developing motor axons and are present at the developing synapse shortly after initial nerve-muscle contact (Kelly and Zacks, 1969; Herrera *et al.*, 2000), and rely on axonally derived neuregulin for survival (Riethmacher *et al.*, 1997; Lin *et al.*, 2000; Wolpowitz *et al.*, 2000). Perisynaptic Schwann cells may additionally rely on muscle-derived NT-3 (Trachtenberg and Thompson, 1997). Perisynaptic Schwann cell processes guide regenerating axon terminals during synaptic remodelling (Ko and Chen, 1996). The role of Schwann cells in ALS is not well understood. Targeted deletion of mutant SOD1 from Schwann cells in mice resulted in the unexpected aggravation of disease (Lobsiger *et al.*, 2009), in contrast with neuroprotective effects of a similar mutant knock-down from astrocytes (Yamanaka *et al.*, 2008b). This is suggestive of a critical role for SOD1 dismutase activity in Schwann cells, however how this contributes to disease remains to be explored.

The role of skeletal muscle in the pathogenesis of ALS remains controversial. Reduced mSOD1 expression in skeletal muscle has no effect on survival in mouse models (Miller *et al.*, 2006; Da Cruz *et al.*, 2012). Additionally, transplantation of skeletal muscle between mSOD1 G93A mice and wild-type mice suggests mSOD1 expression in skeletal muscle alone does not result in ALS pathology (Carrasco *et al.*, 2010). Conversely, others have shown muscle-specific expression of both G37R and G93A SOD1 mutations in mice results in ALS-like motor deficits, with pathology present in the skeletal muscle and also in spinal motor neurons (Wong and Martin, 2010). Upregulating insulin-growth factor-1 (IGF-1) via retroviral delivery to the spinal cord (Kaspar *et al.*, 2003) or muscle-

specific expression (Dobrowolny *et al.*, 2005) delays disease progression in ALS mice. Similarly, muscle-specific expression of transgenic GDNF is neuroprotective in G93A mice (Li *et al.*, 2007). A potential explanation for the discrepancy between studies is that differential aggregation of mSOD1 species between skeletal muscle and spinal motor neurons reflects differing proteasomal and chaperone activity between the two cell types, thus allowing mSOD1 species to preferentially aggregate in spinal motor neurons (Onesto *et al.*, 2011; Wei *et al.*, 2013). Systemic metabolic abnormalities in ALS may extend to the skeletal muscle, alternatively, abnormalities to mitochondria, particularly muscle mitochondria, may drive disease onset (Dupuis *et al.*, 2003). This is particularly evident in further studies by Dupuis et al. (2009), demonstrating that muscle-specific mitochondrial uncoupling in mice is sufficient to destabilise NMJs and trigger degeneration of motor neurons (Dupuis *et al.*, 2009). Abnormal energy homeostasis may also explain the finding that NMJ degeneration in the mSOD1 G93A mouse is preceded by abnormal action potential firing (Magrané *et al.*, 2012). Moreover, abnormal mitochondria accumulate at the peri-junctional skeletal muscle sarcolemma (Zhou *et al.*, 2010).

1.3 EXCITOTOXICITY IN ALS

To date, many mechanisms have been proposed to explain motor neuron degeneration in ALS, including oxidative stress, glutamate excitotoxicity, inflammation, altered functionality of ionic channels, apoptosis, ER stress, defects in the unfolded protein response (UPR), increased protein nitration, defects in axonal transport, mitochondrial dysfunction and energy imbalance (Carriedo *et al.*, 1996; Estevez *et al.*, 1999; Casoni *et al.*, 2005; Kuo *et al.*, 2005; Van Den Bosch *et al.*, 2006; Zona *et al.*, 2006; Guatteo *et al.*,

2007; Boillée and Cleveland, 2008; Foran and Trotti, 2009; Pieri *et al.*, 2009; Carunchio *et al.*, 2010; McCombe and Henderson, 2011) (Figure 1.2). In combination, these mechanisms suggest that the disease is multifactorial in origin (Eisen, 1995; Rothstein, 2009). For the purpose of this thesis, discussion will focus on excitotoxicity, specifically the role of non-neuronal cells in mediating the neuronal response to excitotoxicity.

A substantial body of evidence implicates glutamate excitotoxicity in ALS, however the reasons behind the apparent selective vulnerability to motor neurons and subsequent intracellular damage and axon degeneration are not well understood. Evidence for excitotoxicity in ALS patients is two-fold. Firstly, patients typically demonstrate elevated CSF glutamate levels (Plaitakis and Caroscio, 1987; Rothstein *et al.*, 1990), with glutamate levels corresponding to disease severity (Spreux-Varoquaux *et al.*, 2002). ALS patient CSF induced excitotoxic degeneration in cultured neurons (Olney, 1990), the toxic compound later identified as glutamate (Rothstein *et al.*, 1990). Secondly, the only current therapy for ALS patients is the anti-excitotoxic drug Riluzole (Bensimon *et al.*, 1994; Cheah *et al.*, 2010), proposed to act via a number of anti-excitotoxic mechanisms (Chang *et al.*, 2010). Furthermore, the motor neurons which are resistant to ALS pathology are also resistant to excitotoxicity (Brockington *et al.*, 2013).

1.3.1 Glutamatergic neurotransmission

Glutamatergic neurotransmission accounts for approximately one third of CNS excitatory synaptic activity (Watkins and Evans, 1981; Cotman *et al.*, 1987). Under normal conditions, both intracellular and extracellular concentrations of glutamate remain under tight regulation to ensure abnormal intracellular signalling events do not occur (Orrenius *et al.*, 1989; Baimbridge *et al.*, 1992). Extracellular glutamate is maintained at 0.6μM

(Benveniste *et al.*, 1984), with 2-5μM extracellular glutamate considered pathogenic (Meldrum and Garthwaite, 1990; Rosenberg *et al.*, 1992). Glutamate is rapidly cleared from the synaptic cleft by astrocytic transporters and returned to the motor neuron following temporary conversion to glutamine (Laake *et al.*, 1995). Within the motor neuron, glutamate is resynthesised by glutaminase (Laake *et al.*, 1995) and transported into vesicles by specialised transporter proteins to ensure intracellular levels of glutamate remain constant at 10mM (Kvamme *et al.*, 1985; Bellocchio *et al.*, 2000; Herzog *et al.*, 2001).

1.3.1.1 Glutamate receptors

Glutamate receptors fall into two main categories: ionotropic receptors, or ligand gated ion channels; and g-protein coupled metabotropic receptors (mGluRs) (Van Damme *et al.*, 2005) (Figure 1.5). Ionotropic receptors are further divided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptor subtypes, as classified by their pharmacological activity to the exogenous glutamate analogues NMDA, AMPA and kainic acid (Seeburg, 1993; Hollman and Heinemann, 1994). Ionotropic glutamate receptors are permeable to sodium (Na^+) and potassium (K^+) ions with variable permeability to calcium, depending on the receptor type and subunits present (Van Damme *et al.*, 2005). Specific combinations of glutamate receptors and their subunit composition may influence motor neuron susceptibility to excitotoxicity (King *et al.*, 2006). Both ionotropic and metabotropic receptors are implicated in ALS; classically ionic are associated with direct toxicity via neurons and metabotropic via glial cells (Van Den Bosch *et al.*, 2006), however closer investigation of glial cells, notably oligodendrocytes and astrocytes, indicates they may be direct targets

for ionotropic mediated excitotoxicity (Belachew and Gallo, 2004; Matute *et al.*, 2006; Verkhratsky and Kirchhoff, 2007).

mGluRs act via g-protein coupled second messenger systems to induce release of calcium from intracellular stores or to reduce adenyl cyclase activity (Sladeczek *et al.*, 1985). mGluRs respond slowly following activation and are reportedly involved in regulation of long-term synaptic changes within neurons in response to glutamate neurotransmission (Bortolotto *et al.*, 1999; Heath and Shaw, 2002). Broadly, mGluRs are classified according to their cellular localisation and downstream signalling (Nicoletti *et al.*, 2011). mGluRs are also expressed on glial cells (Ohishi *et al.*, 1994; Berger *et al.*, 2012). Glial mGluRs in particular may be involved in the pathogenesis of ALS, regulating essential functions including cell proliferation, glutamate uptake, neurotrophic support and regulation of inflammation (Berger *et al.*, 2012).

AMPA receptors (AMPA-R) mediate fast synaptic glutamatergic neurotransmission. A critical property of AMPA-Rs is calcium permeability, determined by the calcium impermeable GluR2 subunit. Absent or incorrectly edited GluR2 confers Ca^{2+} permeability to the neuron (Hollmann *et al.*, 1991; Kawahara *et al.*, 2004). Regulation of GluR2 is crucial to the cell in determining susceptibility to excitotoxicity. Motor neurons have decreased expression of GluR2, resulting in an inherent susceptibility to excitotoxicity (Damme *et al.*, 2002). Interestingly, motor neurons that remain spared in ALS have higher expression of GluR2 (Ludolph *et al.*, 2000). GluR2 expression on motor neurons is influenced by surrounding cells, in particular reactive astrocytes via release of TNF-α during prolonged inflammation (Yin *et al.*, 2012). In addition, GluR2 and GluR3 are known to be expressed on microglial cells (Noda *et al.*, 2000). Prolonged activation of these receptors results in release of inflammatory cytokines from microglial cells, further potentiating the excitotoxicity/inflammatory response (De *et al.*, 2005).

Kainate receptors (KA-R) comprise GluR5-7 and share 75-80% sequence homology, yet only approximately 40% with GluR1-4, indicating a potential separate family of receptors (Hollman and Heinemann, 1994). Like AMPA-Rs, and other ligand-gated ion channels, KA-Rs have four putative transmembrane domains and extracellular C terminus. GluRs5-7 undergo alternative splicing and RNA editing, altering channel permeability to ions and affinity to agonists KA and glutamate. An additional two high-affinity KA-Rs KA1 and KA2 share 37% sequence homology with GluR1-4 and 43% with GluR5-7 (Sakimura *et al.*, 1990; Werner *et al.*, 1991; Herb *et al.*, 1992; Hollman and Heinemann, 1994). KA-Rs are implicated in excitotoxicity alongside AMPA-Rs. The two non-NMDA ionotropic receptor subtypes are often considered together, owing in part to the receptor homology, but also that both AMPA-Rs and KA-Rs are activated by kainic acid, thus making it difficult to discern which receptor is mediating excitotoxic damage. Interestingly, KA-R mediated excitotoxicity is associated with activation of the caspases 3 and 9, resulting in apoptosis (Matute and Cavaliere, 2011). This is in contrast with AMPA-R mediated excitotoxicity which results in activation of caspases 8 and 3 and necrosis (Matute *et al.*, 2006; Sánchez-Gómez *et al.*, 2011). Immature oligodendrocytes are vulnerable to KA-R mediated excitotoxicity (Matute *et al.*, 2007; Matute, 2011), however KA-R expression is down-regulated during oligodendrocyte maturation, with the predominant glutamate receptor being the NMDA-R (Káradóttir *et al.*, 2005; Salter and Fern, 2005; Micu *et al.*, 2006).

NMDA-R activation is slower than AMPA-Rs and is associated with the slow excitatory activation and longer-term changes to the synapse such as long-term potentiation (LTP) and long-term depression (LTD) (Kullmann *et al.*, 2000). The NMDA receptor (NMDA-R) forms a calcium permeable membrane pore, sensitive to the excitatory amino acids glutamate and glutamine (Henneberry *et al.*, 1989; Ludolph *et al.*, 2000). Regulation of

NMDA-R calcium permeability occurs via voltage-dependent Mg^{2+} blockade of the channel (Henneberry *et al.*, 1989; Ludolph *et al.*, 2000). Due to their high permeability to calcium, NMDA-Rs are considered the prime receptor for excitotoxicity (Murphy *et al.*, 1988; Frandsen and Schousboe, 1993), which may be mediated by NOX2 signalling (Reyes *et al.*, 2012). The key determinant of NMDA-R excitotoxicity appears to be synaptic vs extrasynaptic localisation of receptors (Bindokas and Miller, 1995). Activation of synaptically localised receptors under normal conditions is typically protective, however activation of extrasynaptic receptors is largely toxic (Hardingham *et al.*, 2002; Hardingham and Bading, 2010). Discrimination between synaptic and extrasynaptic activation may be achieved via phosphorylation of a key amino acid residues encoding the originating location (synaptic vs extrasynaptic) on translocating proteins, such as Jacob (Hardingham and Bading, 2010; Fainzilber *et al.*, 2011).

1.3.1.2 Glutamate transporters

Following neurotransmission, glutamate is rapidly cleared from the synaptic cleft via excitatory amino acid transporters (EAAT) 1-5 located predominantly on presynaptic astrocytes (Arriza *et al.*, 1994; Bosch *et al.*, 2006). EAATs are also located on pre- and post-synaptic neuronal membranes (Lee and Pow, 2010). Astrocytes are able to highly concentrate glutamate via active electrogenic sodium-potassium coupling and conversion of glutamate to glutamine to maintain low extracellular glutamate concentrations (Trotti *et al.*, 1998; Heath and Shaw, 2002). Impaired clearance of glutamate from the synapse has been linked with the development of excitotoxicity. EAAT2 in particular is potentially inhibited by kainic acid (Arriza *et al.*, 1994). Furthermore, the rat EAAT2 homologue GLT1 is down-regulated in cultured neurons (Rodriguez-Kern *et al.*, 2003),

as is GLT1 in human ALS tissue (Rothstein *et al.*, 1995), with corresponding elevated extracellular glutamate levels (Van Damme *et al.*, 2005). A number of studies have shown that glutamate levels in human patient CSF is high enough to induce excitotoxicity in cultured motor neurons (Couratier *et al.*, 1993; Nagai *et al.*, 2007), however these findings remain controversial. Additional studies by Yang and colleagues (2009) demonstrate that impairing the communication between astrocytes and neurons results in impaired astrocyte function and reduced GLT1 expression in a toxic feed-forward pathway. EAATs are also particularly vulnerable to permanent inactivation by oxidation from reactive oxygen species thus resulting in elevated extracellular glutamate (Bruno *et al.*, 2000).

1.3.2 Excitotoxicity

Neuronal vulnerability to excessive glutamate was first described in the 1950s with the observation that application of glutamate triggered retinal degeneration (Lucas and Newhouse, 1957). Excitotoxicity has since been recognised as a pathogenic mechanism in a number of neurodegenerative conditions, including ischaemia, glaucoma, Alzheimer's disease, epilepsy, multiple sclerosis and brain trauma (Hynd *et al.*, 2004; Kostic *et al.*, 2012) (Choi, 1988; Saggu *et al.*, 2008; Saggu *et al.*, 2010). Excitotoxicity occurs by over-stimulation of excitatory amino acid receptors, resulting in calcium influx and consequential pathological changes triggering neuronal loss (Carriedo *et al.*, 1996; Doble, 1996; Van Den Bosch *et al.*, 2000). The response of an individual neuron to excitotoxicity is dependent on a number of factors, including the intensity and duration of the excitatory insult, and the profile of excitatory receptors or downstream signalling molecules expressed by the cell (Arundine and Tymianski, 2003). Neuronal excitotoxic

injury is characterised by the formation of focal swellings, termed varicosities or beads along the dendritic arbour (Ikonomidou *et al.*, 1989), accompanied by loss of spines (Hasbani *et al.*, 2001; Ikegaya *et al.*, 2001). Excitotoxicity has also been implicated in axon degeneration, and in cultured cortical neurons degeneration is preceded by neurofilament loss (Chung et al., 2005). Furthermore, excitotoxicity has been shown to result in a distal axonopathy in retinal ganglion cells (Saggu et al., 2008) and cultured motor neurons (King *et al.*, 2007). The recent discovery of glutamate receptor subunits within myelinated CNS axonal nanocomplexes (Ouardouz *et al.*, 2009a,b) raises further questions into the pathogenesis of ALS. In particular, the notion that the axon itself could be a direct target of excitotoxicity (Stirling and Stys, 2010; Matute and Ransom, 2012). It is currently unknown how excitotoxicity results in axonal degeneration and recent findings have raised questions over exactly how excitotoxicity is induced in neurons.

Excitotoxicity may arise from different mechanisms, predominantly either from direct excitotoxicity or indirect. Direct excitotoxicity occurs following increased activation of glutamate receptors. This may occur due to increased extracellular glutamate or glutamate receptor agonists such as kainic acid (Bar-Peled *et al.*, 1999; Ludolph *et al.*, 2000; Van Damme *et al.*, 2005; Van Den Bosch *et al.*, 2006). Additionally, potent excitotoxic compounds such as quinolinic acid are produced during the inflammatory cascade (Stone and Perkins, 1981; Chen *et al.*, 2011). This mechanism is the predominant cause of excitotoxicity following traumatic brain injury, epilepsy, hypoxia and hypoglycaemia (Ludolph *et al.*, 2000; Van Damme *et al.*, 2005) via release of glutamate from injured and necrotic cells or reduced synaptic glutamate clearance via transporter blockade (Ludolph *et al.*, 2000; Heath and Shaw, 2002; Van Damme *et al.*, 2005; Van Den Bosch *et al.*, 2006). Conversely, indirect excitotoxicity occurs when extracellular levels of glutamate are normal, with the neuron becoming more sensitised to normal

glutamate levels. This process was described by Henneberry and colleagues (1989) after describing that oxygen or glucose stress to cells markedly reduced Mg^{2+} binding to the NMDA receptor, thus conferring increased Ca^{2+} permeability via the receptor (Cox *et al.*, 1989; Henneberry *et al.*, 1989). A similar effect occurs in the absence of the GluR2 AMPA receptor subunit, resulting in greater Ca^{2+} permeability following normal neurotransmission (Ludolph *et al.*, 2000; Van Den Bosch *et al.*, 2000). In addition, a number of environmental toxins such as β-oxalyl-N-amino-L-alanine (BOAA) can trigger excitotoxicity via mitochondrial damage, resulting in oxidative stress and elevated intracellular calcium (Van Damme *et al.*, 2005; Van Den Bosch *et al.*, 2006). This is in contrast with other toxins, such as the cyanobacterial toxin ss-N-methylamino-L-alanine (BMAA) which induces excitotoxic neuronal death by direct stimulation of both NMDA and non-NMDA glutamate receptors (Vyas and Weiss, 2009). Additionally, BMAA has been demonstrated to exert excitotoxicity-independent toxicity via interruption of protein handling (Dunlop *et al.*, 2013). The common mechanism for both forms of excitotoxicity is abnormal intracellular handling of calcium ions.

Calcium is a powerful intracellular signalling molecule and as such, intracellular Ca^{2+} levels are maintained under strict control (Parone *et al.*, 2013). Transient increases in intracellular Ca^{2+} occur following neurotransmission via voltage-gated ion channels during action potential propagation as well as in response to ligands binding to their associated receptors. Such increases are rapidly buffered by calcium binding proteins and mitochondrial sequestration of excess calcium (Berridge *et al.*, 2003; Parone *et al.*, 2013). The toxic action of glutamate in neurodegenerative disease occurs in a calcium-dependent process (Choi, 1987; Arundine and Tymianski, 2003). Motor neurons have reduced Ca^{2+} buffering proteins than less susceptible neuronal sub-types, rendering them more vulnerable to elevated intracellular Ca^{2+} (Ince *et al.*, 1993). Under pathological

conditions, a sustained elevation of intracellular calcium overwhelms the protective mechanisms and can lead to irreversible damage to intracellular components including nuclear genetic material, resulting in apoptotic or necrotic cell death (Orrenius *et al.*, 1989; Orrenius *et al.*, 2003; Van Damme *et al.*, 2005). Such activity is evidenced by activation of calcium effectors such as proteases, endonucleases, some kinases and nitric oxide synthase (Berridge *et al.*, 2003).

1.3.2.1 Vulnerability of motor neurons to excitotoxicity

Motor neurons are sensitive to excitatory amino acid toxicity, demonstrating vulnerability to kainic acid *in vitro* (Rothstein *et al.*, 1993; Carriedo *et al.*, 1996; King *et al.*, 2007) and *in vivo* (Sun *et al.*, 2006). A number of factors contribute to this sensitivity of motor neurons when compared with other neuronal subtypes, including differential expression of glutamate receptors, specifically the AMPA GluR2 subunit (Vickers *et al.*, 1993; Vandenberghe *et al.*, 2000). Mutant mice lacking ADAR, an enzyme required for normal editing of GluR2, develop motor neuron dysfunction attributed to increased permeability of the motor neurons to Ca^{2+} (Hideyama *et al.*, 2010). Additionally, motor neurons are deficient in calcium binding proteins which are required for the generation of rapid Ca^{2+} signals in other neurons (Ince *et al.*, 1993). Instead, Ca^{2+} buffering in motor neurons is almost exclusively performed by the mitochondria (Parone *et al.*, 2013). Induced expression of calcium-binding proteins such as calbindin D, in cultured motor neurons attenuates excitotoxicity by increasing the Ca^{2+} buffering capabilities of the cells (Roy *et al.*, 1998), however there is little evidence to show a similar mechanism occurs *in vivo*. Interestingly, the motor neurons which remain largely spared in ALS, nobably those of the occulmotor and Onuf's nuclei are less vulnerable to excitotoxicity. These resistant

motor neurons do not share the unique receptor or calcium binding protein expression profiles as vulnerable motor neurons (Alexianu *et al.*, 1994).

This specific vulnerability of motor neurons to excitotoxicity has been used as a basis for development of non fALS mouse models of ALS, such as transgenic mice with increased calcium permeability of AMPA receptors which develop a motor phenotype late in life (Kuner *et al.*, 2005). Furthermore, pathology in mSOD1 mouse lines can be reduced by pharmacological blockade of AMPA receptors, specifically targeting the GluR2 subunit (Tateno *et al.*, 2004), suggesting a close relationship between mSOD1 and excitotoxicity. The mechanism for this interaction is not well understood, however mutations to SOD1 are associated with increased production of reactive oxygen species and altered mitochondrial calcium dynamics, both of which are linked with excitotoxicity (Kruman *et al.*, 1999). One proposed mechanism for excitotoxicity focuses on altered RNA editing of glutamate receptors leading to increased vulnerability to excitotoxicity (Kawahara *et al.*, 2004).

Both upper and lower motor neurons exhibit hyperexcitability in the presymptomatic period in human patients (Vucic and Kiernan, 2006; Kiernan and Petri, 2012) and in transgenic SOD1 mice (van Zundert *et al.*, 2008). Hyperexcitability may arise from an imbalance between inhibitory and excitatory neuronal inputs. Whilst the direct consequence of hyperexcitability is not known, it is commonly theorised that hyperexcitability may increase the susceptibility of motor neurons to additional insults, including excitotoxicity (Bae *et al.*, 2013). Hyperexcitability of the motor system is a ubiquitous clinical finding in ALS (Kiernan, 2009), with patients typically presenting with muscle fasciculations and cramps (Kleine *et al.*, 2008), such that fasciculation potentials, detected by electromyography (EMG), are considered a hallmark finding in ALS patients (Swash, 2012). Furthermore, human ALS patients are characterised by an

increase in persistent Na^+ conductances which sensitises the axon to depolarisation (Mogyoros *et al.*, 1998; Kanai *et al.*, 2006; Nakata *et al.*, 2006; Vucic and Kiernan, 2006; Vucic *et al.*, 2007). Regulation of motor neuronal excitability occurs via interneuronal inhibition (Goulding, 2009). Critically, the main inhibitory neurotransmitter γ-aminobutyric acid (GABA), is reduced in ALS patients indicating reduced interneuronal inhibition (Turner and Kiernan, 2012). Unregulated modulation of motor neuron excitability is linked to development of excitotoxicity and subsequent pathology in ALS (Martin and Chang, 2012; Bae *et al.*, 2013).

1.3.2.2 Non-neuronal cells influence the susceptibility of motor neurons to excitotoxicity

Glial cells within the CNS play a key role during excitotoxicity. Specifically, glial cells influence neuronal vulnerability to excitotoxicity and determine neuronal survival (Dugan *et al.*, 1995; Rothstein, 2009). Astrocytes have been intimately linked with excitotoxicity in ALS. Specifically, astrocytes are heavily involved in a number of aspects of excitotoxicity. Astrocytes can alter motor neuron expression of glutamate receptors, likely in their capacity of modulating glutamatergic neurotransmission between motor neurons (Yang *et al.*, 2009). Astrocytic modulation of synaptic activity occurs largely via expression of glutamate transporters (Rothstein *et al.*, 1996; Cleveland and Rothstein, 2001) which are reduced in ALS (Rothstein *et al.*, 1995). Activation of astrocytes correlates with a targeted up-regulation of the calcium-permeable GluR2 subunit on motor neurons via TNF-α release (Van Damme *et al.*, 2007a; Yin *et al.*, 2012). Additionally, the vulnerability of oligodendrocytes to excitotoxicity may result in secondary damage to surrounding neurons (Matute, 2011).

1.4 PROJECT AIMS

The aim of this thesis is to investigate the association between excitotoxicity and axonopathy in ALS, and to better understand the process of NMDA degeneration in this devastating disease.

In recent years, a number of significant advances have been made toward understanding the pathogenesis of ALS (Robberecht and Philips, 2013). Notably, a substantial body of evidence exists to implicate excitotoxicity in ALS. However, a number of key processes still remain to be identified. It remains unclear if distal axonopathy arising from excitotoxicity is mediated directly via the axon or via somatodendritic glutamate receptors. Furthermore, the role of skeletal muscle in the development of motor neuron distal axonopathy is poorly understood. Determining both the primary site and source of excitotoxicity in ALS will provide insight into the initial pathogenesis of excitotoxicity in ALS and subsequent disease progression.

The work in this thesis will address these hypotheses and following aims:

1. ***Excitotoxicity is a pathogenic process in ALS that results in primary degeneration of the axon.***
2. ***The regulation of skeletal muscle Ca^{2+} forms a critical component in the development of distal axonopathy in ALS.***
3. ***Perturbed signalling between motor neurons and non-neuronal cells serves as a trigger for excitotoxicity.***

Aim 1 – To determine the effect of targeted excitotoxicity in a compartmented neuronal culture model.

The mechanisms by how excitotoxicity, primarily considered a somatodendritic event, results in degeneration of the axon, are not well understood; in particular, how this occurs without loss of the cell body. Recently, evidence has suggested the axon itself may be a primary target for excitotoxicity, following the discovery of glutamate receptor subunits in internodal regions within white matter (Ouardouz *et al.*, 2009a,b; Matute and Ransom, 2012). This thesis has utilised novel primary cortical neuron culture techniques to investigate axonally-mediated excitotoxicity. Additionally, this thesis has investigated similarities and differences between axonal excitotoxicity and 'classical' somatodendritic excitotoxicity.

Aim 2 – To investigate pathological changes to the neuromuscular junction of the mSOD1 G93A mouse model of ALS.

The role of skeletal muscle in the pathogenesis of both ALS and excitotoxicity remains controversial, however it has been proposed that altered muscular Ca^{2+} homeostasis may contribute to motor axon instability (Dobrowolny *et al.*, 2005). A better understanding of the distal pathology relating to loss of the NMJ will provide valuable insight into the wider disease process, and may also indicate potential new avenues for therapeutic intervention. Degeneration of the NMJ is considered a key pathological hallmark of ALS, and this progressive degeneration of the NMJ is recapitulated in the mSOD1 G93A mouse model of ALS which develops symptoms in a predictable and consistent manner . This thesis has investigated the NMJ in the mSOD1 G93A mouse to better understand the temporal and structural detail of this degeneration.

Aim 3 – To develop a novel *in vitro* platform for investigation of the lower motor neuron-neuromuscular junction circuit.

Despite a number of *in vitro* and *in vivo* models used to investigate excitotoxicity in motor neuron, the process still remains largely a mystery and none yet completely recapitulate the complexities of motor neuron growth *in vivo*. Perturbed motor neuron-non neuronal cell signalling may form the basis of dysfunction and may prove the tipping point which finally triggers ALS pathology. The interactions between a motor neuron and its associated glial cells and skeletal muscle target are vital for appropriate development, maturation and function of the motor neuron. Thus, a thorough understanding of early changes within the distal motor system is vital for approaching future therapeutic strategies. This thesis describes novel techniques to create a compartmented culture paradigm for motor neurons.

Aim 4 – To investigate targeted excitotoxicity in a novel compartmented model of lower motor neurons *in vitro*.

Despite the well-known connections between excitotoxicity and degeneration of motor neurons, the mechanisms by which this occurs are not well understood. In particular, how glial and muscle cells mediate the response of motor neurons to excitotoxicity. This thesis has investigated the response of motor neurons to focal excitotoxicity in a novel compartmented culture system developed in aim 3.

2 MATERIALS AND METHODS

2.1 ANIMAL CONDITIONS AND CARE

All animal experimentation was performed in accordance with the guidelines stipulated by the University of Tasmania Animal Ethics Committee, in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes. Animals were obtained via Animal Services (University of Tasmania) and housed in microisolator cages under standard conditions (20^{o}C, 12/12 hours light/dark cycle) with *ad libitum* access to food and water. Water, food and bedding were changed twice weekly.

Transgenic YFP mice (strain Tg(Thy1-YFP)16Jrs, Jackson Laboratory, USA) express yellow fluorescent protein (YFP) in a subset of neurons including motor neurons, with expression driven by the neuron-specific *thy1* promoter (Feng *et al.*, 2000). The YFP strain was maintained as a hemizygotic line, obtained from C57Bl/6 females crossed with hemizygous YFP males. Embryos for cell culture were obtained from timed matings, the finding of a mucous plug designated embryonic day 0.5. YFP mice were genotyped at four weeks of age following weaning by detection of YFP fluorescence in ear-clips.

Male transgenic mice expressing a high copy number of the mutant human SOD1 with a Gly-93-Ala substitution (strain B6.Cg-Tg(SOD1*G93A)1Gur/J, Jackson Laboratory, USA) (Gurney *et al.*, 1994) were maintained as a hemizygotic line, obtained from time-mated crosses between C57Bl/6 females and hemizygous mSOD1 G93A males. This mating regime is as suggested by Jackson Laboratories. mSOD1 G93A mice were monitored daily from 112-140 days of age to assess disease progression. Food was placed on the floor of the cage to facilitate feeding. The mice used for these studies were

humanely killed before reaching their ethical end-point, generally 24 weeks of age in this mSOD1 model. Ethical end-point was reached when mice had lost 20% body weight or were unable to right themselves within 30 seconds.

mSOD1 G93A mice were genotyped using standard PCR techniques from tail-clips. DNA was extracted using the Wizard® Genomic DNA purification kit (Promega) following the manufacturers instructions, and the concentration measured at A260. PCR was carried out using GoTaq® green PCR mix (Promega), a minimum of 100ng whole DNA and the following primer sets at a final concentration of 1.33μM; forward: 5'- CAT Cag CCC TAA TCC ATC TgA -3', reverse CgC gAC TAA CAA TCA AAg TgA. PCR cycling was performed on an Eppendorf Mastercycler gradient PCR machine using the following cycles;

95°C 3 minutes

95°C 30 seconds ┐
65°C 30 seconds ├ 35 cycles
72°C 45 seconds ┘

72°C 2 minutes

4°C hold

PCR product was detected with Cybr Safe® on a 2% agarose gel.

2.2 PRIMARY CELL CULTURE

All tissue dissection was performed using microscopic guidance (Nikon SMZ800 fitted with a fibre-optic light source Fiber-Lite, Dolan-Jenner Industries) in a laminar flow hood (Purair-VLF, Airscience). Stainless-steel instruments were autoclaved and sprayed with

70% ethanol prior to use. All tissue processing and subsequent maintenance of cultures was performed in a Class II Biosafety Cabinet (Purifier, Labconco) using aseptic technique.

13mm round glass coverslips (Marienfeld) were etched by immersion in 4M nitric acid for 4 hours followed by 30 minute rinse under running water. Coverslips were rinsed 3x 10 minutes in MilliQ® water. Coverslips were dried and autoclaved for use with cell culture. Treated coverslips were placed in individual wells of 24 well culture trays (Iwaki) using sterile tweezers. Sterility of trays was ensured by exposing to UV radiation for 15 minutes prior to coating coverslips with cell-type specific substrates. Substrates were incubated for 24 hours at room temperature and removed. Wells were filled with cell-type specific medium and trays allowed to equilibrate under standard cell culture conditions ($37^{o}C$, 5% CO_2) for two hours prior to addition of cells.

2.2.1 Microfluidic culture devices

The microfluidic device for culturing neuronal cells was developed by Taylor and colleagues (2005) to provide significant advances over previous techniques to compartmentalise neurons. Microfluidic devices are fabricated from PDMS to create two distinct compartments, separated by microchannels (10µm x 450µm). The design of the devices enables neuronal cells to be plated into one compartment and axons are able to extend into the second compartment. Additionally, such devices maintain two fluidically isolated microenvironments, allowing treatments to be applied to either the cell body or axon of cultured neurons (Figure 2.1).

Microfluidic chambers (SND450, Xona microfluidics) were prepared by removing surface particles with adhesive tape followed by sterilisation in 70% ethanol for 1 hour. Chambers were allowed to dry, attached to 22mm^2 glass coverslips (Livingstone, Germany) and placed in 6 well trays (Iwaki). 22mm^2 coverslips for use with microfluidic chambers were not acid-treated, however coverslips for compartmented rat motor neuron cultures were exposed to plasma treatment (Electro-Technic Products, Inc. Model BD-20V) for 30 seconds to facilitate substrate coating and chamber adhesion. Assembled devices were coated with substrates (refer to Chapters 3.2.1 and 5.2.1) for 24 hours. When different substrates were applied to distal and proximal compartments, a small mark was made on the under-side of the coverslip using permanent marker. Substrates were removed, devices filled with neuron initial medium and allowed to equilibrate under standard cell culture conditions (37^{o}C, 5% CO_2) for 2 hours prior to addition of cells. Culture medium was removed immediately prior to cell plating.

2.3 PHARMACOLOGICAL MANIPULATIONS

Pharmacological manipulations to cell cultures were performed at relative culture maturity (9DIV cortical neurons or 10DIV motor neurons) to either the proximal or distal compartment of microfluidic chambers. Compounds for treatment were diluted in warmed culture medium. Chambers for treatment were prepared by filling the side to be left untreated with warmed culture medium (400µL). 200µL treatments were added to the desired side. Ensuring the volume of the treated side was less than the untreated side creates fluidic isolation between the compartments. When both sides of the device required treatment, volumes were kept identical (200µL) to prevent fluid transfer between compartments.

2.3.1 Live cell imaging

Microfluidic devices were imaged under sterile conditions, returned to incubator conditions and re-imaged at desired timepoints. Live cell imaging was performed on an inverted microscope (Nikon TiE motorised fluorescence microscope fitter with a cooled CCD camera, NIS Software version 3.21) equipped with a temperature controlled chamber. Sterility of cultures was established by sealing chambers with a second sterile coverslip.

2.4 IMMUNOCYTOCHEMISTRY

Cells were fixed in 4% paraformaldehyde (PFA). All culture medium was removed from both sides of microfluidic devices and PFA added to one well of each side of the device. Chambers were incubated for 30 minutes at room temperature on an orbital shaker. PFA was removed and devices filled with PBS. Microfluidic chambers were carefully removed and washed for re-use (refer to section 2.2.1). Cells were rinsed 3 x 10 minutes in PBS and permeabilised using 0.3% Triton-X-100 diluent for 15 minutes. For methanol fixation cells were fixed and permeabilised for 20 minutes at -20^{o}C with ice-cold methanol, followed by 3 x 10 minute PBS washes. Diluent permeabilisation was omitted following methanol fixation. Primary antibodies were added at pre-determined concentrations, diluted in PBS (Table 2.1) and incubated at room temperature for 1 hour followed by overnight at 4^{o}C. Coverslips were rinsed 3 x 10 minutes in PBS and species-appropriate secondary antibodies (Table 2.2) added in PBS. Secondary antibodies were incubated for 2 hours at room temperature in the dark. Immediately following secondary antibody removal, nuclei were counterstained using either nuclear yellow (Hoechst S769121, Sigma) or DAPI (4',6-Diamidino-2-Pheylindole, Sigma) in PBS to visualise

nuclei (Table 2.3) and incubated 15 minutes at room temperature. Cells were rinsed 3 x 10 minutes in PBS and 1x in milliQ® prior to mounting on glass slides using PermaFluor® mounting medium (Dako). Slides were allowed to thoroughly dry at room temperature in the dark.

α-bungarotoxin^{594} (α-BTx) staining of cultures for neuromuscular junction identification was performed prior to diluent permeabilisation. α-BTx was added at 1:400 in PBS for 30 minutes in the dark. Subsequent antibody incubations were also performed in the dark. Phalloidin$^{488/594}$ staining for filamentous actin (f-actin) was performed at 1:200 for the last 30 minutes of secondary antibody staining.

2.5 ANIMAL PERFUSION AND TISSUE PROCESSING

Animals were sacrificed by injection of 140mg/kg sodium pentobarbitone. Transcardial perfusion was performed using 4% PFA in 0.1M phosphate buffer. Hind limbs were removed and post-fixed in 4% PFA for 24 hours post-perfusion. Tissue was washed in PBS and gastrocnemius muscles carefully dissected using microscopic guidance. For cryoprotection, gastrocnemius muscles were transferred to 30% sucrose in PBS for 48 hours at 4^{o}C. Gastrocnemius muscles were snap-frozen in cryomatrix tissue compound (OCT; Thermoscientific, UK), and sectioned transversely into 40µm sections using a cryostat (Leica, Australia). Tissue sections were placed directly onto slides, allowed to air-dry overnight and used immediately for immunohistochemistry.

2.6 IMMUNOHISTOCHEMISTRY

Muscle sections were washed (3 x 1 hour) in PBS to remove OCT. α-BTx (Table 2.3) staining was performed for 30 minutes and tissue washed 3 x 10 minutes. Antigen-retrieval was performed using ice-cold methanol for 30 seconds and sections were permeabilised using 0.3% Triton-X-100 for 2 hours. Primary antibodies were applied in PBS for 1 hour at room temperature and overnight at 4^{o}C. Sections were washed 3 x 10 minutes in PBS, species and isotype specific secondary antibodies were applied for two hours at room temperature in the dark. Nuclei were visualised using DAPI (Table 2.3) diluted in PBS for 15 minutes. Tissue sections were washed 3 x 10 minutes and glass coverslips applied using Permafluor mounting medium (Dako, Australia). Slides were allowed to thoroughly dry at room temperature in the dark.

3 CHRONIC EXCITOTOXICITY INDUCES AXON DEGENERATION IN COMPARTMENTED CORTICAL NEURONAL CULTURES

3.1 INTRODUCTION

Neuronal glutamate receptors, the mediators of excitotoxicity, are found on post-synaptic densities where they are involved in synaptic transmission. However, immunohistochemical techniques have demonstrated the presence of glutamate receptors at numerous extrasynaptic sites including the soma, dendrites and spines (reviewed in Newpher and Ehlers, 2008) and presynaptically (Tovar and Westbrook, 2002). Importantly, electrophysiological techniques have also indicated that these receptors can be functional (Andrasfalvy and Magee, 2001; Bardoni *et al.*, 2004). Dendritic extrasynaptic receptors, and specifically NMDA receptors, have been particularly implicated in excitotoxicity (Sattler *et al.*, 2000). Current evidence supports that notion that synaptic receptor activation promotes neuroprotection through activation of survival genes and suppression of apoptotic genes whereas extrasynaptic stimulation promotes cell death (Hardingham *et al.*, 2002), although this may be due to differences in receptor subunits (Liu *et al.*, 2007). Thus the distribution of synaptic and extrasynaptic NMDA receptors, rather than total calcium load, influence neuronal susceptibility and responses to excitotoxicity (reviewed in Hardingham and Bading, 2010).

Although excitotoxicity is considered primarily a neuronal somatodendritic insult, glutamate toxicity has also been demonstrated to occur in white matter tracts lacking neuronal cell bodies. This type of toxicity, often associated with brain injury, ischemia and glaucoma (Stys and Li, 2000; Saggu *et al.*, 2008), has been attributed to glial cells known to express functional glutamate receptors (Micu *et al.*, 2006; Matute, 2007).

Astrocytes are directly vulnerable to excitotoxicity via NMDA receptor activation (Lee *et al.*, 2010). In addition, oligodendrocytes are directly vulnerable to AMPA mediated Ca^{2+} excitotoxicity resulting in demyelination and secondary axonal pathology (Yoshioka *et al.*, 1995; Bannerman *et al.*, 2007). Excitotoxic activation of glial cells, in particular astrocytes, is associated with increased inflammation (Chen *et al.*, 2010b). A number of inflammatory cytokines and inflammatory metabolites such as quinolinic acid are known to be involved in the excitotoxic process (Stone and Perkins, 1981; Chen *et al.*, 2011). Furthermore, astrocytes play a critical role in modulating extracellular glutamate levels via EAAT2 transport and recycling of glutamate back to neurons (Trotti *et al.*, 1998; Heath and Shaw, 2002). Perturbations to this process are common in the pathogenesis of excitotoxicity.

However, the recent identification of axonal glutamate receptor subunits within axonal internodal nanocomplexes, raises the possibility of direct axonal excitotoxicity (Ouardouz *et al.*, 2009a,b). The functional status of these receptors remains disputed; they may be involved in local regulatory mechanisms within the internodal nanocomplexes they reside in (Matute and Ransom, 2012).

Investigation of the expression of glutamate receptors in different neuronal compartments and their role in excitotoxicity is difficult under standard culture conditions due to the inability to specifically target excitatory agonists. Similarly, *in vivo* investigations are complicated by the presence of glial cells. To overcome this, microfluidic devices (Taylor *et al.*, 2005) were used to establish compartmented embryonic cortical neuron cultures. Such devices allow fluidic isolation of distal axons from cell bodies, thus allowing focal exposure of the axon or soma to excitotoxins. In this study, the maturation of primary mouse cortical neurons was examined within a microfluidic device, in addition to immunocytochemical and western blot analysis of the expression of glutamate receptor

subunits in both the somal and distal axon compartments. To determine if excitotoxin induced axon degeneration can result from somal or axonal exposure to excitotoxin, the effect of a chronic (24hr and 72hr) exposure of glutamate was investigated within both proximal and distal compartments

3.2 METHODS

3.2.1 Cortical neuron culture

Cortical neurons were prepared from the cerebral cortices of embryonic day 14.5 (E14.5) C57Bl/6 mice. Time-mated females were sacrificed by CO_2, embryos removed and decapitated. Heads were kept on ice for the duration of the dissection to minimise tissue degradation. The superficial layers of each cortex were removed under microscopic guidance and transferred to ice-cold Hanks Balanced Salt Solution (HBSS; Gibco). Meningeal layers were removed and tissue transferred to 5ml HBSS. Cortical tissue was trypsinised (0.0125%) for 5 minutes, followed by removal of HBSS and mechanically triturated in 1ml neuron initial medium (Table 8.1). Cell density was determined using the trypan blue dye exclusion assay and the volume adjusted to achieve $8x10^6$ cells per ml. 20µl of the resulting cell suspension was plated into the cell body compartment of prepared microfluidic chambers (refer to Chapter 2.2.1). Plated chambers were returned to incubator conditions to facilitate cell adhesion for 30 minutes before filling with neuron initial medium and returning to the incubator. Medium was replaced with neuron subsequent medium (Table 8.1) at 2 days *in vitro* (2DIV) and maintained with weekly ½ medium changes.

3.2.2 Live cell labelling

To determine the percentage of neurons which extended axons via the microchannels into the distal axon compartment, the lipophilic membrane stain CM-DiI (1µg/mL; Molecular Probes) was added to the distal axon compartment. Fluidic isolation between the distal compartment and proximal compartment was achieved as described in Chapter 2.3.

Microfluidic devices were then incubated for 4 hours under standard cell culture conditions, followed by a medium change. Subsequent experimentation was performed the next day as required.

For morphological analysis of neuronal processes, neurons were transfected with a plasmid expressing Green Fluorescent Protein (GFP; pmax GFP, Lonza). 800ng plasmid DNA was applied to the proximal chamber using the Lipofectamine 2000 reagent (Invitrogen) for 6 hours under standard cell culture conditions and according to manufacturers instructions. Growth medium was replaced and transfected neurons visualised by fluorescence microscopy after 24 hours.

3.2.3 Excitotoxicity

Excitotoxicity was initiated in mature (11DIV) cultures (n = 5 repeats) by a single application 100µM glutamate, kainic acid or NMDA in culture medium (Chung *et al.*, 2005) to either the proximal/cell body or the distal axon chamber. Cultures were incubated under standard cell culture conditions for 24 hours and fluidic isolation between treated and non-treated chambers was achieved as described above. Additional cultures were treated with 100µM glutamate for 72 hours to the axonal chamber, beginning at 10DIV. Treated cultures were fixed with 4% PFA following treatment.

Pharmacological antagonism of kainate and NMDA receptors was achieved with 100µM CNXQ or MK801 diluted in culture medium to either the proximal or distal chamber. Cells (n = 2) were maintained in receptor antagonists for 1 hour prior to treatment with glutamate, kainic acid or NMDA. Treated cultures were fixed with 4% PFA.

3.2.4 Inhibition of caspase activity

The pan-caspase inhibitor Z-VAD-FMK (10μM; R&D Systems) was added to either the distal axon or proximal chamber to inactivate caspase activity. Cultures (n = 3) were pre-incubated with Z-VAD-FMK in culture medium for 2 hours and excitotoxicity subsequently induced as described above.

3.2.5 Cell fixation and immunocytochemistry

Cortical neuron cultures were fixed with 4% PFA as described in Chapter 2.4. Alternatively, cells were fixed in methanol for visualisation of the NR1 antigen (note: AMPA receptor immunoreactivity was not affected by this method of fixation). This was followed by incubation with primary antibodies (Table 2.1) and followed by secondary antibodies (Table 2.2) in PBS. Nuclei were stained using Nuclear Yellow (Table 2.3).

3.2.6 Western blot

For Western blot analysis, all protein from the proximal/somal or distal chambers were harvested from 10x microfluidic device (n=2 repeats) in ice-cold Tris-Trion buffer (10mM Tris, pH 7.4; 1000mM NaCl; 1mM EDTA; 1mM EGTA; 1% Triton-X-100; 10% glycerol; 0.1% SDS and 0.5% deoxycholate) supplemented with protease inhibitor cocktail (Complete™, Roche). Samples were separated by 12.5% SDS PAGE. Coomassie Brilliant Blue staining was also performed on a gel from each experiment. Remaining protein from gels were transferred to a PVDF membrane and blocked overnight in 5% non-fat dried skimmed milk powder. Membranes were incubated in primary antibodies (Table 2.1). Membranes were washed in TBS-Tween. Species appropriate HRP

(horseradish-peroxidase)-conjugated secondary antibodies (Table 2.2) were applied and visualised with chemiluminescent peroxidase substrate (Sigma).

3.2.7 Quantitation of axon degeneration

Four images were randomly captured from each distal axon chamber and immunolabelled for neurofilaments. A 4 x 5 50μm^2 grid was superimposed on each imaging using Adobe Photoshop (CS5) (Figure 3.1). Axons in each square of the grid were scored as either whole, beaded (distinguishable swellings connected by sections of axon) or fragmented (disconnected swellings) (Figure 3.1). Overall degeneration was calculated as the sum of beaded and fragmented axons. Values were expressed as a percentage of total axons. Total values from each square were averaged for each coverslip and analysed using Student's t-Test or one-way ANOVA with Tukey's post-test comparisons. $P<0.05$ considered significant. Data are represented as mean ± SEM.

3.3 RESULTS

3.3.1 Developmental characteristics of compartmented cortical neuronal culture

Mouse cortical neuron cultures were established within compartmented microfluidic culture devices (Taylor *et al.*, 2005) and their growth characteristics were examined over a time course (Figure 3.2). Initial neuronal development (1-3DIV) was restricted to the proximal chamber. By 5DIV, the neurons had extended multiple neurites within the somal chamber and axons were present within the microchannels. At 7DIV long, relatively unbranched axons were present extending from the microchannels into the distal axon chamber, forming an extensively branched network at 11DIV. Isolated axonal health declined from 14DIV, with extensive distal axon degeneration present at 15DIV. Degeneration of axons within the distal axon chamber from 15DIV was not accompanied by degeneration within the somal chamber.

Double immunolabelling verified that axons (NFM immunoreactivity) were present in the distal axon chamber, with neuronal cell bodies and dendrites (MAP2 immunoreactivity) restricted to the somal chamber (Figure 3.3), as previously described by Taylor *et al.* (2005). NFM immunoreactive axons were also present in the somal chamber. Neurons with axons extending into the distal chamber were identified by incubating the distal axon chamber with CM-DiI prior to treatment. CM-DiI was taken up by the axons and transported to the cell body (Perlson *et al.*, 2009) (Figure 3.3). DiI retrograde labelling from the distal axon chamber indicated that approximately 30% of neurons extended axons to the distal chamber.

Neuronal maturity in compartmented cultures was determined by examining the presence of growth cones, synapses and spines. Previous investigations have demonstrated that under standard conditions, immature neurons prior to the development of synapses, have

numerous growth cones (Haas *et al.*, 2004). As neurons mature, punctate synapses and mushroom shaped spines are formed on the dendrites accompanied by the loss of growth cones (King *et al.*, 2006). In the current study, the presence of growth cones in compartmented cultures was examined by phalloidin staining for filamentous actin and synaptic puncta were visualised by immunolabelling with the presynaptic marker synaptophysin, in addition to the dendritic marker MAP2. Dendritic spine morphology was determined by examination of neurons transiently transfected with a GFP expression construct. In the somal chamber at 11DIV, few growth cones were present and synaptophysin positive puncta were immunolabelled along MAP2 immunopositive dendrites (Figure 3.4A), with the number and density of puncta varying between cells, congruent with previous investigations (King *et al.*, 2006). Furthermore, GFP expression revealed short, mushroom-shaped spines (Figure 3.4B) on the dendrites in addition to a number of long filopodial spines (Figure 3.4C). These data indicate that, in the somal chamber, synapses were present in accordance with neuronal maturity in standard cultures (King *et al.*, 2006). However, in the distal axonal chamber at this developmental stage, large growth cones were present on the tips of axons (Figure 3.4D,E), indicating a stage of immaturity not usually seen in standard cultures at this time point *in vitro*.

3.3.2 Glutamate receptor expression in compartmented cultures

To examine whether components of the machinery required for functional glutamatergic signalling, and therefore excitotoxicity, were present, the expression of glutamate receptor subunits was determined. Expression of both NMDA and AMPA receptors was examined within the somal and distal axon chambers of mature (11DIV) cultures by immunocytochemistry using glutamate subunit specific antibodies. The expression

profiles of the receptors varied between neurons within each culture. Within the somal chamber, immunoreactivity for AMPA (GluR1) and NMDA (NR1) receptor subunits was present throughout the soma, with punctate expression along the dendrites (Figure 3.5 A,B). Within the distal chamber, AMPA subunit labelling (GluR4) was frequently present and punctate along distal axons and within the growth cone (Figure 3.5C). Immunoreactivity for NMDA receptors was occasionally present within distal axon growth cones (Figure 3.5D).

To confirm the presence of glutamate receptor subunits in the somal and distal axon chambers, protein was harvested from each chamber and glutamate receptor subunit expression determined by western blot analysis. Coomassie staining of SDS/PAGE gels demonstrated a good yield of protein from both chambers (Figure 3.5 E). Western blots confirmed the presence of NMDA (NR1 labelling at 120kDa) and AMPA (GluR1 GluR2, GluR3 and GluR4 labelling at 100kDa) receptors in the somal chambers (Figure 3.5F). Interestingly, western blot analysis also demonstrated the presence of AMPA receptors in protein harvested from the distal axon chamber. NMDA receptors, however, were not detected in the distal axon chamber. Together, these data indicate differential expression of glutamate receptors subunits on both the somatodendritic compartment and distal axon of the cortical neuron, with strong evidence for the presence of AMPA receptor subunits in the axon.

3.3.3 Functional contribution of expressed glutamate receptors to focal excitotoxicity

To determine the role of the axon in mediating excitotoxicity, mature (11DIV) mouse cortical neurons were treated with 100μM glutamic acid or vehicle control, applied to

either the somal or the distal axon chamber of the microfluidic device, and maintained for 24 hours post-treatment. Following treatment, distal axon degeneration was assessed based on neurofilament immunoreactivity for axonal integrity.

Glutamate applied to the somal chamber resulted in extensive neuronal degeneration, both within the treated chamber and at the untreated distal axon. Specifically, axons in both chambers frequently showed a beaded morphology and additionally complete axon fragmentation was present in some axons. Quantitative analysis of neurofilament immunoreactivity in the untreated distal axon chamber demonstrated that somal glutamate treatment resulted in a significant ($P<0.01$) three-fold increase in total axon degeneration, including the sum of both beaded and fragmented axons (80.5% ± 2.9). Beading (32.2% ± 2.4) and fragmentation (49.5% ± 3.3) were significantly ($P<0.01$) increased relative to untreated controls (26.9 % ± 2.8 total damage; 13.5% ± 1.7 beading; 14.3% ± 1.5 fragmentation) (Figure 3.6A). Widespread axonal degeneration was also present in the treated somal chamber (not quantified) (Chung *et al.*, 2005).

Next, the axons were specifically targeted to determine whether axonal excitotoxicity could also result in degenerative changes. Twenty-four hour glutamate exposure also resulted in beading and fragmentation of the distal axon. Quantitative analysis of neurofilament immunoreactivity demonstrated a significant ($P<0.01$) 1.5 fold increase in total degeneration (46.7% ± 3.2%), axonal beading (21.3% ± 1.0%) and axonal fragmentation (25.5% ± 2.3%) when compared with untreated controls (26.9 % ± 2.8%, total damage; 13.5% ± 1.7% beading; 14.3% ± 1.5% fragmentation) (Figure 3.6A). The degenerative changes in distally treated cultures was significantly ($P<0.01$) 1.5 fold less than the distal degeneration in somal treatment, assessed as both axonal beading and fragmentation.

The neuron-wide effects of somal and axonal excitotoxin exposure were examined to determine the extent of neuronal damage. In addition to distal axon morphology (neurofilament immunoreactivity) (Figure 3.6B-D), changes to dendrites (MAP2 immunoreactivity) (Figure 3.6E-G) and overall cell health were examined, using nuclear yellow to assess nuclear integrity (Figure 3.6H-J). Somal excitotoxicity resulted in severe dendritic beading at 24 hours post-treatment (Figure 3.6E), as has been described previously (Park *et al.*, 1996). There was a significant (20% ± 3.2%, $P<0.01$) increase in apoptotic (condensed) nuclei following somatodendritic excitotoxicity (Figure 3.6I). In contrast to somal exposure, the unexposed somal chambers of distally treated cultures had no change to dendritic morphology relative to controls (Figure 3.6G). The nuclear morphology of CM-DiI stained neurons were assessed, however there was no difference in the percentage of apoptotic nuclei between axonal excitotoxicity at 24 hours and untreated controls (25.4% ± 2.0% 24hr excitotoxicity; 22.7% ± 1.3% control).

To investigate whether delayed apoptosis occurred, through dying-back from the axon, cultures were treated with 100μM glutamate to the distal axon chamber for 72 hours at 10DIV and axon degeneration and cell death assessed. 72 hour treated cultures demonstrated a significant ($P<0.01$) increase in total axon degeneration from untreated controls (52.5% ± 4.3% and 32.5% ± 2.6% respectively), however this was not significantly ($P>0.05$) different to cultures treated for 24 hours (46.7% ± 3.2%) (Figure 3.7A). Additional cultures were labelled with CM-DiI to the distal axon chamber prior to treatment to specifically label neuronal soma with axons in the distal chamber. Following treatment, the nuclear morphology of DiI positive soma was assessed with no significant ($P>0.05$) change to the percentage of apoptotic nuclei relative to controls (13.9% ± 1.9% 72 hour glutamate, 17.8% ± 1.4% controls) (Figure 3.7B).

3.3.4 Both NMDA and AMPA/KA receptors contribute to distal axon degeneration following targeted excitotoxicity

To determine which glutamate receptors are involved in axonal excitotoxicity, 11DIV cultures were treated with 100μM KA or NMDA to either the proximal or distal chamber. Somatodendritic exposure of both glutamate receptor agonists resulted in a significant (P<0.05) increase in distal axon degeneration compared with untreated controls (Figure 3.8A). Distal application of KA did not result in a significant (P>0.05) change in distal axon degeneration, however distal NMDA did resulted in a significant (P<0.05) increase in distal axon degeneration (Figure 3.8A). To further investigate the receptors involved, 11DIV distal axons were pre-incubated in either CNQX or MK801 prior to exposure to 100uM glutamate to either the proximal or the distal compartment. The preliminary results obtained in these analyses suggest distal excitotoxicity may not be mediated solely via NMDA or non-NMDA receptors (Figure 3.8B). Pre-incubation of cells with both CNQX and MK801 reduced distal axon degeneration to similar levels of the vehicle-treated controls (Figure 3.8B).

3.3.5 Mechanisms of focal excitotoxin induced axonal degeneration

To investigate whether axon degeneration following soma or axonal excitotoxicity involved activation of caspases and apoptotic pathways, 11DIV cultures were pre-incubated with 10μM Z-VAD-FMK pan-caspase inhibitor to either the somal or distal axon chambers. Cultures were subsequently treated with 100μM glutamate for 24 hours to induce excitotoxicity. The efficacy of the inhibitor was confirmed by a significant (P<0.05) decrease in apoptotic nuclei following somal excitotoxicity when compared to

the same treatment with no inhibitor (16.06% ± 3.93% glutamate only; 3.93% ± 3.15 inhibition + glutamate).

Somal caspase inhibition prior to somal excitotoxicity significantly ($P<0.01$) decreased distal axon degeneration (31.3% ± 3.2%) when compared with somal treatment alone (54.5% ± 2.3%) (Figure 3.9A). Axonal caspase inhibition combined with somal excitotoxicity also significantly ($P<0.01$) reduced axon degeneration (36.4% ± 2.1%) (Figure 3.9A). Somal caspase inhibition and axonal caspase inhibition similarly significantly ($P<0.01$) decreased axon degeneration in conjunction with axonal excitotoxicity (2.5% ± 4.0% and 9.9% ± 2.4% respectively) compared with axonal excitotoxicity alone (20.6% ± 3.2%) (Figure 3.9B). For both axonal and somal excitotoxicity there was no significant ($P>0.05$) difference between somal and axonal application of caspase inhibition.

3.4 DISCUSSION

In this study, primary cortical neurons were grown in a compartmentalised microfluidic device to determine the expression of glutamate receptors in specific neuronal compartments and to investigate degenerative responses following chronic targeted distal axon excitotoxicity. Of relevance to the current study, previous investigations (Choi, 1987; Liu *et al.*, 1996; King *et al.*, 2006) have demonstrated that neuronal vulnerability to excitotoxicity is dependent on neuronal maturity and expression of glutamate receptor subunits, which is variable between cell types. Thus, for the current study it was important to determine the maturity of neurons within the microfluidic culture chambers.

Under standard growth conditions, development of primary murine cortical neurons occurs via a sequence of predetermined steps that include neurite outgrowth, polarisation and elongation, followed by movement of glutamate receptors into the synapses and loss of immaturity markers such as growth cones (Dotti *et al.*, 1988; Haas *et al.*, 2004). The timing of these developmental stages is dependent on culture density (de Lima *et al.*, 1997; Rao *et al.*, 1998). Assessment of neuronal growth of cultures in microfluidic chambers indicates that development occurs in a similar manner to standard cortical culture within the somal chamber, including the presence of mature spines and punctate glutamate receptor subunits at 11DIV. However, in the axonal chamber, even at relative culture maturity (11DIV), numerous axonal growth cones remained present. The presence of growth cones is likely to be due to the inability of presynaptic neurons to find post-synaptic partners in this culture system and may affect the axonal expression of glutamate receptor subunits.

The current data indicate that, in addition to the well-documented expression of glutamate receptor subunits on the somatodendritic compartment of cultured primary cortical

neurons (King *et al.*, 2006), glutamate receptors, and in particular AMPA receptors, were also present on axons. Excitotoxic stimulation of axonal glutamate receptors resulted in axonal degeneration, which, unlike somatodendritic exposure, was confined to the exposed segment of the axon and did not cause retrograde degeneration or apoptotic cell death, even at extended time-points of 72 hours. The application of both KA and NMDA to the distal axon compartment resulted in distal axon degeneration, however there was no significant protective effect following pharmacological blockade of either NMDA or non-NMDA receptors. These data also suggest the presence of functional glutamate receptor complexes on the axons in this chamber.

3.4.1 Axon degeneration and excitotoxicity

Axon degeneration following glutamate receptor stimulation has been previously reported in a number of studies. *In vitro* studies have indicated that axon degeneration occurs as a result of chronic excitotoxicity in cultured motor (King *et al.*, 2007) and cortical (Chung *et al.*, 2005) neurons. In these studies, however, glutamate or other agonists were globally applied to the cells and so it is unclear if axon degeneration occurred from toxicity to somatodendritic glutamate receptors, or receptors present on axons, growth cones or pre-synaptic terminals. *In vivo* studies have allowed focal excitotoxin exposure of neuronal compartments. Somal glutamate exposure to retinal ganglion cells resulted in a degeneration of the distal axon (Saggu *et al.*, 2008), confirming excitotoxic axonal degeneration in the unexposed axon segment, consistent with the current study in cortical neurons. *In vivo* glutamate exposure to myelinated axons also resulted in axonal damage to the optic nerve (Matute, 1998) and external capsule (Fowler *et al.*, 2003). The vulnerability of myelinating oligodendrocytes to excitotoxicity has been well documented

and myelin has been demonstrated to play a role in axonal excitotoxicity (Fowler *et al.*, 2006). However, studies using myelin-deficient Shiverer mice show that compact myelin is not required for AMPA toxicity to axons (Pitt *et al.*, 2010).

A study by Underhill and Goldberg (2007) utilised a Campenot style compartmentalised culture system to directly examine the role of glutamate receptor activation in axon degeneration in the absence of glial cells. Their data demonstrated that brief (two hour) axonal exposure to glutamate receptor agonists, NMDA or AMPA, did not result in significant axonal degeneration. Conversely, the current study using a compartmentalised microfluidic culture system, demonstrates the novel finding that chronic (24 hour) glutamate exposure results in axonal blebbing and fragmentation in a proportion of axons. The reasons for these conflicting results may be the length of time of the exposure, the agonists used or differences in the culture systems, which may select for isolation of axons from specific cell types. Interestingly, *in vivo* studies also suggest difference in chronic versus acute excitotoxic axonal exposure (Matute, 1998). These data suggest that excitotoxicity can be mediated through glutamate receptors expressed on the axon.

3.4.2 Excitotoxicity and glutamate receptor expression

In the current study excitotoxicity could be mediated through glutamate receptors expressed either on the axon shaft or on the growth cones. Glutamate receptors can be trafficked to the axonal compartment and in particular their presence within growth cones during neuronal development has been well documented (King *et al.*, 2006), with a proposed involvement in pathfinding (Zheng *et al.*, 1996). Recently, subunits for AMPA and kainate receptors have been shown to be present on myelinated axons (Ouardouz *et al.*, 2009a; 2009b), although the functional activity of these receptors is currently

disputed. This data confirms the presence of AMPA receptors in the axonal compartment of cultured axons by immunocytochemical labelling and Western blot analysis. The role of these axonal glutamate receptors is unclear, however the expression of functional NMDA and non-NMDA receptors on glial cells (Gallo and Russell, 1995; Verkhratsky and Kirchhoff, 2007) raises the possibility that axonal glutamate receptor subunits may facilitate glutamatergic signalling between axons and myelinating oligodendrocytes or astrocytes.

A limitation of the current study is that cultured distal axons were unable to synapse on other neurons, preventing the formation of the presynaptic terminal. This excludes the possibility that excitotoxicity was mediated through presynaptic receptors. *In vivo*, both NMDA and AMPA receptors are found presynaptically, where they are thought to regulate glutamate release (recently reviewed in Pinheiro and Mulle, 2008) and could potentially be targets for excitotoxic stimulation. Although the expression pattern of axonal glutamate receptors may differ *in vivo*, the current study suggests that excitotoxicity can be mediated through extra-synaptic glutamate receptors expressed on the axon.

The demonstration of the extra-synaptic expression of glutamate receptor subtypes (Passafaro *et al.*, 2001; Tovar and Westbrook, 2002; Kane-Jackson and Smith, 2003; van Zundert *et al.*, 2004), suggests a wider role for glutamate than solely as an inter-neuronal excitatory transmitter (Araque and Perea, 2004). Glutamate receptor subunits at non-synaptic sites on dendrites and spines are thought to act as a reserve supply of synaptic receptors (for recent review see Newpher and Ehlers, 2008). However, electrophysiological recordings indicate that these extrasynaptic receptors are functional (Andrasfalvy and Magee, 2001) and a modulatory role has been suggested, through activation by glutamate spillover from the synapse or glial derived glutamate (Diamond

and Jahr, 2000; Jourdain *et al.*, 2007). In terms of pathological stimulation of glutamate receptors, extrasynaptic and synaptic receptors have been reported to play a significantly different role in excitotoxicity. Stimulation of synaptic NMDA receptors is neuroprotective, whilst activation of extrasynaptic NMDA receptors triggers neuronal degeneration (Hardingham *et al.*, 2002). Although, some authors demonstrate preferential expression of receptor subunit types at extrasynaptic sites is responsible for this effect rather than localisation itself (Liu *et al.*, 2007).

3.4.3 Mechanisms of axonal glutamate excitotoxicity and implications to disease

Mechanism of axonal degeneration, including potential differences between axonal and somal glutamate stimulation, can not be fully determined in the current study, however, the protective effect of a pan-caspase inhibitor suggests the involvement of pathways associated with apoptosis. It is of particular interest to note that application of inhibitors to either the soma or axon provided protection from degeneration. The role of axonal caspases in axonal degeneration is being increasingly recognised and has been reported in a number of models (Schoenmann *et al.*, 2010; Smith *et al.*, 2011). This study also suggests that retrograde signalling to the soma is involved in axon degeneration following axonal excitotoxicity, without inducing frank apoptosis. Further elucidating the mechanisms of excitotoxin induced axon degeneration will be the subject of future studies using this model.

3.4.4 Conclusions

The findings of the current study have a number of implications for our understanding of neurodegenerative disease. Excitotoxicity within the white matter has been shown to occur in a number of degenerative conditions including glaucoma (Saggu *et al.*, 2008) and multiple sclerosis (Pitt *et al.*, 2003), and is also common following injury (reviewed in Lau and Tymianski, 2010). Furthermore, axonal excitotoxicity may be involved in any condition involving excitotoxic pathogenesis including, potentially, amyotrophic lateral sclerosis (Van Damme *et al.*, 2005) and Alzheimer's disease (Hynd *et al.*, 2004), with local regions of excitotoxicity triggering axon degeneration and synaptic loss. At present, excitotoxic damage is attributed to neuronal soma and glial cells, and secondary axon degeneration via axon-glia signalling. The current study suggests that axon degeneration could occur from direct exposure of the axon to excitotoxins. However, axonal excitotoxicity did not result in a dying back, even 72 hours following exposure. The relative immaturity of the axons through lack of presynaptic targets and therefore retrograde signals from postsynaptic cells may be a factor in the lack of die-back in this study. It is currently unknown how axonally mediated excitotoxicity affects a cell's survival in mature cells *in vivo* in the long-term. Additionlly, further investigations are required to determine which receptor complex is primarily responsible for axonally mediated excitotoxicity. Determining both the receptors involved and the signalling mechanisms involved in distal axon degeneration following excitotoxicity may have therapeutic implications as intervention may need to be directed specifically to induce axon protection.

4 DEGENERATION OF THE UNDERLYING STRUCTURAL PROTEINS OCCURS DURING DEGENERATION OF THE MSOD1 G93A NMJ

4.1 INTRODUCTION

There is increasing evidence that the distal axon and synapse are an early and important cellular target for pathology in ALS (Raff *et al.*, 2002; Wishart *et al.*, 2006). Loss of NMJ is considered the first pathophysiological event in ALS and occurs from loss of the motor neuron axon resulting in loss of the post-synaptic apparatus (Dupuis and Loeffler, 2009). Consequently, degeneration in ALS is regarded by many as a distal pathology which develops from 'dying back' of the motor neuron distal axon (Fischer *et al.*, 2004; Fischer and Glass, 2007).

Clinical distal defects have also been characterised in human ALS, indicative of dying back axonopathy (Fischer *et al.*, 2004; Fischer and Glass, 2007). Electromyography (EMG) demonstrates loss of motor neurons accompanied by fasciculation potentials and spontaneous fibrillation in human skeletal muscles (Maselli *et al.*, 1993). Similarly, quantitative morphometric analysis has established distal-to proximal progression of axonopathy in ALS patient phrenic nerves (Bradley *et al.*, 1983). Histological analysis of human skeletal muscle biopsies from confirmed ALS cases reported loss of presynaptic components from the NMJ (Tsujihata *et al.*, 1984). Difficulties in accessing high-quality pre-symptomatic or early symptomatic human skeletal muscle samples has hindered investigation of early distal changes in ALS, however many of these processes are replicated by murine models of ALS, including the commonly used mSOD1 G93A mouse (Fischer *et al.*, 2004). In the mouse, post-synaptic AChRs slowly dissipate from

the initial healthy 'pretzel' organisation and become increasingly shrunken and fragmented over several weeks following denervation (Li and Thompson, 2011). This is proposed to occur via progressive destabilisation of the basal lamina (Marshall *et al.*, 1977; Li and Thompson, 2011). Human skeletal muscle biopsy samples from diagnosed ALS patients indicate a similar slow process of AChR dissociation (Tsujihata *et al.*, 1984).

In addition to distal degeneration, mSOD1 models have revealed a number of pre-symptomatic changes, including vulnerability to environmental stresses such as hypoxia during the presymptomatic period (David *et al.*, 2007). Such environmental triggers have been demonstrated to act in multiple neuronal compartments, including the NMJ (David *et al.*, 2007). Murine models for peripheral motor neuropathy, bax deletion and mSOD1 G93A have also indicated that the triggering and regulation of degeneration in ALS occurs in a compartmented manner, with prevention of somal degeneration rarely extending to protection of the distal axon or improvement in clinical outcomes (Sagot *et al.*, 1995; Gould *et al.*, 2006; Dewil *et al.*, 2007). Thus, degeneration of the distal components is a key process in ALS, however the sequence of changes is not fully known. Furthermore, understanding the post-synaptic changes will help determine the role of muscle versus motor neuron in the mechanisms of degeneration of the NMJ.

The NMJ is a complex synapse with numerous interconnected signalling pathways involved in coordination between synaptic input and muscle contraction (Wu *et al.*, 2010). Development of the NMJ requires precise signalling between the motor terminal and skeletal muscle fibre to coordinate localisation of post-synaptic AChRs to enable synaptic transmission (reviewed in Wu *et al.*, 2010). Much of the information regarding the structural components of the NMJ has been gathered in developmental models utilising a variety of protein knock-down and gene silencing techniques (reviewed in Wu

et al., 2010). Consequently, little is known about many of the structural proteins in the context of aging and degenerative models. Briefly, formation of the NMJ centres around three main pathways; 1) agrin/LRP4/MuSK signalling to promote aggregation of AChRs at the developing synapse, 2) NRG1/ErbB signalling to stabilise the synapse and promote AChR expression by synaptic nuclei and 3) ACh voltage-dependent neurotransmission and signalling to downregulate AChR expression by non-synaptic muscle nuclei (Sanes and Lichtman, 1999; Wu *et al.*, 2010; Nishimune, 2012). For the purpose of this project, a number of proteins that perform a range of functions in these signalling cascades were selected for investigation in a timecourse of the mSOD1 G93A mouse.

The mSOD1 G93A mouse develops ALS-like symptoms around 86 days of age, reaching end-point between 160 and 175 days of age (Gurney *et al.*, 1994) and is widely used in ALS research. The mSOD1 G93A mouse has a rapid disease progression, thus making it a suitable model to investigate pathology in a time and cost effective manner. In mSOD1 models, the timing of NMJ loss varies depending on the muscle fibre composition (Fischer *et al.*, 2004; Pun *et al.*, 2006). The role of skeletal muscle in degeneration of the NMJ remains controversial in both ALS and in associated animal models (Dupuis and Loeffler, 2009). Specific down-regulation of mSOD1 in transgenic mSOD1 models has no effect on the disease process (Miller *et al.*, 2006; Towne *et al.*, 2008). Conversely, muscle-specific expression of mSOD1 produced some key features of ALS, notably spinal cord astrocytosis and inflammation, and also severe pathology within the skeletal muscle (Dobrowolny *et al.*, 2008; Wong and Martin, 2010). Despite the controversy, some still regard the skeletal muscle as a primary target for mSOD1 mediated toxicity (Dobrowolny *et al.*, 2005; Dobrowolny *et al.*, 2008; Wong and Martin, 2010), with beneficial effects of IgF-1 and exercise noted (Kaspar *et al.*, 2003). Others have

suggested an active role of the muscle fibre in development of ALS (Cappello *et al.*, 2012).

One of the disadvantages to a mSOD1 model is the potential for the rapid progression to obscure some of the more subtle aspects of degeneration. Onset of degeneration in the mSOD1 G93A mouse consistently begins with the hind-limbs, leaving the forelimbs relatively spared (Gurney *et al.*, 1994; Chiu *et al.*, 1995; Azzouz *et al.*, 1997; Bendotti and Carri, 2004). The forelimb muscles in the mouse represent a currently understudied region of murine musculature (Mathewson *et al.*, 2012) and may represent a novel insight into presymptomatic alterations that occur at the NMJ in the mSOD1 mouse model.

In this study, pre-synaptic motor terminals, skeletal muscle proteins nestin, rapsyn and dystrophin and Schwann cells were examined over a time-course of mSOD1 G93A and wild-type control mice. Although we know that the presynaptic axons die back before the AChRs are lost it is unclear if this is preceded by loss of postsynaptic structural proteins or alteration to the terminal Schwann cells. Analysis of pre-synaptic terminals indicated a significant loss of colocalisation with post-synaptic α-BTx staining at 56 days of age. At 84 days, presynaptic terminals, nestin, rapsyn and Schwann cells were significantly less colocalised with α-BTx compared with controls. Dystrophin colocalisation was significantly reduced from α-BTx by 112 days. Analysis of the forelimbs, however only indicated a decrease in pre-synaptic terminals late in disease, at 140 days. These results indicate that loss of pre-synaptic terminals is likely the main factor in development of ALS pathology however these results also indicate that loss of α-BTx is preceded by alterations to the underlying postsynaptic structure.

4.2 METHODS

All animal use was approved by the Animal Ethics Committee of the University of Tasmania. Mice were housed in 12 hour light/dark cycles and monitored daily for signs of distress or illness. mSOD1 G93A (Chapter 2.1) mice (Gurney *et al.*, 1994) were checked daily from 16 weeks of age to monitor disease progression, with additional food placed on the floor of the cage as required. To minimise suffering, animals were sacrificed if considered to have reached end-point, determined by either a 20% loss from maximal body weight or inability to right once rolled onto their back (Schutz *et al.*, 2005).

4.2.1 Tissue collection

WT and high copy number mSOD1 G93A mice at 28, 56, 84, 112 and 140 days of age (n=4 each group) were transcardially perfused with 4% paraformaldehyde. The hind-limb lateral gastrocnemius muscle and forelimb extensor muscles were dissected (refer to figure 4.1) and cryoprotected prior to sectioning. The forelimb extensors incorporate the extensor carpi radialis longus, extensor digitorum communis, extensor digitorum lateralis and extensor carpi ulnaris (Elashry *et al.*, 2009; Mathewson *et al.*, 2012) (Figure 4.1) and was selected as a region analogous to the gastrocnemius muscle in the absence of a directly comparable muscle mass. Muscles were cryo-sectioned at 40µm, beginning 1mm from the lateral surface in gastrocnemius and 100µm from the lateral surface in forelimb muscle. Sections were placed directly onto coated FLEX immunohistochemistry slides (Dako) and washed 4 hours in PBS at room temperature to remove OCT compound for immunohistochemistry. Tissue sections were processed identically to minimise batch variation.

4.2.2 Immunohistochemistry

Sections were stained for acetylcholine receptors (AChRs) using α-BTx (Table 2.3). α-BTx was applied to each section (1:200) for 30 minutes. Sections were washed 1x 1min in ice-cold methanol to reveal epitopes, followed by 4 hours in 0.1% Triton-X-100 diluent to permeabilise cells. Neurofilaments were labelled using a cocktail of mouse-anti phosphorylated neurofilaments (SMI312) and mouse-anti dephosphorylated neurofilaments (SMI32). Sections were incubated in primary antibodies or isotype controls (Table 2.1) for 1 hour at room temperature followed by overnight at 4^{o}C. Excess antibodies were removed in PBS washes (3x 10 min). Species-specific Alexa488 antibodies (Table 2.3) were added and incubated for 2 hours at room temperature. Sections were incubated in DAPI (Table 2.3) for 20 minutes at room temperature. Sections were washed 3x30 minutes and coverslips mounted using PermaFluor (Dako) mounting medium. Slides were allowed to fully dry prior to microscopy.

4.2.3 Quantitation

Each immunohistochemical marker was assessed relative to α-BTx. For the purpose of this investigation, NMJs were considered "occupied" if there was evidence of complete or partial association with pre-synaptic markers and α-BTx (Cappello *et al.*, 2012) (Figure 4.2). Similarly, assessment of post-synaptic structures relative to α-BTx staining were scored as "colocaslied" or "non-colocalised". Analysis was performed using a Leica upright fluorescence microscope (refer to Chapter 2.6) and using a dual 488 and 594 cube/filter to visualise both labels simultaneously. Gastrocnemius NMJs were counted from 3 fields of view (20x) from comparable regions of the muscle. All NMJs in the forelimb muscles were counted. All counts were performed blinded to the genotype and

age of the animal. N=3 for all time-points and genotypes. Statistical analysis was performed using two-way ANOVA with Tukey's post-hoc test, $P<0.05$ considered significant (GraphPad Prism version 5). Data represented ± standard error of the mean (SEM).

4.3 RESULTS

To determine changes to the NMJs over time, muscle sections were obtained from mSOD1 G93A and wild-type littermate controls over the life-span of the mSOD1 G93A mouse, from late post-natal development to endstage disease. The time-points chosen reflect a number of stages in the progression of ALS-like disease in these mice, with 28 and 56 days representing the presymptomatic period, 84 days representing onset, 112 days representing mid-stage disease and 140 days representing late disease (Gurney *et al.*, 1994; King *et al.*, 2012). Transgenic mice reached ethical end-stage around 150 ± 4 days of age, similar to previous studies (Cowin *et al.*, 2011). As is well characterised in this mouse model, the transgenic and wild-type mice were morphologically indistinguishable from birth until around 100 days of age, where mild leg tremors became visible in the hindlimbs when handled by the tail. At the 112 day point, mSOD1 G93A mice were noticeably slimmer than their wild-type littermates and had an observably abnormal gait. Consistent with the literature, by 140 days, the mSOD1 G93A mice had marked muscle weakness particularly in the hindlimb (Cowin *et al.*, 2011).

4.3.1 Denervation of mSOD1 G93A NMJs in the gastrocnemius muscle is apparent prior to overt symptom onset

In order to characterise the sequence of changes resulting in denervation of mSOD1 G93A NMJs, lateral gastrocnemius muscle sections were immunolabelled with a cocktail of antibodies to neurofilaments and synaptophysin to visualise the pre-synaptic terminal. Innervation was defined as NMJs sharing evidence of either complete or partial colocalisation of presynaptic elements with α-BTx. In contrast, denervated NMJs had no presynaptic labelling colocalised with α-BTx staining. There was no significant (P>0.05)

difference in the proportion of NMJs with presynaptic terminal colocalisation with α-BTx in wild-type and mSOD1 G93A at 28 days of age. However at 56 days of age, there was a significant (P<0.05) 14.5% decrease in the proportion of NMJs with colocalised lablleing for presynaptic immunolabelling and α-BTx (97.3% ± 1.45% wild-type; 83.1% ± 1.10% mSOD1 G93A) (Figure 4.3). At 84 days of age, there was a significant (P<0.05) 44% decrease in the proportion of NMJs with colocalised labelling for presynaptic immunolabelling and α-BTx (88.7% ± 2.74% wild-type; 49.5% ± 5.26% mSOD1 G93A). Furthermore, at 112 and 140 days of age there were significant (P<0.05) decreases in the proportion of NMJs with colocalised presynaptic labelling and α-BTx, representing a 50% and 70% loss from age-matched wild-type MNJs respectively (112 days: 96.5% ± 1.51% wild-type; 58.0% ± 5.77% mSOD1; 140 days: 96.4% ± 2.0% wild-type; 23.4% ± 6.79% mSOD1 G93A).

4.3.2 The structural proteins nestin, rapsyn and dystrophin are progressively lost from mSOD1 G93A hind-limb NMJs

To further determine the sequence of post-synaptic changes in mSOD1 tissue, muscle sections from a range of ages were examined using immunohistochemistry to key NMJ-associated proteins. At 28 and 56 days of age there was no significant (P>0.05) difference in colocalisation of either nestin, rapsyn or dystrophin with α-BTx in the gastrocnemius muscle of wild-type and mSOD1 G93A mice (Figure 4.4). However, at 84 days of age, there was a significant (P<0.05) decrease in the proportion of NMJs showing nestin (43.6% ± 4.24%) and rapsyn (43.0% ± 7.68%) colocalisation with α-BTx in mSOD1 gastrocnemius muscles compared to controls (89.1% ± 3.56%; 76.9% ± 4.4% nestin and rapsyn controls, respectively). There was no significant (P>0.05) difference in the

proportion of NMJs with dystrophin colocalised with α-BTx. Decreased nestin colocalisation with α-BTx was associated with a change in immunoreactivity to a more dispersed expression throughout the muscle fibre as previously reported following denervation (Vaittinen *et al.*, 1999). At 112 days of age, the proportion of NMJs with dystrophin colocalised with α-BTx staining in mSOD1 gastrocnemius was significantly ($P<0.05$) decreased from wild-type (44.09% ± 5.19% mSOD1; 90.58% ± 5.62% wild-type). Rapsyn and nestin also maintained a significant ($P<0.05$) decrease in the proportion of NMJs colocalised with α-BTx compared to wild-type NMJs (Figure 4.3). By 140 days of age, the proportion of NMJs with each marker colocalised with α-BTx was further decreased, reflecting a 70%, 63% and 66% loss of nestin, rapsyn and dystrophin colocalisation with α-BTx respectively. These decreases were significantly ($P<0.01$) reduced from age-matched wild-type mice (Figure 4.3). These results indicate that the organisation of key structural components is affected over time in mSOD1 G93A skeletal muscles following presynaptic loss, likely reflecting progressive destabilisation of the post-synaptic receptor complexes.

4.3.3 Schwann cells progressively lose S100 immunoreactivity over a time-course in mSOD1 G93A hind-limb muscles

The peri-synaptic Schwann cell performs an important role during formation, maturation and maintenance of the NMJ (Balice-Gordon, 1996; Koirala and Reddy, 2003). To assess NMJ Schwann cells, mSOD1 G93A and wild-type skeletal muscles were immunolabelled for the calcium binding protein S100, a marker for Schwann cells and co-stained with α-BTx. Mature Schwann cells envelope the NMJ, strongly colocalising with α-BTx staining. At 28 days of age there was no significant ($P>0.05$) change in the proportion of

NMJs with S100 colocalised with α-BTx. However by 56 days there was a significant (P<0.05) decrease in the proportion of NMJs with S100 colocalised with α-BTx (76.6% ± 3.97%), reflecting an 18% loss of colocalisation from α-BTx from age-matched wild-type NMJs (93.1% ± 1.79%). By 84 days of age, the proportion of NMJs with S100 colocalised with α-BTx was further decreased to 44% compared with wild-type, signifying a significant (P<0.05) decrease (51.5% ± 8.36%, 91.3% ± 0.85% mSOD1 and wild-type respectively). The proportion of NMJs with S100 colocalised with α-BTx was reduced to 57% at 112 days in mSOD1 tissue compared with wild-type, with a 68% reduction in the proportion of NMJs colocalised with S100 and α-BTx compared with wild-type NMJs by 140 days of age (Figure 4.3).

To determine whether the loss of S100 colocalisation with α-BTx at the NMJ reflected a loss of Schwann cells or the S100 protein, S100 stained sections were counter-stained with DAPI to visualise nuclei. Schwann cell nuclei were visible around α-BTx staining in healthy muscles, colocalised with S100 staining. The distribution of nuclei surrounding α-BTx staining was not altered in mSOD1 G93A tissue at any age-point despite loss of S100 immunoreactivity (Figure 4.5). These preliminary results suggest that the loss of S100 immunoreactivity in the skeletal muscles of aging mSOD1 G93A mice may represent a loss of the S100 protein rather than initial loss of cells however further investigations into this process are needed.

4.3.4 NMJ pathology is delayed in the forelimbs of mSOD1 G93A mice

In the forelimb, a significant (P<0.05) decrease in the proportion of NMJs with presynaptic terminals colocalised with α-BTx was only present at 140 days of age, reflecting a 27% decrease in colocalisation (95.1% ± 2.17% wild-type; 69.8% 4.75%

mSOD1 G93A) (Figure 4.6). Analysis of nestin, rapsyn and dystrophin in the forelimb tissue demonstrated no significant (P>0.05) change in the proportion of NMJs with either protein colocalised with α-BTx compared to wild-type animals at any age. There was no significant (P>0.05) difference in the proportion of NMJs with S100 colocalised with α-BTx in the forelimb muscles of mSOD1 G93A mice at any age. The organisation of nuclei surrounding α-BTx staining in the forelimbs was unaffected. These results indicate that NMJ pathology in the forelimbs is delayed relative to the hindlimbs.

4.4 DISCUSSION

In this study, a number of post-synaptic structural components of the NMJ were examined over a time-course in the mSOD1 G93A mouse model of ALS. Quantification of NMJs with colocalisation of pre- and post-synaptic structures in this transgenic model indicates that die-back of motor neuron axons is apparent from 56 days of age, with a dramatic decrease around 84 days, or disease onset. Further investigations using immunohistochemical labelling of a number of structural proteins were performed to determine whether denervation is preceded by changes to the post-synaptic structures. The proportion of NMJs with nestin and rapsyn colocalisation with α-BTx in the hindlimbs of mSOD mice were not significantly different from wild-type tissue at the earlier time-points investigated, however demonstrated a significant ($P<0.05$) decrease at 86 days of age, showing further decreases at 112 and 140 days. The proportion of NMJs with colocalisation between dystrophin and α-BTx in mSOD1 mice was significantly decreased at 112 and 140 days of age compared to wildtype. S100 staining for Schwann cells revealed the proportion of NMJs showing colocalisation of S100 with α-BTx was significantly ($P<0.05$) decreased from 56 days onwards. Co-staining with DAPI indicated that the loss of S100 staining represented a loss of the S100 protein rather than degeneration of Schwann cells. Finally, analysis of forelimb musculature did not indicate changes to structural organisation, save for a significant 27% reduction of the proportion of NMJs with presynaptic terminals colocalised with α-BTx at 140 days.

4.4.1 Early deficits in colocalisation of pre-synaptic motor terminals with α-BTx may reflect compensatory remodelling of neuromuscular connections

The loss of pre-synaptic proteins from some NMJs at 56 days of age may reflect a very early degeneration which is compensated for by sprouting of neighbouring fibres. In healthy muscle, denervation is rapidly corrected by compensatory plasticity (Magrassi *et al.*, 1987; Balice-Gordon *et al.*, 1990; Fluck, 2006; Li and Thompson, 2011). If the reinnervating axon is of a different type to the previous axon, the skeletal muscle fibre switches type to match the innervating neuron (Pette, 2001). However, the process is incomplete in ALS, with a failure of the skeletal muscle fibre-switching mechanism resulting in mixed atrophic groups in contrast with the grouped atrophy which is usually seen in progressive denervation (Baloh *et al.*, 2007) Similar fibre-type grouping, indicative of this process can be seen in human ALS and Charcot-Marie-Tooth disease patients (Brooke and Engel, 1969; Telerman-Toppet and Coers, 1978). Indeed, attempted reinnervation is present in skeletal muscle biopsies from human ALS patients (Hansen and Ballantyne, 1978; Fischer and Glass, 2007). Consequently, it remains highly likely that degeneration of motor neurons in ALS occurs before the onset of symptoms, with the inherent plasticity of NMJ remodelling compensating for the loss (Mead *et al.*, 2011). This theory is supported by the present study, indicating a small but significant decrease in the proportion of NMJs with colocalisation between motor neuron terminals and post-synaptic receptors at a time-point prior to disease onset. Previous analyses using comparable approaches have reported similar findings (Cappello *et al.*, 2012). Others have noted a more dramatic and early loss of presynaptic structures (Fischer *et al.*, 2004), however differences in protein labelling and the muscles examined may account for the discrepancies between studies. Additionally, in this study, ‘colocalisation’ was defined as both partial and complete overlap of immunohistochemical labels with α-BTx staining.

The importance of using both pre- and post-synaptic NMJ components to determine synaptic connectivity was highlighted by Li and Thompson (2011) who demonstrated that complete dissociation of AChRs from NMJs can take up to 11 weeks following denervation in wild-type mice.

4.4.2 Loss of structural proteins precede loss of post-synaptic receptors

To probe the mechanisms underlying degeneration of the NMJ in the mSOD1 mouse, further investigations were performed into some of the underlying structural components of the NMJ. Rapsyn is a critical effector for AChR aggregation (Frail *et al.*, 1987; Zuber and Unwin, 2013). In addition to AChR binding, rapsyn is critical for a number of molecular interactions (Phillips *et al.*, 1991; Wu *et al.*, 2010; Piguet *et al.*, 2011), including binding to the cytoskeleton (Antolik *et al.*, 2007) and molecules such as calpain (Chen *et al.*, 2007), β-catenin (Zhang *et al.*, 2007) and α-actinin (Dobbins *et al.*, 2008). The significant decrease in the proportion of NMJs showing reduced rapsyn colocalisation with α-BTx was unexpected, however other proteins, including dystroglycan may also interact with AChRs (Pilgram *et al.*, 2010). Loss of rapsyn colocalisation has not been investigated in other denervation models, so it remains unknown whether the pathology as occurs in mSOD1 G93A tissue is common to denervation or is related to mSOD1 toxicity.

Nestin, an intermediate filament, is highly expressed throughout the developing nervous system and performs a large number of roles across a very diverse range of cells (Capetanaki *et al.*, 2007). In the mature fibre, nestin is highly localised to the NMJ and myotendinous junction in a voltage-dependent manner (Lendahl *et al.*, 1990; Vaittinen *et al.*, 1999). Nestin associates with AChR clusters (Vaittinen *et al.*, 1999; Kang *et al.*,

2007) and unlike other skeletal muscle intermediate filaments, binds with microtubules or microfilaments (Herrmann and Aebi, 2000) for stabilisation of AChRs (Bloch, 1983; Weston *et al.*, 2000; Luo *et al.*, 2002; Lee *et al.*, 2009). Nestin is involved in negative regulation of AChR expression via regulation of Cdk5 activation (Yang *et al.*, 2011). Cdk5 activity is altered in mSOD1 mouse models, and the toxic p25 fragment of p35 cleavage by Cdk5 is upregulated in mSOD1 G93A muscles (Dobrowolny *et al.*, 2008). In this study, a decrease in NMJs showing nestin coloclaised with α-BTx coincided with a more 'dispersed' pattern of nestin immunolabelling throughout the central region of the muscle fibre, similar to that noted by Vaittinen and colleagues (1999). Such alterations in protein organisation suggest nestin is no longer associated with AChR clusters and microtubules, which may affect nestin regulation of pathways such as Cdk5 kinase activity in these NMJs.

Dystrophin is a member of the dystrophin glycoprotein complex (DGC) required for maintenance of the NMJ (Lyons and Slater, 1991; Knuesel *et al.*, 1999). Specifically, the DGC is responsible for organisation and stabilisation of the synaptic folds (Sanes and Lichtman, 1999; Banks *et al.*, 2003; Shiao *et al.*, 2004) linking the muscle plasma membrane to the F-actin component of the cytoskeleton (Ervasti and Campbell, 1993; Banks *et al.*, 2009). The DGC also putatively acts as a scaffold for signalling proteins including nNOS, calmodulin, phosphoinositol triphosphate 3 and may also be involved with mitogen activated protein kinase (MAPK) signalling (Rando, 2001) and maintaining Ca^{2+} homeostasis (Gillis, 1996; Carlson, 1998). Activity of the dystrophin associated protein complex is not restricted to the NMJ, also performing similar stabilisation of central synapses and is present in astrocytic end-feet surrounding CNS blood vessels (Na *et al.*, 2013). Dystrophin mutations are associated with the lethal X-linked muscle disorder Duchenne muscular dystrophy (DMD)(Koenig *et al.*, 1987; Anderson *et al.*,

2002). The symptoms of DMD result from loss of mature dystrophin activity, primarily by progressive destabilisation of the NMJ rendering the skeletal muscle increasingly vulnerable to contractile injury (Pilgram *et al.*, 2010). These studies indicate that whilst loss of dystrophin is not immediately deleterious to the NMJ, it does result in progressive destabilisation. In the current study, the proportion of NMJs with reduced dystrophin and α-BTx colocalisation was not significantly different until 86 day of age, or around symptom onset, suggesting that loss of dystrophin from the NMJ occurs much later during disease in mSOD1 G93A mice and may reflect disassembly of the AChR complex from the skeletal muscle membrane. It is currently unknown whether the reduced colocalisation of rapsyn, nestin and dystrophin with α-BTx at the NMJ in mSOD1 mouse occurs via the same mechanisms or reflect different disease and degenerative pathways. However, the role of dystrophin in maintaining Ca^{2+} homeostasis is of particular interest in ALS, whereby subtle reorganisation of dystrophin and potentially other NMJ structural components may exacerbate Ca^{2+} mediated damage.

4.4.3 Altered Ca^{2+} and energy homeostasis may be mediated by the NMJ

The results from this study indicate that there is a significant and progressive loss in the proportion of NMJs showing colocalisation with S100 and α-BTx in aging mSOD1 mice compared with wildtypes. The S100 protein family comprises 24 calcium binding and signalling members (Donato, 2001; Marenholz *et al.*, 2004; Santamaria-Kisiel *et al.*, 2006). As a group, S100 proteins perform a diverse range of function, including regulation of Ca^{2+} homeostasis, energy metabolism, cell proliferation, apoptosis, inflammation and cell migration (Heizmann *et al.*, 2002; Donato *et al.*, 2013). In contrast

with other Ca^{2+} binding proteins, many S100 proteins are not restricted to the intracellular environment, with noted extracellular and paracrine activity (Donato *et al.*, 2009).

S100β is predominantly expressed by glial cells, including astrocytes and Schwann cells (Bhattacharyya *et al.*, 1992) and is commonly used as an immunohistchemical marker for the latter. Additionally, activated cells such as astrocytes or dying cells have been reported to secrete S100β (Shashoua *et al.*, 1984; Van Eldik and Zimmer, 1987), however the role of the secreted protein remains controversial (Van Eldik and Wainwright, 2003; Donato *et al.*, 2009). In human ALS patients, CSF and serum levels of S100β are reduced (Otto *et al.*, 1998; Sussmuth *et al.*, 2003), however immunoreactivity is increased within spinal motor neurons (Migheli *et al.*, 1999).

A recent investigation using human ALS and mSOD1 G93A tissue also indicated that S100 loss in skeletal muscles was due to a loss of protein, and additionally noted that levels of S100 immunoreactivity varied between vulnerable and resistant skeletal muscles (Liu *et al.*, 2013). This recent finding supports the results from the current study whereby S100 loss was only significant in the hindlimb muscles and remained spared in the forelimb over the timecourse studied. Liu and colleagues (2013) proposed that loss of S100 protein is likely to result in subsequent dysfunction in homeostasis at the NMJ. In addition to regulation of Ca^{2+}, S100β is a potent proliferative signal for skeletal myoblasts in the presence of basic fibroblast growth factor (Sorci *et al.*, 2003; Riuzzi *et al.*, 2011) via inhibition of p38 MAPK (Riuzzi *et al.*, 2006).

The role of the skeletal muscle in the pathogenesis of ALS is being increasingly recognised. In particular, the hypothesis that skeletal muscle energy dysfunction may precede NMJ degeneration (Dupuis and Loeffler, 2009). Initial evidence for this theory arose from characterisation of an early deficiency in body-weight in mSOD1 mice which

was not attributed to muscular atrophy (Dupuis *et al.*, 2004). This was later described as a chronic energy deficiency, a condition which is partially ameliorated by feeding mSOD1 mice a high-fat diet (Dupuis *et al.*, 2004; Mattson *et al.*, 2007). This state of chronic energy deficiency has also been described in many human ALS patients, who are hypermetabolic with increased lipidaemia (Dupuis *et al.*, 2008).

At present, the biological significance of S100 loss in ALS patients and mouse models remain unclear, however it is likely that the effects of S100β may extend to perturbed energy regulation and the NMJ, and may additionally affect the skeletal muscle.

4.4.4 The forelimb as a model of presymptomatic degeneration in the mSOD1 G93A mouse

The onset of disease in the mSOD1 G93A mouse is well-characterised and consistently begins with hind-limb weakness which(Hampson and Manalo, 1998) progresses to a severe motor disability and finally paralysis of respiratory muscles leading to death whilst the forelimb muscles remain relatively spared (Gurney *et al.*, 1994; Chiu *et al.*, 1995; Azzouz *et al.*, 1997; Bendotti and Carri, 2004). The mouse forelimb muscles are composed entirely of fast fibres, characterised by type II myosin heavy chain isoforms (IIA, IIB and IIX) (Elashry *et al.*, 2009; Mathewson *et al.*, 2012). This is in contrast with the gastrocnemius muscle which is composed of both type I and type II fibres (Schiaffino and Reggiani, 2011). Mathewson et al., (2012) have suggested that the forelimb muscles are relatively comparable between humans and mice, despite differences in limb function and skeletal muscle biomarkers between humans and mice. Specifically, mice have an additional myosin isoform (IIB) which is not present in humans (Mathewson *et al.*, 2012; Tirrell *et al.*, 2012). An interesting paradox in the mSOD1 G93A mouse model is that the

forelimbs remain relatively spared during disease, however the muscles are composed entirely of type II fibres, known to be preferentially vulnerable in ALS (Hegedus *et al.*, 2008).

In this present study, investigation of pre-and post-synaptic components indicated that the phenotypic sparing of the forelimbs in mSOD1 G93A mice is supported by delayed histological pathology. There was a significant decrease in the proportion of NMJs with colocalisation between presynaptic terminals and α-BTx at 140 days of age, however there was no difference with any other marker or time-point examined. This finding is indicative that pathology in the forelimbs may be similar but delayed from that of the gastrocnemius muscle. The cause for this phenotypic phenomenon is not well understood, however the most probable cause is differential activity of mutant SOD1 rather than the composition of the skeletal muscle fibres.

The site of onset of muscle weakness in human ALS is generally distributed evenly between lower limb, upper limb and bulbar onset (Leigh and Ray-Chaudhuri, 1994; Jackson and Bryan, 1998; Ravits and La Spada, 2009; Turner *et al.*, 2013), however human fALS cases with mutations in mSOD1 are more likely to present with lower-limb onset (Millecamps *et al.*, 2010). This finding supports the notion that the mSOD1 mouse model remains an excellent model for ALS, particularly for human patients with mSOD1.

The delay in disease in the mSOD1 G93A forelimb provides a unique stage to investigate subtle pathology which may otherwise be masked in the aggressive degeneration characteristic of the hindlimb musculature. Studies have demonstrated that spinal pathology in the mSOD1 mouse is widespread throughout both the lumbar and thoracic regions (Graffmo *et al.*, 2013), however the delayed pathology in the forelimb muscles is suggestive of potential differences in the disease process which may be localised to

specific regions of the spinal cord. Additionally, the results from this study indicate development of the NMJ in the mSOD1 G93A mouse appears to occur normally, with degeneration occurring from progressive dysfunction within the organism as opposed to an inherent instability of the NMJ.

4.4.5 Conclusions

The findings from this study highlight the importance of a range of proteins at the NMJ, indicating that mislocalisation of key structural components may play a role in the progressive dysfunction of this specialised syanpse. In addition to structural roles, each of the proteins examined have critical functions in skeletal muscle signalling, with increasing evidence to suggest that these processes become dysfunctional in ALS. Furthermore, these results support a growing acknowledgement that non-neuronal energetic dysfunction is a key process in ALS. These data suggest that such dysregulation may be affected by loss of Schwann cell S100 in addition to dysfunction within the skeletal muscle and motor neuron. Finally, these data indicate that the comparatively under-studied murine forelimb musculature may provide a unique opportunity to investigate subtle pathology in the mSOD1 mouse.

5 DEVELOPMENT OF A COMPARTMENTED CULTURE MODEL FOR INVESTIGATING THE LOWER MOTOR NEURON – NEUROMUSCULAR JUNCTION CIRCUIT

5.1 INTRODUCTION

Lower motor neurons are a unique neuronal subtype. The lower motor neuron is characterised by a long axon which connects the neuronal soma within the spinal cord to the NMJ within peripheral skeletal muscle. As such, the motor neuron axon extends through a range of anatomically distinct locations of the body and is supported by specific environments with regards to both the cellular milieu and extracellular matrices. The highly specialised NMJ is situated within the peripheral nervous system, spatially separated from the motor neuron soma, which is located centrally within the spinal cord, surrounded by glial cells. A second defining characteristic of the motor neuron is its specific vulnerability to pathology in ALS. Whilst a large array of potential mechanisms for this vulnerability has been proposed, it still remains unclear how these interact to result in degeneration of the motor neuron. This is especially when considering how excitotoxicity, a widely recognised pathogenic process in ALS (Van Damme *et al.*, 2005; Van Den Bosch *et al.*, 2006), results in degeneration of the axon and loss of the NMJ.

Neuro-muscular signaling is a two-way process involving an anterograde electrochemical signal resulting in muscle contraction, and determination of muscle fiber type (Naya *et al.*, 2000) and retrograde neurotrophic signaling to maintain neuronal health (Kablar and Belliveau, 2005). Thus, the health and function of the lower motor neuron and the muscle tissue that is innervated are co-dependent. Degeneration of the NMJ is a key and early pathological feature of many motor neuron diseases and myopathies. In motor neuron

disease, dysfunction of the spinal motor neuron results in muscular atrophy, however the role of neuron-muscle interactions is yet to be fully deciphered (Dupuis *et al.*, 2003; Dobrowolny *et al.*, 2005; Wong and Martin, 2010). Similarly, the muscular dystrophies, which involve cycles of muscle necrosis and attempted regeneration affect the health of the motor neuron and the functioning of the NMJ (Wallace and McNally, 2009).

The development of distal neuropathy in ALS is closely associated with motor neuron axon degeneration, such that axon degeneration is considered by many to be a primary pathogenic process in ALS (Chapter 1.2). The mechanisms by which axon degeneration occurs in ALS is not well understood, however the ALS motor neuron is characterised by a large number of abnormal cellular processes (protein misfolding, energy deficit, axonal neurofilament inclusions, slowing of axonal transport, abnormal mitochondria, particularly in the distal segment), any one of which could cause axon degeneration (Chapter 1.2.2). Degenerating axons in ALS share features in common with both Wallerian degeneration and developmental axon pruning, including beading and fragmentation similar to Wallerian degeneration (Chapter 1.2.2.1). However there are key differences in the mechanisms underlying Wallerian degeneration and developmental pruning. Critically, developmental axon pruning occurs in a caspase-dependent mechanism whereas Wallerian degeneration does not (Parson *et al.*, 1997; Finn *et al.*, 2000; Chen *et al.*, 2012a). Axon degeneration in ALS has been termed Wallerian-like degeneration and occurs in a caspase-dependent manner in mouse models (Li *et al.*, 2000; Pasinelli *et al.*, 2000; Vukosavic *et al.*, 2000) and *in vitro* (Pasinelli *et al.*, 2000). *In vitro* degeneration of the distal axon in ALS is characterised by formation of distal axon swellings and comparative preservation of the long motor neuron axon (King *et al.*,

2007). The formation of distal axon swellings in cultured motor neurons is accompanied by mislocalisation of dephosphorylated neurofilaments (King *et al.*, 2007).

Cell culture models offer the potential to examine the complex cellular interactions of the lower motor neuron within controlled environments. However, many current techniques for culture of motor neurons are based on techniques traditionally used to culture cortical or hippocampal neurons, consisting of a monolayer of primary neurons grown on varied substrates (Daniels *et al.*, 2000; King *et al.*, 2011). Such models typically involved co-culture of spinal motor neurons on either glial or muscle-derived cells and have provided a considerable insight into the interactions that occur between motor neurons and non-neuronal cells. However, these models are not able to recapitulate the unique spatial organisation of the lower motor neuron *in vivo*.

The Campenot chamber was developed to overcome this problem (Campenot, 1977). The Campenot chamber consists of a teflon divider which is attached to a scratched glass coverslip with grease sealant to form three compartments. Neurons plated into the central compartment extend axons into the outer compartments, aided by the addition of exogenous growth factors (Campenot, 1977). Whilst this culture model has been previously used to create compartmented motor neuron cultures (Guo et al., 2010; Guo *et al*., 2011), the addition of exogenous growth factors introduces a potentially confounding issue for studies aimed at probing physiological mechanisms. The growth factors which are commonly added, BDNF and GDNF alter the cell-surface receptors which are expressed by the motor neuron, thus potentially altering the subsequent response to receptor activation (Mattson *et al.*, 1993; Cheng *et al.*, 1995; Mattson, 2008). Furthermore, neurotrophins including BDNF have been shown to be neuroprotective during excitotoxicity (Cheng and Mattson, 1994; Mattson, 2008). The development of the

microfluidic chamber (Chapter 2.2.1) provides the best solution to create compartmented motor neuron cultures with minimal external manipulation. To date, no studies have used these devices to investigate spinal motor neuron function or dysfunction.

There is increasing evidence to suggest that the neuronal microenvironment is vital for neuronal function *in vivo* and that the interactions between motor neurons and the surrounding cells play a critical role during development of pathology in ALS (Millet and Gillette, 2012). Therefore, the aim of this investigation was to develop and characterise a novel method for culturing spinal motor neurons that incorporates non-neuronal cell populations and the unique temporal organisation of the lower-motor neuron circuit as occurs *in vivo*. Once developed, this model would be suitable for a large range of downstream applications to study both normal and disease-linked motor neuron physiology. In this thesis, the compartmented cultures were used to investigate excitotoxicity in motor neurons. Specifically, the aim of this thesis was to investigate whether excitotoxic degeneration in motor neurons can occur through the axon or NMJ, and whether this occurs via similar mechanisms to the excitotoxic degeneration in cortical neurons, as shown in Chapter 3.

5.2 METHODS

5.2.1 Primary cell culture

13mm^2 glass coverslips and microfluidic chambers were prepared for culture as described in Chapter 3. Glass coverslips were coated with poly-L-lysine (PLL) for glial cells, collagen for primary skeletal muscle cells, or poly-L-lysine/laminin (PLL-L) for C2C12 myoblasts. Microfluidic chambers were filled with PLL-L on the proximal side and laminin/collagen to the distal side (Figure 5.1). Flasks were prepared with either PLL or collagen. All substrates were incubated overnight at room temperature, removed and devices filled with cell-specific medium. All cell culture substrates were allowed to equilibrate for 2 hours prior to addition of cells.

5.2.1.1 Primary glial cells

Spinal glial cells were obtained from the spinal cords of two postnatal day 2 (P2) Sprague-Dawley rats or four P2 C57Bl/6 mice. Neonates were decapitated using sharp scissors. The skin was sterilised using 70% ethanol and pups placed in a clean petri dish with the back side facing up. The skin was opened using sterile large spring-loaded scissors and the spinal cord exposed. The spinal cavity was opened with a single horizontal cut at the base of the tail and the cavity opened using forceps to one side of the spinal cord. Spinal cords were collected in 100µl drop of HBSS in a sterile petri-dish and stripped of meninges. Tissue was trypsinised (0.0125% trypsin) for 5 minutes. Trypsin was removed and tissue triturated in 2ml glial cell medium (Table 8.1) and filtered through 80nm mesh. Cells were pelleted (300 x g, 5 minutes) and plated into 2x prepared PLL coated P25 flasks (Iwaki). Cultures were incubated under standard cell culture

conditions for 24 hours followed by a complete medium change with warmed glial cell medium. Mixed glial cultures were maintained for 2-3 weeks until confluent with weekly ½ medium changes.

Glial cells were passaged by washing the cells once in warmed trypsin/EDTA (refer to Chapter 8.2), followed by incubation in trypsin/EDTA for 5 minutes. Cells were dislodged from flasks by firmly hitting with the heel of the hand. Cells were pelleted (300 x g, 5 minutes) and plated either onto 24x PLL coated glass coverslips (rat) or uncoated coverslips (mouse), or into the proximal chamber of 24x microfluidic devices.

5.2.1.2 Primary skeletal myocytes

Primary myocyte cultures were prepared from the hind limbs of two P2 Sprague-Dawley rats. Neonates were decapitated using sharp scissors. Skin was sterilised in 70% ethanol and hind limbs removed. The feet were severed using scissors and tweezers used to remove skin. Muscle tissue was dissected from hind limb bones and finely minced using spring-loaded scissors, and transferred to 2mL HBSS. Tissue was incubated in 500µl collagenase/dispase (refer to Chapter 8.2) for 40 minutes and mechanically triturated. After trituration, 2ml primary myoblast growth medium (Table 8.1) was added and cells passed through fine gauze. Cells were pelleted (350 x g, 10 minutes), resuspended in 6ml myoblast growth medium and plated into two prepared collagen-coated P25 flasks and grown under standard cell culture conditions. Culture medium was replaced in full after 24 hours and myocytes allowed to grow for a further 4-5 days, or until 70% confluent.

Semi-confluent myoblasts were passaged onto either collagen-coated glass coverslips (density of 50,000 cells per coverslip) or into the distal compartment of microfluidic

chambers containing a substrate of laminin/collagen. Primary myoblast growth medium was removed and cells rinsed 1x (10 seconds) with 1ml warmed trypsin-EDTA. Cells were incubated for 2 minutes with a second 1ml of trypsin-EDTA and dislodged from the flask by hitting the flask firmly with the heel of the hand. Cells were pelleted (350 x g, 10 minutes) and added to coverslips or microfluidic chambers. Passaged myoblasts were allowed to grow for 1 day in primary myoblast medium following passaging, after which they were transferred to neuron initial growth medium (Table 8.1) to promote differentiation.

5.2.1.3 C2C12 myoblasts

C2C12 mouse myoblast cells (American Type Culture Collection (ATCC), Virginia, USA) were maintained at a low passage number (<10) in C2C12 growth medium as recommended by the manufacturers suggestions (Table 8.1). C2C12 myoblasts were transferred onto PLL-L coated coverslips or the distal compartment of microfluidic chambers using the trypsin-EDTA method described above. C2C12 myocytes were differentiated by serum-shock in C2C12 differentiation medium 1 (CDM1) (Table 8.1) for 2 days, and transferred to C2C12 differentiation medium 2 (CDM2) (Table 8.1) for three days thereafter. Cultures were maintained in serum-free neuronal subsequent medium (Table 8.1) for the duration of cultures.

5.2.1.4 Primary rodent motor neurons

Primary spinal motor neurons were obtained from the spinal cords to E14.5 Sprague-Dawley rats or E13.5 C57Bl/6 mice. Time-mated females were killed by CO_2, embryos

removed and decapitated. Embryo bodies were maintained on ice for the duration of the dissection to minimise tissue degradation. Spinal cords were removed by placing the bodies in a sterile petri dish with the spinal cord facing up. Using microscopic guidance, tweezers were used to score along both sides of the spinal cord to remove skin. The spinal cord was then removed and transferred to a drop of ice-cold HBSS. Meninges and dorsal root ganglia were removed using fine forceps and cleaned spinal cords transferred to 5ml HBSS. Tissue was trypsinised (0.00625%) for 5 minutes. Trypsin was removed and cells triturated in 2mL HBSS. The cell suspension was pelleted in 3.5% bovine serum albumin (BSA) and enriched for motor neurons in 1.06g/L Optiprep™ gradient for 25 minutes at 400 x g with the brake off. Motor neurons were pelleted at 700 x g and resuspended in 250µl. Cell density was estimated using the trypan blue dye exclusion assay and the volume adjusted to achieve $8x10^6$ cells per ml. 10µl motor neurons ($8.5x10^4$ motor neurons) were plated into the proximal compartment of prepared microfluidic chambers (Chapter 2.2.1) or onto prepared 13mm coverslips with glial cells, myocytes or PLL. Plated cells were returned to incubator conditions. Medium was replaced with neuron subsequent medium (Table 8.1) at 2DIV (rat) or 7DIV (mouse) and maintained with weekly ½ medium changes.

5.2.2 Addition of glia and muscle cells to microfluidic chambers

For primary rat cultures, spinal glial cells were grown in tissue culture flasks to 70% confluency, before harvesting and adding to the proximal side of microfluidic devices at motor neuron 2DIV (MN-2DIV) (Figure 5.2A). Neuron initial growth medium (Table 8.1) was removed and glia added at $1.5x10^5$ cells. Glial cells were allowed to adhere for 15 minutes under standard growth conditions, followed by addition of neuronal initial

medium to fill devices. Devices were returned to the incubator and medium replaced with neuron subsequent medium at motor neuron 5DIV. Cultures were observed daily to determine axonal growth into the distal chamber, skeletal muscle cells were added following secondary neurite extension (MN-7-10DIV). The distal chamber was maintained in neuron initial growth medium to encourage myoblast proliferation under standard culture conditions. Growth medium was replaced with neuron subsequent medium following myotube differentiation and cultures maintained with weekly ½ medium changes thereafter.

For mouse-derived cultures, spinal motor neurons were plated directly to PLL-L coated microfluidic chambers or onto confluent mouse spinal glial cells or C2C12 cells on coverslips (Figure 5.2B). On both coverslips and in microfluidic chamber, spinal motor neurons were maintained in neuron initial medium until MN-7DIV, then transferred to neuron subsequent medium. C2C12 myoblasts were added to the distal compartment of mouse microfluidic chambers at MN-2DIV. Distal C2C12 myocytes were differentiated at MN-4DIV with CDM1 for 2 days, then CDM2 for 3 days and finally maintained in serum-free neuronal subsequent medium for the duration of the culture.

5.2.3 Pharmacological intervention

Excitotoxicity was induced in compartmented lower motor neuron cultures by the application of 100μM kainic acid in subsequent medium to either the proximal (motor neuron cell bodies + glia) or the distal (motor neuron distal axons + C2C12s) compartment. Cells were treated for 24 hours followed by paraformaldehyde fixation (Chapter 2.4).

5.2.4 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and permeabilised with 0.3% Triton X-100 in PBS. Primary antibodies (Table 2.1) were diluted in PBS and incubated at room temperature for 1 hour followed by overnight at 4^{o}C. Species-specific secondary antibodies were incubated for 2 hours at room temperature (Table 2.2). α-BTx staining, for evidence of NMJ formation, was performed for 30 minutes prior to cell permeabilisation and primary antibody incubation (Table 2.3). Nuclei were visualised with Nuclear Yellow (Sigma, 0.001%, 15min) staining following secondary antibody labelling. Images were obtained using a Leica DMLB2 upright fluorescence microscope fitted with a cooled CCD optronics camera. Images were adjusted for brightness and contrast using Adobe Photoshop (CS5).

5.2.5 Image acquisition and data analysis

Motor neurons grown on spinal glia or muscle were fixed at 3DIV and immunolabelled for beta-III-tubulin. Four images per coverslip were captured from four separate cultures for each growth condition. Quantitation was performed blinded to experimental conditions. Axonal identity was confirmed by positive-tau and negative-MAP2 immunoreactivity. Neurite counts were performed with all neurites present, regardless of axonal or dendritic identity. Cell survival was counted as the number of motor neurons per mm^2. Axon measurements, neurite numbers and cell numbers were averaged and analysed using one-way ANOVA with Tukey's post-test correction for multiple comparisons, P<0.05 considered significant. For all motor neuron analysis, data was obtained from a minimum of n=4 separate cultures.

Formation of NMJs in MN-18DIV compartmented cultures was quantified by differences to α-BTx clustering along myotubes in the presence of neurons. α-BTx staining for AChRs was used to differentiate widespread prepatterned AChR clustering from discrete synaptic clusters. Cultures were labelled with α-BTx and NFM and images captured (40x). Images were thresholded in ImageJ, region of interest (200μm^2) selected either within 20μm of an axon or no axons within 20μm and α-BTx stained particles analysed. Mean area of α-BTx staining was analysed using Student's 2-Tailed t-test (GraphPad). $P<0.05$ was considered significant.

Images for quantification of axon degeneration were captured ensuring image setting remained constant between image sets. Four distal images were captured for each coverslip, with a minimum of three individual culture repeats for each group. Images were superimposed with a 50μm grid using Image-J (National Institute of Health, USA).

5.2.6 Electron microscopy

Microfluidic chamber cultures for EM analysis were fixed in 4% paraformaldehyde/2.5% glutaraldehyde. Microfluidic chambers were removed and cultures dehydrated in acetone. Coverslips were critical-point dried, sputter coated with platinum and examined using a Hitachi SU-70 field emission scanning electron microscope.

5.3 RESULTS

A mixture of immortal cell lines and primary cells were used for this study. Unless specified, results were in both rat and mouse-derived cultures.

5.3.1 Non-neuronal cellular interactions determine growth characteristics of primary motor neurons

To determine the effect of substrate or non-neuronal cell interactions on the growth and development of motor neurons, rat neurons were grown on feeder layers of spinal glial cells or skeletal muscle cells for 3 days. Co-cultures were fixed and immunolabelled with neuron-specific beta-III-tubulin to identify neuronal morphology. Motor neurons demonstrated significantly ($P<0.01$) increased survival when co-cultured with spinal glia (19.5 ± 1.2 cells/mm^2) when compared with skeletal muscle feeder layers (13.9 ± 1.2 cells/mm^2) (Figure 5.3A). Motor neurons co-cultured with glial cells had significantly ($P<0.01$) more neurites per neuron (4.0 ± 0.2) compared with motor neurons cultured with muscle cells (3.0 ± 0.1) (Figure 5.3B). Additionally, motor neurons cultured on skeletal muscle had significantly ($P<0.05$) longer axons (408.9 ± 50.5μm) than motor neurons co-cultured with glial cells (280.0 ± 18.8μm) (Figure 5.3C). These data indicate that motor neuron growth is highly sensitive to the presence of different non-neuronal cells.

5.3.2 Compartmented, fluidically isolated spinal glia cells and myotubes support motor neuron growth

To mimic the cellular interactions that occur *in vivo*, compartmented co-cultures were developed, incorporating spinal glial cells positioned with motor neuron cell bodies in one chamber, and motor neuron axons extending to a second chamber containing skeletal muscle cells (Figure 5.4). The growth of cells in the chambers was monitored daily by light microscopy. At 2DIV, motor neurons were visible in the proximal chamber, with short neurites extending from the soma (Figure 5.4A). Motor neuron axons extended into the microchannels that divide the proximal and distal chambers at 8DIV (Figure 5.4B) and extend from the microchannels into the distal chamber at 10DIV (Figure 5.4C). Rat motor neuron distal axons formed a dense network by 14DIV (Figure 5.4D).

Cultured primary spinal glial cells were added to the proximal compartment of rat-derived cultures at 2DIV. Glial cells which were added to the chambers prior to motor neurons or simultaneously, resulted in blocked microchannels, thus necessitating delayed addition. Excessive proliferation of glial cells was controlled by transferring the cell body compartment to serum-free medium from 5DIV. Failure to add glial cells resulted in extensive death and degeneration of motor neurons from 3DIV, with no surviving cells after 6DIV. Primary myoblasts were added to the distal compartment at 14DIV in rat-derived primary cultures following the formation of the dense axonal network (Figure 5.4D). In contrast, mouse motor neurons degenerated rapidly from the distal compartment when C2C12 myocytes were absent, necessitating the much earlier addition of myocytes in these cultures. Earlier addition of rat primary myocytes resulted in microchannels becoming blocked. Myocytes from both rat primary cultures and C1C12 cells rapidly differentiated into myotubes, characterised by morphology (elongated shape) and

physiology (spontaneously contractile) by MN-18DIV (rat) or MN-7DIV (mouse) (Figure 5.4E).

The compartmentalisation of cell types was confirmed in mature cultures at MN-14DIV (rat) or MN-10DIV (mouse) using immunocytochemistry. Within the proximal chamber, motor neurons were immunoreactive for MAP2 (dendrites) and NFM (axons) (Figure 5.5A), with axons retaining NFM immunoreactivity for the duration of the culture. Critically, the neurons in compartmented culture were immunoreactive for dephosphorylated neurofilaments by 10DIV, had cell bodies greater than 20µm and had an extensive dendritic arbour. Additionally, motor neurons were strongly immunoreactive for dephosphorylated neurofilaments. These characteristics are strongly indicative of identification as motor neurons (Sternberger and Sternberger, 1983; Carriedo *et al.*, 1996; Tsang *et al.*, 2000). MAP2 staining also revealed development of an appropriate number of neurites, as seen with motor neurons co-cultured with glial cells on coverslips. Glial cells immunoreactive for GFAP were confined to the proximal chamber (Figure 5.5B). Immunocytochemical analysis demonstrated spinal glial cultures comprised a mixture of cells consisting predominantly of GFAP-positive astrocytes, however minor populations of O1-positive oligodendrocytes and Iba1-positive microglia were also present.

Motor neuron axons immunoreactive for NFM were present within the proximal chamber (Figure 5.5A), microgrooves (Figure 5.5C) and distal chamber (Figure 5.5D,E). Myoblasts were present in the distal chamber shortly after plating (Figure 5.5D) and remained within the distal chamber after differentiation into myotubes (Figure 5.5E). Immunocytochemical analysis demonstrated both populations of myotubes were immunoreactive for the intermediate filament desmin (Figure 5.5E) and nuclear staining indicated myotubes were multinucleated (Figure 5.5F).

5.3.3 Characterisation of NMJs in compartmented culture

To determine the extent of interactions between distal axons and cultured myotubes, compartmented cultures were assessed using both SEM and immunocytochemical techniques. Light microscopy visualisation of cultures containing differentiated myotubes indicated that distal axons were interacting with the myotubes. This physical interaction was confirmed with SEM. Motor neuron axons were visible extending towards and making physical contact with differentiated skeletal muscle fibers (Figure 5.6A,B).

The extent of these interactions was further probed using immunocytochemistry to analyse AChR distribution along the skeletal myofibre. Previous studies have demonstrated that myotubes rapidly switch from "pre-patterned" AChR clustering characterised by diffuse AChR expression punctated with very small dense clusters, to synaptic AChR clusters comprising few, discrete clusters and minimal AChR staining outside these regions (Fischbach and Cohen, 1973; Lin *et al*., 2001). To visualise receptors, AChRs were stained with α-BTx and the pattern of receptor localisation was analysed. α-BTx staining indicated large, discrete α-BTx clusters in close contact with motor neuron axons (Figure 5.6C, D) as distinct from diffuse α-BTx staining populated with very small bright puncta characteristic of pre-patterning. Synaptophysin positive puncta were also present in the vicinity of synaptic-like α-BTx staining (Figure 5.6E).

Synaptic localisation of α-BTx staining was quantified by changes to mean area of α-BTx particles. α-BTx clusters within 20μm of motor neuron axon terminals had significantly (P<0.01) smaller mean particle area when compared with myotubes >20μm from motor neurons (97.53 ± 7.36px, 3777.67 ± 339.36px with and without motor neurons respectively) (Figure 5.7). No difference was detected in α-BTx staining of AChR pre-

patterning on cultured muscle fibers without added motor neurons when compared with muscle fibers in co-cultures >20μm away from motor neuron terminals.

Further characterisation of NMJ formation in the distal compartment was assessed by α-BTx staining relative to immunolabelling for rapsyn, nestin and dystrophin at 10DIV in mouse-derived cultures. Control myocyte cultures, grown in the absence of motor neurons, were similarly labelled to differentiate between synaptic and pre-patterned AChR clustering. In co-cultures, synaptic-like α-BTx staining was colocalised with plaque-like arrangement of rapsyn immunoreactivity (Figure 5.8). This was not evident in C2C12s cultured without motor neurons (Figure 5.8).

In control C2C12 cultures, nestin immunoreactivity in myocytes was distributed uniformly throughout the myofibres, with stronger immunoreactivity at the ends of myofibres (Figure 5.9). In co-cultures, nestin immunoreactivity was less pronounced throughout the myocyte, with more intense regions of staining around the middle of the fibre (Figure 5.9). However, alterations in nestin immunoreactivity were not always associated with direct motor neuron axon contact.

The organisation of dystrophin immunolabelling was additionally altered in the presence of motor neurons. In control C2C12 cultures, dystrophin labelling was dispersed throughout the myocytes (Figure 5.10). In motor neuron + C2C12 co-cultures, dystrophin was more localised to the myocyte periphery, with distinct labelling in the centre of the fibres (Figure 5.10). Distinct central immunoreactivity of dystrophin was particularly prominent in myofibres with plaque-like α-BTx staining and contact with motor neurons.

These data indicate the motor neuron axon terminals not only initiate NMJ-like AChR clustering on primary myotubes under these culture conditions, but the NMJ-like

connections occur in conjunction with pre-synaptic vesicle accumulation in motor terminals.

5.3.4 Cultured motor neurons are resistant to axonally targeted excitotoxicity

To investigate the response of compartmented spinal motor neurons to excitotoxicity, 100μm kainic acid was added to either the proximal or distal compartment of mouse-derived cultures for 24 hours. Excitotoxicity induced in the proximal compartment resulted in extensive neuronal degeneration, evidenced by fragmentation of neurofilament-positive axons, reduced MAP2 immunoreactive dendrites and extensive cell death (Figure 5.11). Quantitation of degeneration of distal axons resulted in a significant ($P<0.05$) increase in percentage axon degeneration (combined beading and fragmentation) from untreated controls (55.8% ± 2.58% proximal excitotoxicity; 43.4% ± 2.08% control) (Figure 5.12). Widespread axonal degeneration was present within the distal compartment. Closer investigation into the type of axonal damage indicated that proximal kainic acid excitotoxicity initiated a significant ($P<0.05$) increase in axonal fragmentation (31.2% ± 1.60% proximal kainic acid, 20.9% ± 1.90% control) however there was no significant ($P>0.05$) change in axonal beading (25.6% ± 2.00% proximal kainic acid, 22.5% ± 1.65% control).Additional cultures were treated with proximal kainic acid for 4 hours, resulting in mild somatodendritic pathology (Figure 5.11).

Kainic acid was then applied to the distal compartment. There was no detectable change in neuronal morphology within the untreated proximal compartment (Figure 5.11). Within the treated distal compartment there was also no change to axonal morphology or immunoreactivity (Figure 5.11). Quantitation of axonal pathology resulted in no

significant (P>0.05) change in axonal morphology from control cultures (41.0% ± 3.22% distal excitotoxicity; 43.4% ± 2.08% control) (Figure 5.12). These data indicate that spinal motor neuron axons in co-culture with C2C12 cells are not vulnerable to kainic-acid induced degeneration via the distal axon.

5.3.5 Caspase activation in cultured motor neurons demonstrates two distinct patterns of distribution

To further probe the mechanisms of excitotoxicity in these compartmented cultures, preliminary investigations were performed where both distally and proximally treated cultures were immunolabelled for activated caspase 3, a putative effector of excitotoxin-induced axon degeneration (Nikolaev *et al.*, 2009; Simon *et al.*, 2012; King *et al.*, 2013). In untreated control cultures within the proximal compartment, active caspase 3 smoothly distributed along axons, with very few apoptotic cell bodies present (Figure 5.13A, E). Kainic acid excitotoxicity in the proximal compartment almost completely abolished smooth axonal caspase distribution, with the large majority of axons demonstrating caspase activation localised to swellings and fragmented axons (Figure 5.13 B). This was also evident after 4 hours of proximal kainic acid treatment, with almost no smooth caspase labelling evident (Figure 5.13 C). Within the distal compartment, control cultures had few caspase-positive axons, with a small number of caspase-positive axonal swellings (Figure 5.13D). Following proximal KA exposure, the many axons demonstrated caspase-positive swellings in the distal compartment (Figure 5.13F, G). Furthermore, distal kainic acid treatment resulted in the development of caspase-positive puncta, despite the absence of overt degeneration (Figure 5.13H). These results indicate

that targeted axonal kainic acid exposure induces changes in caspase localisation within spinal motor in the absence of frank degeneration.

5.4 DISCUSSION

The primary aim of this chapter was to develop a novel neuronal culture system in which to study the lower motor neuron and cellular mechanisms relevant to ALS pathogenesis. Initial investigations utilising standard neuronal culture techniques indicated that different non-neuronal cells affected development of motor neurons. Consequently, techniques were developed to spatially and temporally organise motor neurons within the compartmented chambers to recreate the organisation and cellular interactions of the *in vivo* circuit. Within these cultures, immunocytochemical analysis indicated that the lower motor neurons developed to relative culture maturity. Furthermore, compartmented lower motor neuron cultures were characterised by the formation of putative NMJs between the isolated motor neuron distal axons and skeletal myocytes. Critically, these interactions were achieved using cells derived from a number of sources. The second aim of this study was to use this model to investigate excitotoxicity in cultured motor neurons. In contrast with cortical neurons (Chapter 3), spinal motor neurons are predominantly susceptible to excitotoxicity via the somatodendritic neuronal compartment, however, targeted axonal kainic acid did induce subtle changes in the distribution of activated caspase 3 in the absence of overt axonal degeneration.

5.4.1 Communication between non-neuronal cells and motor neurons is required for appropriate development of spinal motor neurons *in vitro*

Signaling between non-neuronal cells and motor neurons is crucial for *in vivo* development of the nervous system. In addition to neurotrophins released by CNS glia, the survival of motor neurons relies on their ability to locate and form synapses with

skeletal muscle fiber (Oppenheim, 1991; Ekestern, 2004). Glial cells provide factors that are required for motor neuron development and survival *in vivo*. Astrocytic release of glial-cell-line derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), receptor ligands and neuropoietic cytokines is needed for appropriate growth of motor neurons *in vivo* (Ekestern, 2004) and influences vulnerability of motor neurons via $P75^{NTR}$ mediated cell death (Taylor *et al.*, 2007). Furthermore, many of these factors, particularly BDNF and GDNF are added to dissociated cultures of motor neurons to promote survival (Digby *et al.*, 1985). Skeletal muscle cells release trophic factors to promote axonal extension through the developmental extracellular milieu (Taylor *et al.*, 2007), culminating in formation of neuromuscular junctions. Motor neurons that fail to form neuromuscular junctions eventually die during *in vivo* development (Ekestern, 2004).

The data in this thesis demonstrates a differential effect of cell substrate on the growth of cultured motor neuron. Interaction of motor neurons with specific non-neuronal cell substrates can significantly affect their survival, dendritic arborisation and neurite outgrowth. Previously, aspects of this neuronal and non-neuronal signaling can be mimicked in 'traditional' co-cultures of dissociated neurons with glia and/or muscle cells (Daniels *et al.*, 2000; King *et al.*, 2011). Although axons readily extended into the distal axon compartment in this study, the presence of skeletal muscle cells was crucial for long-term survival of motor neuron axons when isolated from the cell body environment. This was especially clear in mouse-derived motor neuron cultures which rapidly degenerated when no distal synaptic targets were present.

An interesting feature of this culture model is that motor neuron maturity appears accelerated, and this was particularly evident in the mouse-derived cultures. Previous

investigations utilising primary rat motor neurons in co-culture with glial cells indicates that relative culture maturity is not reached until around 21DIV, evidenced by expression of dephosphorylated neurofilaments (King *et al.*, 2007). Absence of dephosphorylated neurofilaments is considered indicative of immaturity in cultured motor neurons (Vartiainen *et al.*, 1999). Within the present study, motor neurons were strongly immunoreactive for dephosphorylated neurofilaments at 10DIV. A number of factors may potentially cause this earlier maturation of motor neurons, including an increased density of cultured cells and the presence of skeletal muscle in the distal compartment. However, it is likely that the presence of distal skeletal myocytes plays a key role potentially via retrograde signalling from the distal terminus.

5.4.2 Fluidically isolated motor neuron axons can form NMJs *in vitro*

The development of a neuromuscular synapse involves contact and bidirectional signaling between a motor neuron axon terminal and skeletal muscle fiber (Lin *et al.*, 2008; Ferraro *et al.*, 2011). When grown in dissociated co-culture with myotubes (primary or stem cell derived), motor neurons form rudimentary neuromuscular junctions evidenced by dense clustering of AChRs on the myotube surface in areas adjacent to motor neuron terminals (Dutton *et al.*, 1995; Guo *et al.*, 2011). This clustering occurs in conjunction with increased immunoreactivity for the protein synaptophysin, a presynaptic vesicle marker, within motor neuron terminals (Dutton *et al.*, 1995). Cultured NMJs can also be grown using spinal cord explants placed such that motor neuron axons extend towards cultured muscle monolayers (Kobayashi *et al.*, 1987; Mars *et al.*, 2001). In the absence of spinal motor neurons, skeletal muscle cells are characterised by 'prepatterned' expression of AChRs. Such prepatterning develops independently of innervation via

MuSK-rapsyn interactions (Ferraro *et al.*, 2011). Presynaptic AChR clustering was evident in our compartmented cultures prior to contact with motor neuron axon terminals. This data indicate that distal motor axon terminals are sufficient to induce synaptic clustering of AChRs, which is consistent with studies that demonstrate clustering induced by the neuronal release of agrin in conjunction with neurotransmitter (ACh) release from the distal synaptic terminal.

Within this chapter, NMJ formation occurred between isolated distal axons and primary skeletal muscle fibers. Similarly, a number of experiments demonstrated AChR clustering induced by motor neurons grown within Campenot compartmented devices (Nelson *et al.*, 1992; Lanuza *et al.*, 2000), however the effect of the addition of exogenous factors to promote outgrowth of motor neuron axons in this culture system need to be considered. In this respect, neurotrophins including BDNF have been shown to alter AChR clustering on cultured muscle cells and affect subsequent formation of neuromuscular junctions (Peng *et al.*, 2003; Li *et al.*, 2012). The response to many of these factors is highly concentration-specific in motor neurons, implicating local axon protein synthesis and a location-specific component in trophin responses (Nedelec *et al.*, 2012). This novel microfluidic model demonstrates formation of neuromuscular junctions in cell culture is possible in a model recapitulating the *in vivo* cellular organisation.

5.4.3 Differential effects of excitotoxic-induced axon degeneration in spinal motor neurons compared with cortical neurons

Previous investigations in this thesis have demonstrated that cultured cortical neurons are vulnerable to excitotoxicity directed to the axon *in vitro* (Chapter 3), however this did not

occur following similar treatment to compartmented spinal motor neurons in this present study. There are a number of likely explanations for this difference. Firstly, the axons of cortical neurons are proposed to communicate directly with oligodendrocytes via glutamatergic signalling (Matute, 2011; Matute and Ransom, 2012). In contrast, communication between the motor neuron axon and Schwann cell does not occur via glutamate, instead communication occurs via reciprocal NRG-1 signalling (Nave and Salzer, 2006; Salzer, 2012). At present, there is little evidence regarding the role of NRG-1 in excitotoxicity. However, a number of recent studies have proposed that in sensory neurons, NRG-1 signalling may be neuroprotective from excessive glutamate (Liu *et al.*, 2011; Li *et al.*, 2013). This may occur via reduction of caspase (Liu *et al.*, 2011) or by modulation of NMDA-R localisation (Schrattenholz and Soskic, 2006). Schwann cells are not regarded to be vulnerable to excitotoxicity, however are neuroprotective to cultured motor neurons in experimental excitotoxicity (Ragancokova *et al.*, 2009).

5.4.4 Atypical caspase distribution in cultured motor neurons exposed to excitotoxicity

Activation of the caspase signalling pathway, in particular casapase-3, is traditionally considered to result in imminent cell death (Maghsoudi *et al.*, 2012). However, there is increasing evidence that caspases are involved in a wider range of cellular processes beyond apoptotic pathways, including axonal pruning (Nikolaev *et al.*, 2009; Schoenmann *et al.*, 2010; Simon *et al.*, 2012) and synaptic reorganisation (D'Amelio *et al.*, 2011). More recently, axonal localisation of caspases has been described in a number of neurodegenerative conditions, including Alzheimer's disease (Nikolaev *et al.*, 2009), injury (Chen *et al.*, 2004a) and following excitotoxicity in cultured cortical neurons (King

et al., 2013). There is increasing evidence to suggest that excitotoxic axon degeneration is a distinct process from Wallerian degeneration, a proposal which is supported by these findings (Buki and Povlishock, 2006). The role of these axonally localised caspases is not known, however a number of relevant substrates for caspase-3 cleavage have been identified, including cytoskeletal proteins and dynein/dynactin complexes (Simon *et al.*, 2012). Cytoskeletal cleavage by activated caspase-3 in the axon has been proposed as a mechanism for formation of axonal beading following excitotoxicity (Schoenmann *et al.*, 2010).

However, both pre-caspase-3 and activated caspase-3 have also been identified in healthy axons (Schoenmann *et al.*, 2010), suggestive of a normal axonal function. Within the present study, activated caspase-3 was detected in motor neuron axons. However, the distribution of immunoreactivity was unusual, with a proportion of motor neuron axons demonstrating smooth and dense caspase-3 immunolabelling. In contrast, activated caspase-3 activation has been previously described to occur in discrete axonal puncta (Schoenmann *et al.*, 2010; King *et al.*, 2013) or localised to abnormal nuclei (Maghsoudi *et al.*, 2012). The significance of this atypical caspase distribution is not clear in the current study. Furthermore, atypical caspase localisation did not correlate with classical signs of damage within those neurons, suggesting the activation of caspase-3 was not detrimental to the neuron.

5.4.5 Spatial organisation of cultured cells is relevant for investigating neurodegenerative disease

Disruption of signaling between motor neurons and their non-neuronal cells plays a crucial role in the development of neuromuscular disorders and ALS in particular. The non-cell autonomous theory of ALS is strongly supported by the impact of glial cells in progression of motor neuron degeneration (Boillée *et al.*, 2006a). The close interplay between motor neurons and astrocytes in ALS is evident in chimera studies where mice with astrocyte-specific expression of mSOD1 develop characteristic motor neuron pathology, whereas mSOD1 expression restricted to motor neurons only does not (Clement *et al.*, 2003). Increasing astrocytic expression of Nrf2 in a number of SOD1 mutants delays onset (Vargas *et al.*, 2008). Additionally, astrocyte expression of mSOD1 increases motor neuron susceptibility to excitotoxicity via downregulation of GluR2 expression (Van Damme *et al.*, 2007b). Conversely, reducing astrocytic mSOD1 expression in mouse models reduces microglial activation, prolonging survival (Yamanaka *et al.*, 2008b).

Whereas, increasing evidence implicates astrocytes as being important contributors to ALS pathogenesis, the role of muscle in ALS onset/progression remains controversial. Studies have shown specific mSOD1 expression in muscle initiates ALS-like dysfunction within the muscle (Dobrowolny *et al.*, 2008), although others have demonstrated no effect of decreasing mSOD1 expression within mSOD1 mutant muscle (Miller *et al.*, 2006; Towne *et al.*, 2008). Nevertheless, muscle presents an attractive target for therapeutic intervention in ALS. Increasing skeletal muscle expression of IGF1 increases survival in G93A mice, even resulting in reduced inflammation within the spinal cord (Dobrowolny *et al.*, 2005). mSOD1 G93A mice demonstrate altered mitochondrial

morphology and signs of energy depletion without immediate axon degeneration or loss of neuromuscular functionality prior to symptom onset (Da Cruz *et al.*, 2012). In such mice, the subsequent onset of muscle weakness occurs later, indicating a point at which compensatory mechanisms become overwhelmed and neuromuscular/distal axon degeneration begins. However, it still remains unclear what this trigger is. Furthermore, such a 'tipping point' could be encountered at any point within the lower motor neuron circuit. At present, cell culture investigations into motor neuron and non-neuronal cell interactions in ALS-like conditions have been performed using traditional monolayer culture techniques. The current data reports a distinct effect of cellular substrates on the growth of motor neurons *in vitro*. This study has implications both in determining the relevance of specific cell types to the mechanisms of ALS and also for modeling aspects of the disease in cell culture to include cell types and replicate their unique *in vivo* organisation.

5.4.6 Conclusions

The culture paradigm developed in this thesis is characterised by normal growth of motor neurons and formation of NMJs between motor neuron distal axons and skeletal myocytes. The ability to experimentally manipulate motor neurons and associated non-neuronal cells within this compartmented co-culture system will facilitate experimentation into motor neuron and NMJ physiology. Furthermore, such investigations will be conducted within physiologically relevant microenvironments, shown to be critical for appropriate neuronal development (Millet and Gillette, 2012). The data presented in this study indicate that cultured spinal motor neurons respond to excitotoxicity via different mechanisms to cortical neurons. In particular, this study has

demonstrated that excitotoxicity resulting in motor neuron axon degeneration in these preparations is likely to be mediated via the somatodendritic neuronal compartment. Additionally, this investigation demonstrated the presence of atypical axonal caspase immunolabelling, the physiological relevance of which is currently unknown. This model presents a unique platform for screening potential therapeutic agents for neurodegenerative disease prior to utilising mouse models. A compartmented culture approach also allows the addition of cells from a wide variety of sources and will be suitable for use with induced pluripotent stem cell derived motor neurons from human patients (Dimos *et al.*, 2008; Egawa *et al.*, 2012). This compartmented motor neuron culture model represents an exciting opportunity to investigate motor neuron pathophysiology with the greatest *in vivo* relevance.

6 FINAL DISCUSSION

The development of the mSOD1 G93A mouse model of ALS has provided a wealth of information into the mechanisms involved in the pathogenesis of ALS (Gurney *et al.*, 1994). Investigations using this model and others have lead to development of a range of new potential therapies, however at this time, none have successfully progressed through clinical trials in humans. The repeated failure of such trials has highlighted the fact that many aspects of ALS pathogenesis remain unexplained. It is still not known how excitotoxicity results in axon degeneration, nor is the process of distal axon and NMJ degeneration, thought to be one of the initial pathological alterations in ALS. Until we have a better understanding of the mechanisms underlying the pathology and progression of ALS, it remains unlikely that effective therapies will be discovered. The central aim of this thesis was to investigate axon degeneration in ALS, with a specific focus on how excitotoxicity is involved in the development of this distal axon pathology.

Glutamate excitotoxicity is a known pathological process in a number of neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease, traumatic brain injury and motor neuron disease (Chapter 1.3). Similarly, a conserved pathological hallmark of these diseases is axonal degeneration. How excitotoxicity, primarily considered to occur through somatodendritic glutamate receptors, results in degeneration, often of the distal axon, is not known. The recent discovery of glutamate receptor subunits along myelinated axons raises the question of whether excitotoxicity is limited to the somatodendritic compartment, or whether the axon may be a primary target for excitotoxicity as well (Ouardouz *et al.*, 2009a,b; Matute, 2011). Thus, the first aim of this thesis was to investigate how excitotoxicity results in distal axon degeneration. Data

within this study (Chapter 3) demonstrates that the axons of cortical neurons are directly vulnerable to excitotoxicity.

Excitotoxicity following injury is often considered to occur via NMDA-Rs. In contrast, excitotoxicity in ALS is proposed to occur via AMPA-Rs (Chapter 1.3.2). This may reflect key differences in the pathogenesis of excitotoxicity, being primarily acute excitotoxicity in the former and chronic in the latter (Chapter 1.3.2). Alternatively, this may reflect the fact that AMPA-R permeability to calcium and thus vulnerability to excitotoxicity can be altered via inclusion or exclusion of the Ca^{2+} impermeable GluR2 subunit. As previously discussed, a number of factors can alter GluR expression, and of particular interest is that neuronal expression of GluR2 can be modulated by glial cells (Chapter 1.3.2.1). Excitotoxicity in the white matter was considered to occur primarily via NMDA-Rs on oligodendrocytes, resulting in secondary damage to axons (Matute, 1998; Fowler *et al.*, 2003; Fowler *et al.*, 2006; Saggu *et al.*, 2008). Following the discovery of GluRs on myelinated axons (Ouardouz *et al.*, 2009a,b), the hypothesis that axons may respond directly to excitotoxicity was proposed by Matute (2011). The data in this study (Chapter 3) indicates that this can occur in preparations of cortical neuron axons which are devoid of oligodendroglia. Furthermore, evidence gained from this investigation indicates that axonally mediated excitotoxicity is likely to occur via AMPA-Rs (Chapter 3). The neuronal response to excitotoxicity may be mediated via different pathways depending on axonal vs somatodendritic exposure, thus protective mechanisms aimed at the soma may have little effect on protection from axonal excitotoxicity.

Excitotoxin-induced neuronal degeneration has been shown to involve activation of caspases, the effector proteases for apoptosis (Favaloro *et al.*, 2012; King *et al.*, 2013). The data in this study demonstrates that both somatodendritic and axonally induced excitotoxicity results in caspase-mediated cortical axonal degeneration. Widespread

blockade of caspase activity reduced proximally induced excitotoxic axon degeneration and prevented distal excitotoxic axon degeneration in these cortical cultures, critically, this process can be achieved by either axonal or somatodendritic caspase inhibition (Chapter 3).

Neurons are unique cells with highly specialised sub-domains. This compartmented specialisation is critical for neuronal function and enables neurons to span great distances within the body. Neuronal compartmentalisation is particularly evident when considering the axon, with studies increasingly highlighting the ability of the axon to respond to environmental cues through modulation of local mRNA synthesis independently of somal responses (Cosker *et al.*, 2013). Such processes may be additionally influenced by local interactions with non-neuronal cells (Cosker *et al.*, 2013). In this study, neuronal compartmentalisation was evident by simultaneous presentation of markers of maturity and immaturity in different cellular compartments of cultured cortical neurons (Chapter 3). Cultured cortical neurons developed morphological features of neuronal maturation in culture (dendritic spines, synaptic puncta, mature-like organisation of GluRs and response to excitotoxicity), however also retained distal axonal growth cones. A plausible explanation for this seemingly paradoxical finding is that many axons remained within the proximal compartment and formed synaptic connections with dendrites.

Neuronal compartmentalisation may also play a critical role in mediating excitotoxicity. Within the dendrites, localisation of NMDA-Rs to synaptic or extrasynaptic localisations affects the response of the neuron to subsequent receptor activation (Chapter 1.3.1.1). In this thesis, it was demonstrated that activation of axonal glutamate receptors results in degeneration of the axon without overt somatodendritic involvement, highly suggestive of local modulation of excitotoxicity (Chapter 3). At the dendrites, activation of differentially localised NMDA-Rs yields either neuroprotective or neurotoxic results

(synaptic and extrasynaptic NMDARs, respectively) (Hardingham and Bading, 2010). From this present study it is not clear whether activation of axonal AMPA-Rs under physiological conditions is likely to be protective or deleterious *in vivo*. If axonally localised AMPA-Rs are normally involved in crosstalk with oligodendrocytes during myelination (Matute, 2007), it is possible that low-level activation of such receptors could be protective for the axons, however this remains purely speculative. Independent of the normal functioning of these receptors, the current data indicates that chronic high-level activation is locally deleterious.

Taking the compartmented nature of neuronal growth into account is especially pertinent when considering the motor neuron, whereby different neuronal compartments are located in physically different regions of the organism and interact with a vast range of cell types including CNS glia (proximal) and the skeletal muscle (distal). Each of these cell types are being increasingly recognised for the roles they play during disease in ALS, with widely acknowledged involvement of glial cells and accumulating evidence that the skeletal muscle may also contribute. The loss of the distal neuro-muscular synapse has been described as one of the first pathological events in humans and mice. It is therefore critical to fully understand the series of changes which lead to loss of the NMJ, especially in ALS.

Despite a wealth of information regarding the complex protein interactions involved in development and maturation of the NMJ (Chapter 1.2.3), very little is known about how these components contribute to disease outside of neuromuscular diseases arising from specific genetic causes. Consequently, the second aim of this thesis was to characterise degeneration of the NMJ in a widely used mouse model of ALS. Loss of the NMJ has been demonstrated in both human ALS patients and in mutant SOD1 models, and is generally attributed to onset of symptoms (Chapter 1.2.3) (Figure 6.1). Previous studies

have focused on pathology within the motor neuron distal axon and used loss of colocalisation between motor neuron axons and post-synaptic AChRs to indicate degeneration of the NMJ (Chapter 1.2.3). However, little is known about the sequence of changes that occurs during NMJ breakdown and this could give some clues as to the initial cause of degeneration. Furthermore, whilst the role of skeletal muscle in ALS is being increasingly discussed (Dobrowolny *et al.*, 2008; Wong and Martin, 2010), it remains unclear if degeneration is initiated in the muscle or occurs as a consequence of motor neuron dysfunction, such as from hyperexcitability or axonal starvation from the soma.

The mSOD1 mouse develops clinical symptoms in the hind-limbs first, with the forelimbs remaining relatively spared until later in the disease (age) (Chapter 4). In this study, both the hindlimb and forelimb skeletal muscles were examined to determine the precise pathological timing of NMJ degeneration. In the hindlimbs, the earliest detectable changes occurred at 56 days of age, representing a reduced proportion of NMJs with colocalised presynaptic motor terminals and S100 with α-BTx. This reduction in the proportion of colocalised α-BTx with motor terminals and S100 further decreased throughout the times examined. By 84 days, there was a reduction in the proportion of NMJs showing colocalisation between α-BTx and rapsyn or nestin. By 112 days, the proportion of NMJs with colocalised dystrophin and α-BTx was significantly reduced. This contrasted with the findings from the forelimbs where the only pathology in this study was a loss in colocalisation between α-BTx and presynaptic motor terminals at 140 days of age (Chapter 4). These results support the previous theories from Fischer *et al.* (2004) proposing degeneration in ALS reflects a distal "die-back" axonopathy. The gradual loss of rapsyn, nestin and dystrophin from NMJs is therefore likely to represent secondary degenerative changes resulting in dismantling of the post-synaptic complex. In

the hindlimbs, loss of S100 from terminal Schwann cells was detectable at the same age-point as loss of presynaptic terminal localisation with α-BTx, however this was not observed in the forelimbs. The delayed nature of loss of the other markers examined in the forelimbs suggests that the forelimb skeletal muscle in the mSDO1 mouse may be useful for investigating subtle degenerative or remodelling events which are otherwise obscured by the pathology that occurs in the mouse hind-limbs.

This thesis demonstrates that loss of the NMJ occurs in a progressive manner (Chapter 4). Evidence gained in this study and in others (Chapter 1.2.3) further demonstrates that loss of neuromuscular connectivity occurs in conjunction with altered organisation of a number of key NMJ structural components. Such loss of structural proteins is likely to result in instability of the NMJ and contribute to fragmentation of post-synaptic AChR clusters (Chapter 4). The loss of structural proteins in this study may indicate a number of different processes. The simplest explanation for which is that loss of structural proteins colocalised to membrane AChR clusters represents progressive dismantling of the NMJ following denervation and lack of reinnervation. From mouse injury models, there is evidence that disassembly of the post-synaptic apparatus is delayed for weeks following denervation as a likely result of the skeletal muscle remaining prepared for reinnervation (Li and Thompson, 2011).

The loss of the investigated proteins from the NMJ is likely to affect signalling between the different proteins that comprise and support the NMJ (Chapter 4). The interactions between these proteins remain relatively unexplored in the context of ALS. Furthermore, it is possible that such perturbations to this signalling may occur very early in ALS and may subtly contribute to destabilisation of the NMJ and weakening muscle-motor neuron signalling. The ALS NMJ is characterised by abnormal action potential conduction (Magrané *et al.*, 2012). Additionally, the skeletal muscle is characterised by synaptic

accumulations of mitochondria, likely to reflect perturbed Ca^{2+} homeostasis (Zhou *et al.*, 2010). The relationship between these processes is not well understood, however it remains possible that dysfunction at the NMJ could arise from a positive feedback loop involving both the motor neuron and skeletal muscle.

From investigations into Duchenne muscular dystrophy (DMD), occurring from dystrophin mutations, it has become evident that whilst some proteins such as dystrophin are not critical for initial formation of the NMJ, they play a key role in the maturation and function of the synapse (Chapter 4). In the case of dystrophin, the truncated mutated protein fails to incorporate into the dystrophin glycoprotein complex (DGC), rendering the skeletal muscle increasingly vulnerable to contractile injury (Pilgram *et al.*, 2010). Although mutations to the investigated proteins have not been linked with ALS, it remains possible that altered post-synaptic signalling and abberant protein interactions may result in mislocalisation of these proteins, thus rendering the NMJ increasingly unstable. This study serves to highlight the importance of further investigation of the NMJ and the role of skeletal muscle in ALS. Furthermore, the forelimb muscles in the mSOD1 mouse may provide a suitable location in which to further investigate whether such effects occur prior to degeneration of the motor neuron axon.

Schwann cells play an important role in myelination of the motor neuron and maturation of the NMJ (Chapter 1.2.3). The Schwann cell also assists during reinnervation of skeletal muscle fibres by guiding the regenerating axon to the recently denervated NMJ site (Koirala and Reddy, 2003). This is in contrast with development of the NMJ where 'pre-patterned' post-synaptic receptor clustering does not dictate where the NMJ will form (Chapter 1.2.3). The results of this thesis indicate an early and progressive loss of peri-synaptic S100 staining in aging mSOD1 neuromuscular junctions, and suggest that the loss of S100 staining reflects a loss of the proteins and not the cells themselves

(Chapter 4). A similar result has been seen in other studies using both mouse and human tissue (Liu *et al.*, 2013). The implication for the early loss of S100 is not known, however could be involved in abnormal neurotransmission at the NMJ (Liu *et al.*, 2013).

The preferential degeneration of the lower limbs in the mSOD G93A model prior to degeneration of the upper limbs has previously received little attention. There are a number of potential causes for this variable onset of phenotype. Firstly, the axons which innervate the hind-limb muscles are longer than those of the forelimb and thus may represent a more vulnerable population of motor neurons. Whilst this remains a possibility, the same pattern degeneration of hind-limb muscles does not occur in mSOD1 G93A transgenic rats (Matsumoto *et al.*, 2006). Peculiarly, the mSOD1 G93A rats demonstrate preferential degeneration of the forelimb motor neurons, leaving the hindlimbs less affected (Matsumoto *et al.*, 2006). A second possibility is that the motor neurons that innervate the hindlimbs are different from those of the forelimbs and may have differential vulnerability to mSOD1 mediated toxicity. In both human ALS patients with mSOD1 mutations and in mSOD1 mouse models, there is a preferential site of onset in the lower limbs (Gurney *et al.*, 1994; Chiu *et al.*, 1995; Azzouz *et al.*, 1997; Bendotti and Carri, 2004; Millecamps *et al.*, 2010). There are well-described differences in the types of motor neurons which innervate the fore- and hindlimbs in both mice and humans, notably, the gastrocnemius muscle comprises a mixture of type I slow and type II fast motor neurons, with the forelimbs predominantly type II in humans and entirely type II in mice (Elashry *et al.*, 2009; Schiaffino and Reggiani, 2011; Mathewson *et al.*, 2012). Type II fibres are preferentially vulnerable to degeneration in a number neurodegenerative conditions including ALS, sarcopenia and aging (Hegedus *et al.*, 2008; Gordon *et al.*, 2009; Jang and Van Remmen, 2011). Based on these results, it would be interesting to investigate differences in the motor neuron populations within different

levels of the spinal cord as has been done for the ocular motor neurons compared with limb skeletal muscles (Chapter 1.3.2.1). Moreover, it would be interesting to investigate how any differences in the motor neurons reflects pathology in the spinal cord and thus determine which types of pathology are associated with functional loss.

The purpose of aim 2 (Chapter 4) was to further define the process of NMJ degeneration in the mSOD1 G93A mouse model of ALS. This study indicates that the distribution of a number of structural proteins within the skeletal muscle fibre is altered following degeneration of the motor neuron distal axon. The slow process is likely to result in destabilisation of the AChR membrane complex and may also result in perturbed signalling. The results from this thesis indicate that the interactions of a large number of NMJ proteins warrant further investigation in ALS and perhaps degeneration of the NMJ could be delayed or prevented by therapeutic targeting of these structures.

However degeneration of the NMJ is a complex process to model. Current *in vitro* techniques are reliant on either motor neurons cultured as a monolayer with glia or muscle, or in Campenot chambers. The former approach lacks the temporal and fluidic organisation of the spinal motor neuron *in vivo*, and the latter necessitates the addition of exogenous factors which is known to affect receptor expression (Chapter 5). To address these shortcomings, the third aim of this thesis was to develop cell culture techniques using the microfluidic chambers to combine spinal motor neurons with glial and muscle cells. In isolation, cultured glia and muscle each promoted different neuronal phenotypes *in vitro*, likely representing the fact that neither cell population is normally in contact with the entire motor neuron (Chapter 5). The combination of glia, muscle and motor neurons within the microfluidic device resolved this issue and resulted in mature and stable cultures for investigating the compartmented response of motor neurons to excitotoxicity (Chapter 5).

Excitotoxicity is a known pathogenic process in ALS (Chapter 1.3) however it is not known whether excitotoxin-induced axon degeneration occurs via the same pathways that cause degeneration of cortical neurons (Chapter 3). The final aim of this thesis was to investigate the mechanisms of excitotoxic axon degeneration in a compartmented cell culture model. The results from this thesis indicate that excitotoxicity in motor neurons does not occur via the distal axon (Chapter 5). The localisation of GluRs on cortical axons is proposed to serve the primary function of communication with oligodendrocytes (Chapter 1.2), however communication with motor neuron axons and Schwann cells occurs via different mechanisms, primarily via NRG-1 signalling in contrast with the voltage- and possibly glutamate-dependent signalling between oligodendrocytes and axons.

6.1 LIMITATIONS AND ALTERNATIVE INTERPRETATIONS

The investigation in Chapter 3 provided evidence for a direct role of the axon in excitotoxicity, which suggests that the distal axon may be capable of independently responding to excitotoxicity. These interactions could be further investigated using other amino acid neurotransmitters or analogous excitatory agents. Preliminary evidence suggests that distal axon excitotoxicity in this study was mediated via both NMDA and non-NMDA glutamate receptors, more work is needed to fully elucidate this response. This study utilised the glutamate receptor antagonists CNQX and MK801 to block the ionotropic NMDA and non-NMDA receptors, however there are many other receptor antagonists which could be trialled. Whilst this study strongly suggests that distal axons may respond to direct excitotoxicity, it is still unclear which receptors are involved. The

use of advanced imaging techniques and electrophysiology should be considered during furture studies.

Another limitation of this work is the reliance on the mSOD1 G93A mouse model of ALS. Whilst this mouse model recapitulates some aspects of the human disease, there are some key differences. The failure of promising therapeutic agents from mouse trials in human clinical trials has raised questions over the validity over the mSOD1 model. However recent investigations using advanced molecular techniques have demonstrated that there may be more similarities between human disease and the mSOD1 model than previously thought. It is then alternatively proposed that the lack of successful therapeutic candidates may reflect a problem of relying heavily on murine disease models that only represent a small fraction of human ALS cases. As such, it is important to focus on the development of non-transgenic models or to validate findings across a range of transgenic lines of ALS (Swarup and Julien, 2011). Within the mSOD1 G93A lines, it is well established that the number of transgene copies can dramatically affect disease outcome in the mice. Additionally, the failure of promising drug candidates in human clinical trials highlight the importance of understanding the underlying biology of disease to better target potential new therapies.

The investigations performed into the loss of structural proteins from the NMJs of mSOD1 G93A mice should be repeated using different analysis techniques, such as automated colocalisation algorithms to remove human error from these studies. Additionally, protein levels should be investigated using western blot or mRNA expression levels probed to determine whether the loss of marker colocalisation with α-BTX reflects loss of protein or structural reorganisation. Simiarly, the results regarding the loss of the S100 protein from terminal Schwann cells should be interpreted with caution at this current time. Further analysis of Schwann cell nuclei, the use of other

Schwann cell markers such as $P75^{NTR}$ and triple-labelling with motor terminals may reveal a different result. Whilst others have suggested the loss of S100 protein from terminal Schwann cells does not immediately occur from loss of cells, there is no direct quantitative evidence from this study to fully support the conclusion at this time.

The results of Chapter 5 of this thesis present a novel method for coordinating a number of different primary cell types to produce compartmented cultures of the lower motor neuron-neuromuscular junction circuit. There is great potential for this model to be used in conjunction with a range of human patient-derived cells, however no such cells were trialled in this project. The formation of putative NMJs in this model is an excellent outcome, however the functionality of these synapses should be tested using electrophysiology or direct manipulation of the circuit. As for limitations in Chapter 3, the response of these cultures to excitotoxicity should be further investigated using a wider range of excitatory amino acid neurotransmitters.

6.2 IMPLICATIONS

Gaining a better insight into neuronal function is critical to understanding complex neurodegenerative disorders including ALS. In particular, the mechanisms by which excitotoxicity results in neuronal damage and degeneration of the motor neuron distal axon and NMJ. The data obtained in this thesis reveals that whilst excitotoxicity can be initiated through the axon in cortical neurons, in ALS excitotoxicity is likely to be initiated through the soma and dendrites, with axon degeneration occurring following somatodendritic excitotoxicity. Furthermore, the results from this thesis highlight the differences between mouse cortical and spinal motor neurons. Whilst differences in function, localisation and susceptibility to disease in ALS are well known, it is likely that

these differences are not as well appreciated as they should be. This is particularly relevant when considering excitotoxic degeneration. The results from this thesis raise the possibility that excitotoxin-mediated neurodegeneration occurs via different mechanisms in cortical and spinal motor neurons, a finding which may influence the discovery of future drug targets for excitotoxicity. Furthermore, whilst non-neuronal cells are being increasingly recognised in the pathogenesis of ALS, little is still known about the role of the skeletal muscle. In particular, whether the skeletal muscle plays an active role in the development of distal motor neuron axonopathy. The results from this thesis demonstrate that structural changes to the NMJ can occur early in the disease process.

A potentially exciting avenue for further research is the potential for the novel compartmented culture model developed for aim 3 (Chapter 5) to be used with cells derived from human patients. Such cells will provide a wealth of information into specific disease processes from individual patients. Additionally, the use of cells derived from individual patients may serve as a useful validation tool for results gained from animal and cell culture experiments. In a disease where 90% of cases are still considered to be sporadic in onset, investigation of disease mechanisms and possible therapeutic targets may need to be approached on a more personal level.

6.3 CONCLUSIONS

In summary, the studies within this thesis have provided insight into the mechanisms of glutamate excitotoxicity in cultured neurons, with particular regard to excitotoxicity resulting in axon degeneration (Figure 6.2). This thesis has demonstrated that the axons of cortical neurons are directly vulnerable to excitotoxicity, whereas the axons of spinal motor neurons are not. Additionally, this thesis has demonstrated that post-synaptic

changes occur in the NMJ prior to loss of the post-synaptic AChRs. These results indicate a new understanding of some of the disease mechanisms involved in ALS. However, critically, provide two new models in which to investigate the pathogenesis of ALS. The first, is a novel compartmented culture paradigm which enables exquisite control over the development of motor neuron-co-cultures and may be used with a large variety of cells from different sources. Secondly, this thesis has demonstrated that the forelimbs of the mSOD1 G93A mouse may represent an appropriate location in which to investigate very early and subtle pathological change. The results from this thesis both provide direct evidence for mechanisms of toxicity in ALS, and additionally provide new models in which to further investigate pathology. It is hoped that the data within this thesis and future experiments utilising these models will aid in the development of new drug targets to combat this devastating disease.

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8 APPENDICES

8.1 COMMON LABORATORY REAGENTS

0.01M Phosphate Buffered Saline (PBS) – 1.0L

850mL MilliQ® water
100mL 90.0g/L Sodium chloride (NaCl; BDH, USA)
40mL 28.0g/L Di-sodium hydrogen orthophosphate (Na_2HPO; BDH, USA)
10mL 31.2g/L Sodium di-hydrogen orthophosphate ($NaH_2PO_4.2H_2O$; Ajax, Australia)

4% Paraformaldehyde – 1.0L

40g granulated paraformaldehyde (PFA)
500mL MilliQ® water
400mL 28.0g/L Na_2HPO
100mL 31.2g/L $NaH_2PO_4.2H_2O$
1.0M NaOH & 1.0M HCl to pH

Heat MilliQ® to 80^oC, add granulated PFA and 5 drops of NaOH. Stir until PFA dissolved, add $NaH_2PO_4.H_2O$ and Na_2HPO. Filter and pH to 7.4.

Tissue Storage Solution – 500mL

500mL 0.01M PBS
0.5g sodium azide

Cryoprotectant Solution (30% sucrose) – 500mL

500mL 0.01M PBS
150g sucrose
0.02% sodium azide

0.3% Triton-X-100 Diluent – 200mL

200mL 0.1M PBS
600µl Triton-X-100 diluent (Sigma, USA)

8.2 REAGENTS FOR CELL CULTURE

1.06g/L Optiprep

4mL HBSS
1mL Optiprep™

Mix well immediately prior to use.

0.001% Poly-L-lysine

450ml 0.01M PBS
50ml (10%) Poly-L-lysine

2.5ng/ml Poly-L-laminin

10ml 0.001% Poly-L-lysine
25µl Laminin (Sigma)

0.01% Collagen

100ml 0.01M PBS
10ml (10%) Calf-skin collagen (Sigma)

0.25% Trypsin

20mL 0.01M PBS
0.05g Trypsin

Combine and filter-sterilise. Store 1mL aliquots -20^{o}C.

0.05% Trypsin EDTA

100ml 0.01M PBS

0.05g Trypsin
0.0186g EDTA

Combine and filter-sterilise. Store 10mL aliquots -20^{o}C.

Collagenase/Dispase

1mg/ml Collagenase
2.4U/ml Dispase I
2.5mM $CaCl_2$

Store 250µl aliquots collagenase and dispase -20^{o}C; $CaCl_2$ stock 4^{o}C. Combine immediately prior to use.

Table 1.1 – Genes involved in ALS

Gene	Protein	Normal function	Mutant activity	Reference
SOD1	Superoxide dismutase 1	Antioxidant	Toxic gain of function	Rosen *et al*., 1993
ALS2	Alsin	Endosome trafficking	Uncertain	Yang *et al*., 2001
VEGF	Vascular endothelial growth factor	Induced by hypoxia	Loss of function	Oosthyse *et al*., 2001
Angiogenin	Angiogenin	Related to VEGF	Loss of function	Greenway *et al*., 2004; 2006
Peripherin	Peripherin	Cytoskeleton	Loss of function	Gross-Louis *et al*., 2004
SETX	Senataxin	DNA processing and repair	Loss of function	Chen *et al*., 2004
VAPB	Vesicle-associated membrane protein	Axon transport	Deficits in axon transport	Nishimura *et al*., 2004
TARDBP	TAR-DNA binding protein (TDP-43)	DNA binding, associated with half mammalian transcription	Loss of function and/or mislocalisation to cytoplasm. Aggregation.	Kabashi *et* al., 2008; Sreedharan *et al*., 2008
FUS	Fused in sarcoma	RNA binding	Loss of function and/or mislocalisation to cytoplasm. Aggregation.	Kwiatkowski *et al*., 2009; Vance *et al*., 2009
ATXN-2	Ataxin-2	Uncertain	Poly-Q repeats, toxicity in a dose-dependent manner	Elden *et al*., 2010
OPTN	Optineurin	Negative regulation of TNF-α activation of NF-κB	Loss of function and/or intracellular aggregates	Maruyama *et al*., 2010
VCP	Valosin-containing protein	Diverse range of intracellular functions including cell cycle and protein degradation	Intracellular aggregates and loss of function	Johnson *et al*., 2010
C9orf72	C9ORF72	Unknown	Uncertain. Formation of dipeptide repeat proteins by RAN translation	DeJesus-Hernandez *et al*., 2011; Renton *et al*., 2011
UBQLN2	Ubiquilin-2	Delivery of ubiquitinated proteins to the proteasome for degradation	Impaired protein degradation	Deng *et al*., 2011
Eph4	Tyrosine kinase ephrin receptor 4		Disease modification, missing Eph4 slows disease progression	Van Hoeke *et al*., 2012
PFN-1	Profilin-1	Conversion of monomeric actin to filamentous actin	Dysfunction	Wu *et al*., 2012a
1p34.1	Unknown	Unknown	Age of onset modification	Ahmeti *et al*., 2013

Table 2.1 – Primary antibodies

Name	Immunogen	Species	Dilution	Fixation	Supplier
β-III tubulin	Neuron-specific cytoskeletal marker	Mm	1:2000	4% PFA	Promega
Desmin	Myocyte intermediate filament	Mm	1:400	4% PFA	Dako
Dystrophin	NMJ scaffolding protein (postsynaptic)	Rp	1:100	4% PFA	Abcam
Fibronectin	Fibroblast specific protein	Rp	1:1000	4% PFA	Sigma
GFAP	Glial fibrillary acidic protein	Mm	1:1000	4% PFA	Chemicon
GFAP	Glial fibrillary acidic protein	Rp	1:2000	4% PFA	Dako
GluR1	AMPA receptor subunit 1	Rp	1:100	4% PFA	Chemicon
GluR2	AMPA receptor subunit 2	Mm	1:1000	4% PFA	Chemicon
GluR4	AMPA receptor subunit 4		1:500	4% PFA	Chemicon
MAP2	Microtubule-associated protein 2	Mm	1:1000	4% PFA	Millipore
Nestin	Developmental intermediate filament	Mm	1:500	4% PFA	BD Transduction
NFM	Neurofilament medium chain	Rp	1:1000	4% PFA	Serotec
NR1	NMDA receptor subunit 1	Rp	1:100	Methanol	BD Pharmingen
Rapsyn	NMJ scaffolding protein (postsynaptic)	Mm	1:200	4% PFA	Sigma
S100	Schwann cell marker	Rp	1:500	4% PFA	Dako
SMI32	Dephosphorylated neurofilaments	Mm	1:2000	4% PFA	Sternberger monoclonals
SMI312	Phosphorylated neurofilaments	Mm	1:1000	4% PFA	Sternberger monoclonals
Synaptophysin	Pre-synaptic vesicle marker	Rp	1:500	4% PFA	Millipore
Vimentin	Mesenchymal cell intermediate filament	Mm (IgM)	1:100	4% PFA	Chemicon

*Mm = mouse monoclonal (IgG unless specified), Rp = rabbit polyclonal

Table 2.2 – Secondary antibodies

Name	Fluorophore	IcC dilution	IhC dilution	Supplier
Anti-mouse IgG	Alexa 488	1:1000	1:750	Molecular Probes
Anti-mouse IgM	Alexa 488	1:1000	1:750	Molecular Probes
Anti-rabbit IgG	Alexa 488	1:1000	1:750	Molecular Probes
Anti-rabbit IgG	Alexa 594	1:1000	1:750	Molecular Probes
Anti-mouse HRP (horseradish peroxidase)		1:1000 (Western blot)		Dako
Anti-rabbit HRP (horseradish peroxidase)		1:1000 (Western blot)		Dako

Table 2.3 – Stains

Name	Labels	Fluorophore	Dilution	Supplier
DAPI	Nuclei	UV	1:10,000	Sigma
Nuclear Yellow	Nuclei	UV	1:10,000	Sigma
Phalloidin	Filamentous actin	Alexa 488 or 594	1:400	Molecular Probes
α-Bungarotoxin (α-BTx)	Acetylcholine receptors (AChR)	Alexa 594	1:200	Molecular Probes

Table 8.1 – Growth media for cell culture

Medium name	Used for	Components
Neuron initial medium	Primary neurons (cortical and motor)	500mL Neurobasal Medium (Gibco) 50mL (10%) Fetal Bovine Serum (FBS) (Invitrogen), heat inactivate at 65^{o}C for 1 hour 10ml (2%) B27 Neuronal Supplement (Invitrogen) 5ml (1%) Antibiotic/Antimycotic (Invitrogen) 0.5µM Glutamic acid 25nM Glutamine
Neuron subsequent medium	Primary neurons (cortical and motor) and co-cultures coverslip and microfluidic chambers)	500ml Neurobasal Medium (Gibco) 10ml (2%) B27 Neuronal Supplement (Invitrogen) 5ml (1%) Antibiotic/Antimycotic (Invitrogen) 25nM Glutamine
Glial growth medium	Primary spinal glial cells (mouse and rat derived)	500ml Dulbecco's modified Eagle Medium (DMEM) 50ml (10%) Heat-inactivated FBS 5ml (1%) Antibiotic/antimycotic
Primary myoblast growth medium	Primary rat myocyte cultures	80ml (80%) Ham's F-10 Nutrient Mix (Gibco) 20ml (20%) heat-inactivated FBS 1mL (1%) Antibiotic/Antimycotic 50µl (2.5ng/ml) basic human fibroblast growth factor (FGF, Sigma). Stock solution 5µg/ml in 0.01% BSA
C2C12 myoblast growth medium	C2C12 myoblasts	500ml DMEM 50ml (10%) non-heat inactivated FBS 5ml (1%) antibiotic/antimycotic
CDM1(C2C12 differentiation medium 1)	C2C12 myoblasts and C2C12 myoblast co-culture with motor neurons	90ml Neuron subsequent medium 10ml (10%) non-heat inactivated Horse serum (Invitrogen)
CDM2 (C2C12 differentiation medium 2)	C2C12 myoblasts and C2C12 myoblast co-culture with motor neurons	93ml Neuron subsequent medium 7ml (7%) non-heat inactivated FBS (Invitrogen)

Figure 1.1 – Upper and lower motor neurons

Upper motor neurons (UMNs) originate in the motor cortex and project axons via corticospinal tracts to the spinal cord, forming connections with lower motor neurons (LMNs) in the anterior horns of the spinal cord. LMNs extend via peripheral nerves and synapse with peripheral skeletal muscle. Bulbar motor neurons for control of speech and swallowing originate in the brain stem. Both UMNs and LMNs are affected in ALS.

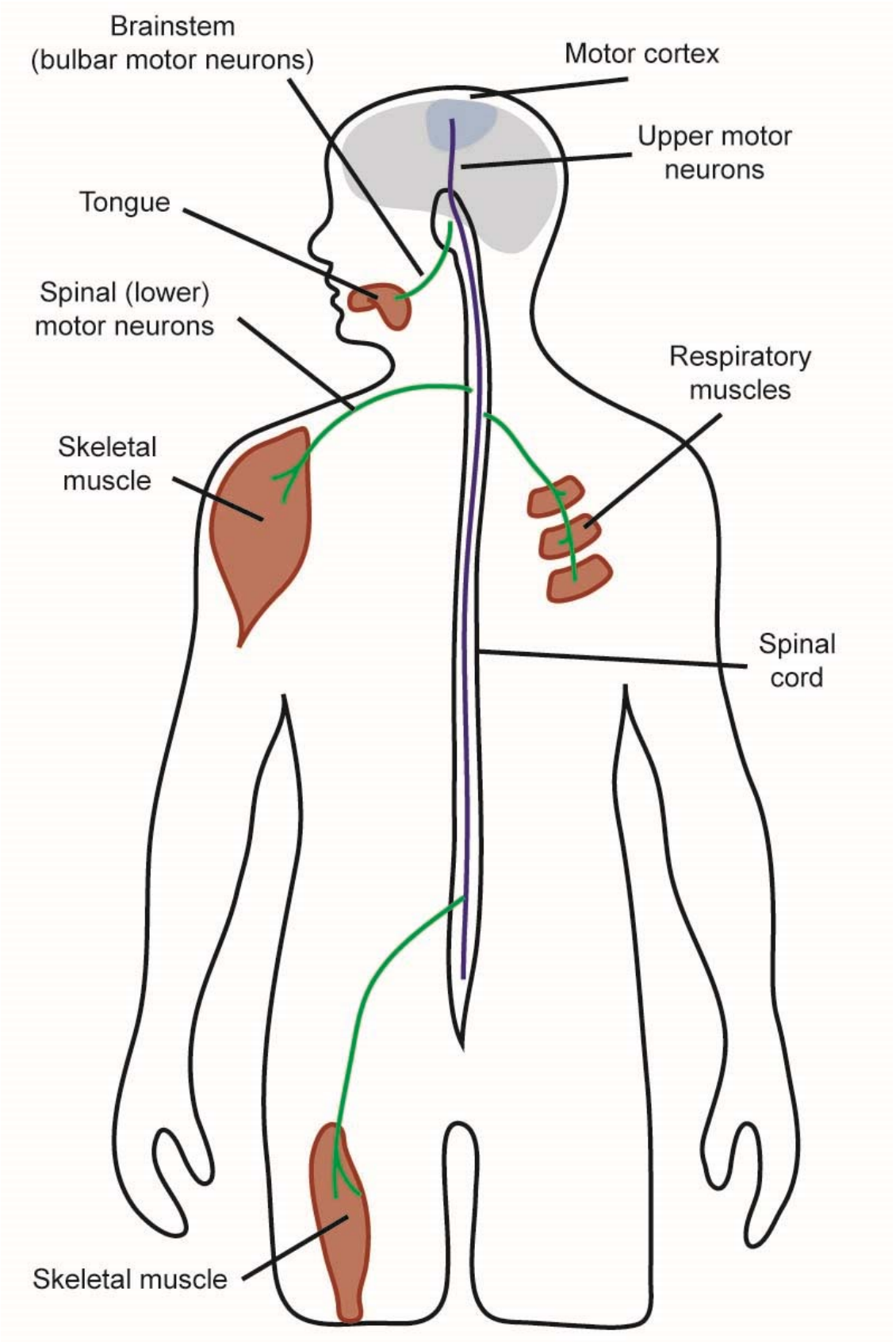

Brainstem
(bulbar motor neurons)
Motor cortex
Upper motor
neurons
Tongue
Spinal (lower)
motor neurons
Respiratory
muscles
Skeletal
muscle
Spinal
cord
Skeletal muscle

Figure 1.2 – Potential Mechanisms in ALS.

ALS pathology may arise from any number or combination of potential mechanisms. The ALS brain and spinal cord contain a large number of activated glial cells, including astrocytes and microglia. Activated astrocytes are associated with reduced glutamate uptake from the synapse, increasing the chance the neuron will succumb to excitotoxicity. Activated microglial cells are associated with an inflammatory response that may result in subsequent apoptosis of the motor neuron. Protein aggregation and misfolding are typical signs of the ALS motor neuron, with a number of mutations within the protein handling system likely to exacerbate stress. Additionally, ALS is characterised by degeneration of the motor neuron distal axon and loss of the neuromuscular junction. **(**Adapted from Turner *et al*., 2013).

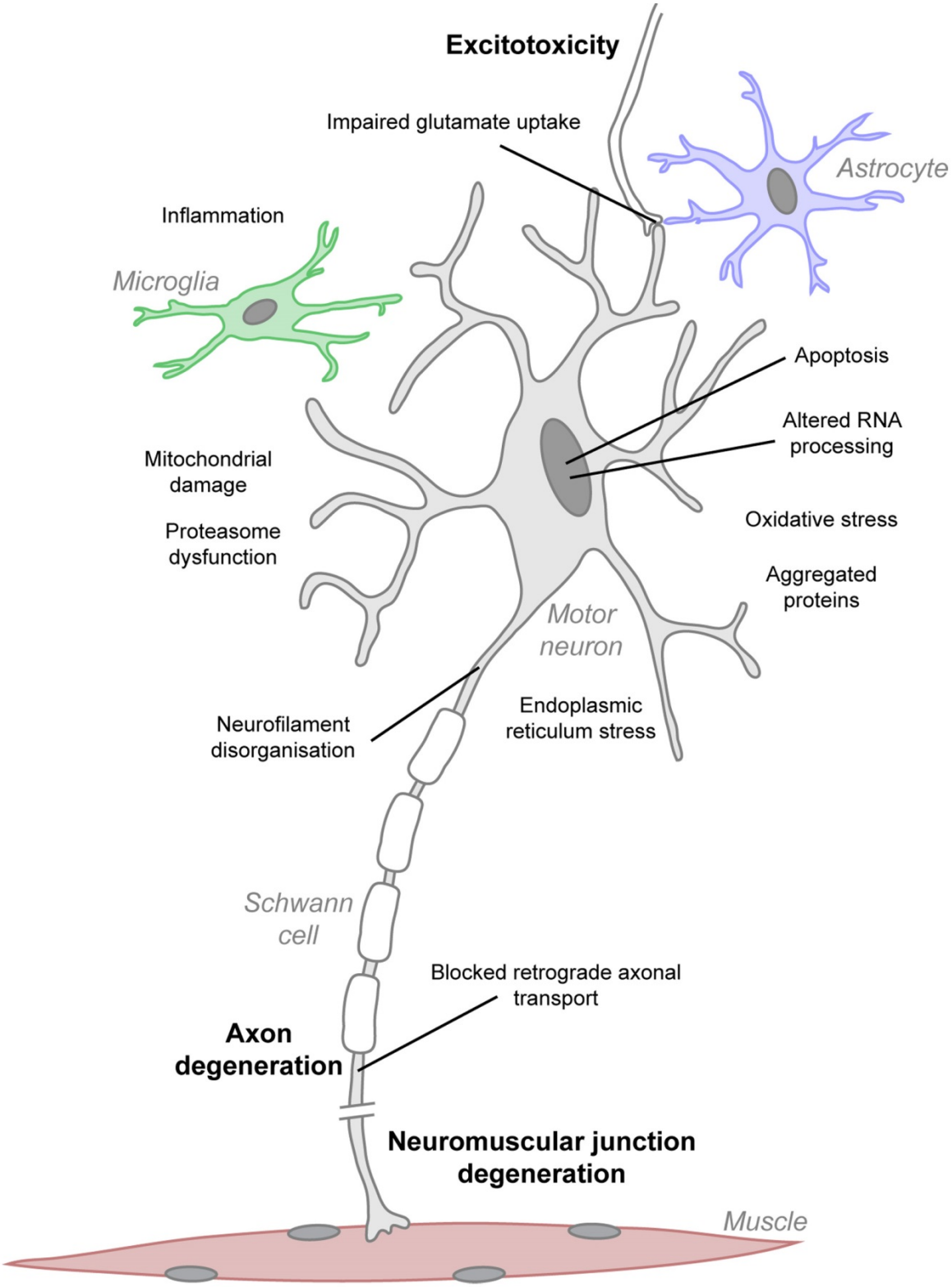

Excitotoxicity
Impaired glutamate uptake
Astrocyte
Inflammation
Microglia
Apoptosis
Altered RNA processing
Mitochondrial damage
Proteasome dysfunction
Oxidative stress
Aggregated proteins
Motor neuron
Endoplasmic reticulum stress
Neurofilament disorganisation
Schwann cell
Blocked retrograde axonal transport
Axon degeneration
Neuromuscular junction degeneration
Muscle

Figure 1.3 – Theories of axon degeneration

Degeneration of the neuronal axon may occur during a wide range of conditions, including disease, injury and during development. Axons may degenerate in a 'dying forward' pattern, characterised by initial degeneration of the proximal portion of the axon, proceeding distally. Alternatively, an axon may degenerate in a 'dying back' pattern, whereby the distal portions of the axon degenerate first and proceed proximally. A third form of degeneration, Wallerian degeneration, occurs following axon transection or crushing injury, with degeneration of the axonal portion distal to the injury site following formation of localised axonal swellings or 'beads'. Any form of axon degeneration can occur without neuronal loss. Development of axon degeneration is considered to occur following convergence on a common pathway involving impaired axon transport, mitochondrial failure and a rise in axonal Ca^{2+} resulting in activation of calpain and degradation of axon components.

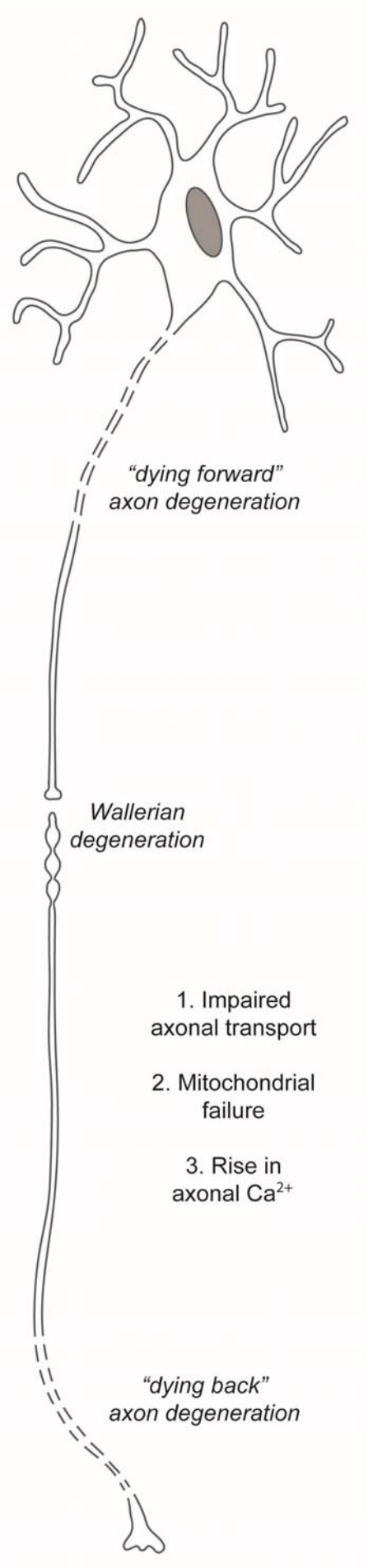

"dying forward"
axon degeneration
Wallerian
degeneration
1. Impaired
axonal transport
2. Mitochondrial
failure
3. Rise in
axonal Ca^{2+}
"dying back"
axon degeneration

Figure 1.4 – Signalling pathways at the NMJ.

During development of the neuromuscular system, three main pathways regulate formation of the NMJ at the post-synaptic membrane, notably agrin/LRP4/MuSK signalling to promote AChR clustering via interactions with rapsyn, NRG-1/ErbB signalling to promote AChR expression by sub-synaptic nuclei, and ACh voltage-dependent downregulation of AChR expression by non-synaptic myonuclei. In addition to these processes, a large number of other proteins interact to stabilise the NMJ, including the dytsrophin glycoprotein complex (DGC) comprising dystrophin and related proteins. The DGC is crucial for development of synaptic folds and interacts with intracellular, extracellular and laminar proteins. Additional protein interactions link the NMJ post-synaptic apparatus with the skeletal cytoskeleton, including interactions with nestin and F-actin. (Adapted from Shi *et al*., 2012).

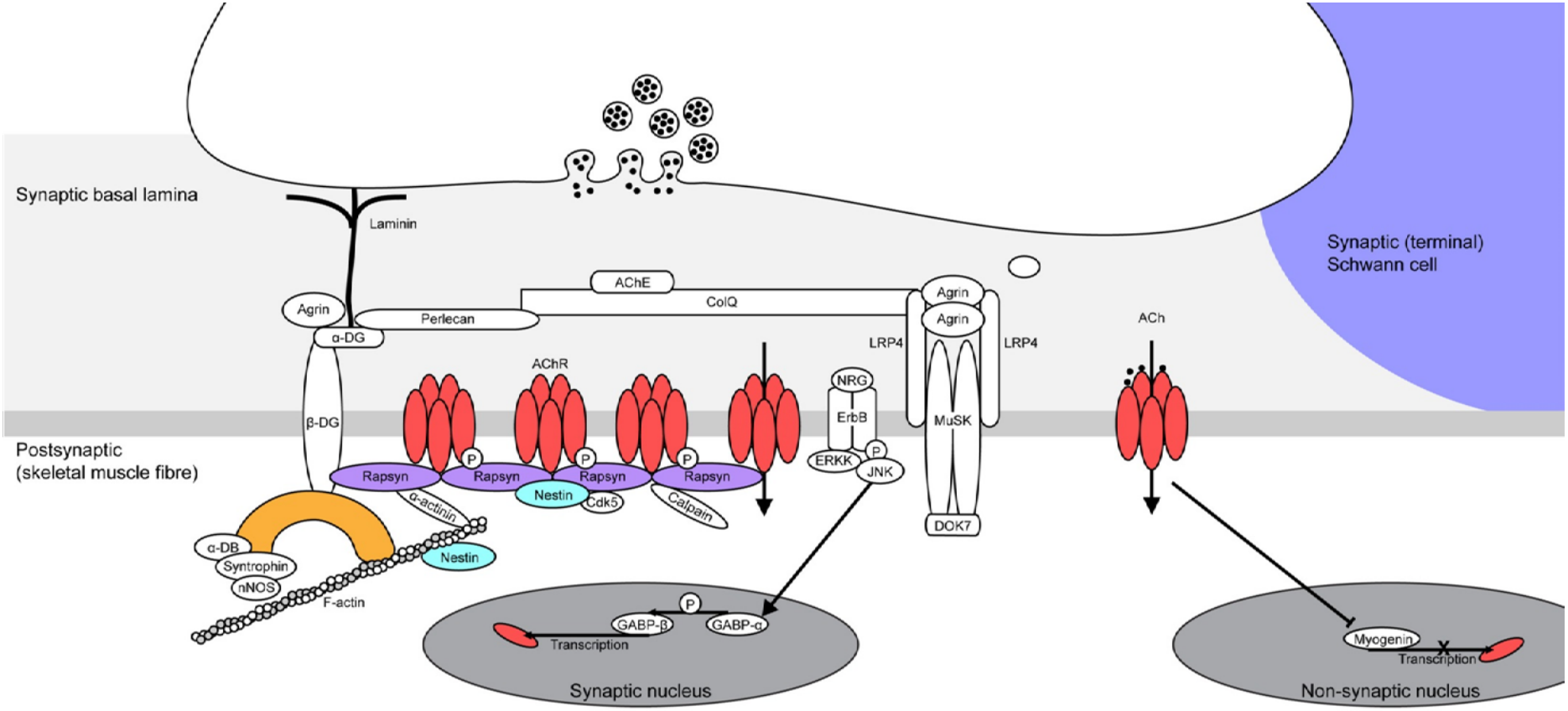

Synaptic basal lamina
Laminin
Synaptic (terminal)
Schwann cell
AChE
ColQ
Agrin
Perlecan
α-DG
Agrin
Agrin
LRP4
LRP4
ACh
AChR
NRG
ErbB
ERKK
P
JNK
MuSK
β-DG
Postsynaptic
(skeletal muscle fibre)
Rapsyn
Rapsyn
Rapsyn
Rapsyn
Nestin
Cdk5
Calpain
α-actinin
α-DB
Syntrophin
nNOS
F-actin
Nestin
DOK7
GABP-β
GABP-α
Transcription
Synaptic nucleus
Myogenin
Transcription
Non-synaptic nucleus

Figure 1.5 – Glutamate receptors and transporters

Depolarisation of the presynaptic neuron (1) results in vesicle translocation to the axon terminal membrane to release the neurotransmitter glutamate. Glutamate diffuses across the synaptic cleft to activate receptors (AMPA, kainate, NMDA and mGluR) on the post-synaptic neuron (2) activate voltage- and calcium-dependent signalling cascades within the post-synaptic neuron. Activation of mGluR receptors on the presynaptic neuron (3) inhibits translocation of additional vesicles to the membrane, terminating neurotransmission. Excitatory amino acid transporters (EAAT2) on perisynaptic astrocytes actively and swiftly remove glutamate from the synapse (4) for conversion to glutamine by glutamine synthase (5). The resulting glutamine is transferred to the presynaptic neuron and converted back to glutamate (glutaminase) (6). Glutamate is packaged into vesicles by the vesicular transporter VGLUT in preparation for the next neurotransmission (7). (Adapted from Sanacora *et al*., 2008).

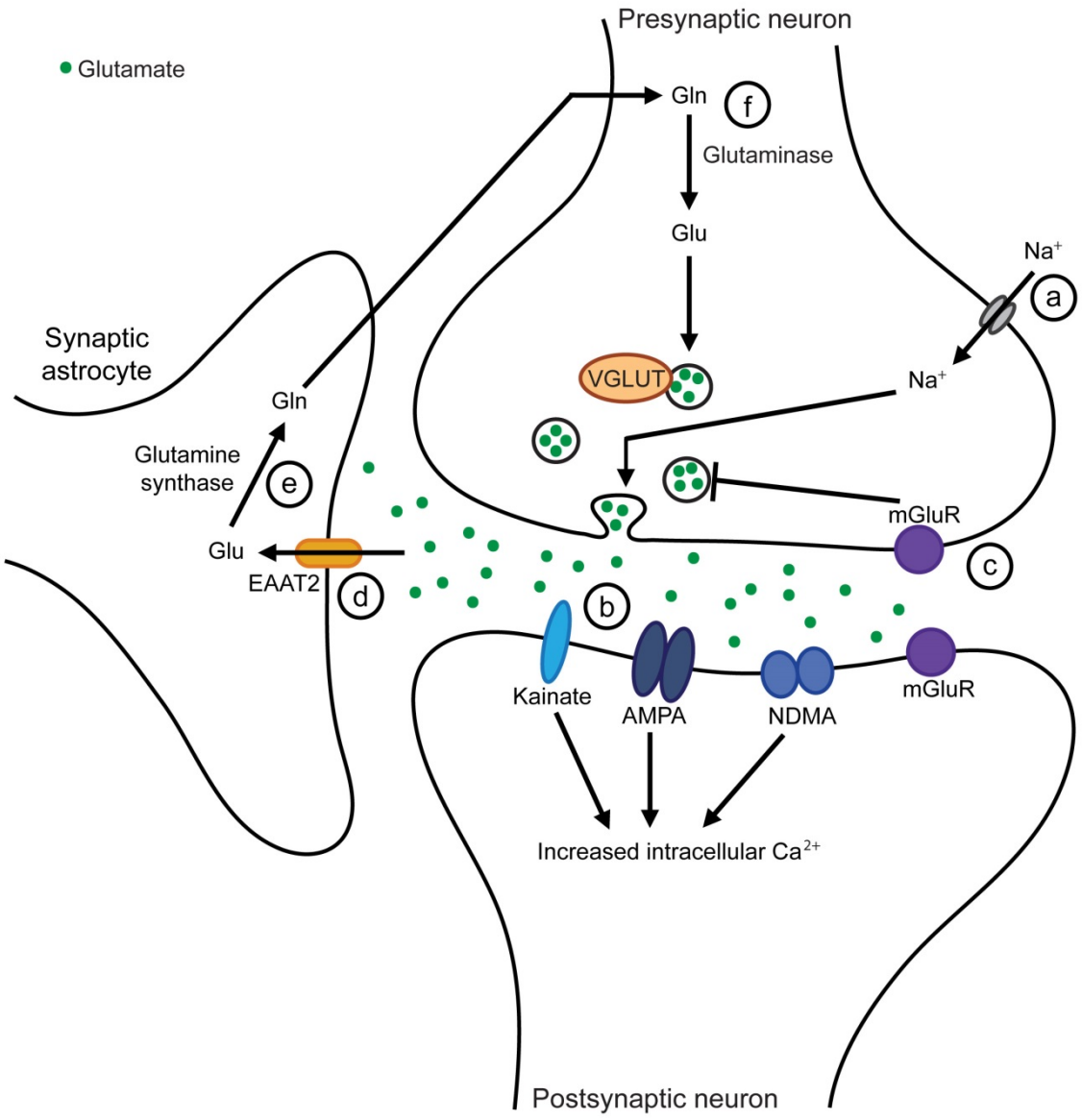

Presynaptic neuron
Glutamate
Gln
f
Glutaminase
Glu
Na+
a
Synaptic astrocyte
VGLUT
Na+
Gln
Glutamine synthase
e
mGluR
Glu
EAAT2
d
c
b
Kainate
AMPA
NDMA
mGluR
Increased intracellular Ca2+
Postsynaptic neuron

Figure 2.1 – Microfluidics schematic.

(**A**) Schematic of the microfluidic chamber, depicting neuronal organisation and dimensions (**B**). (**C**) Cell bodies are restricted to the proximal chamber, and axons extend into the distal chamber. (**D**) Arrows indicate selective glutamate treatment (yellow) of either the proximal or distal chamber, fluidic isolation is maintained by manipulating fluid levels. Not drawn to scale.

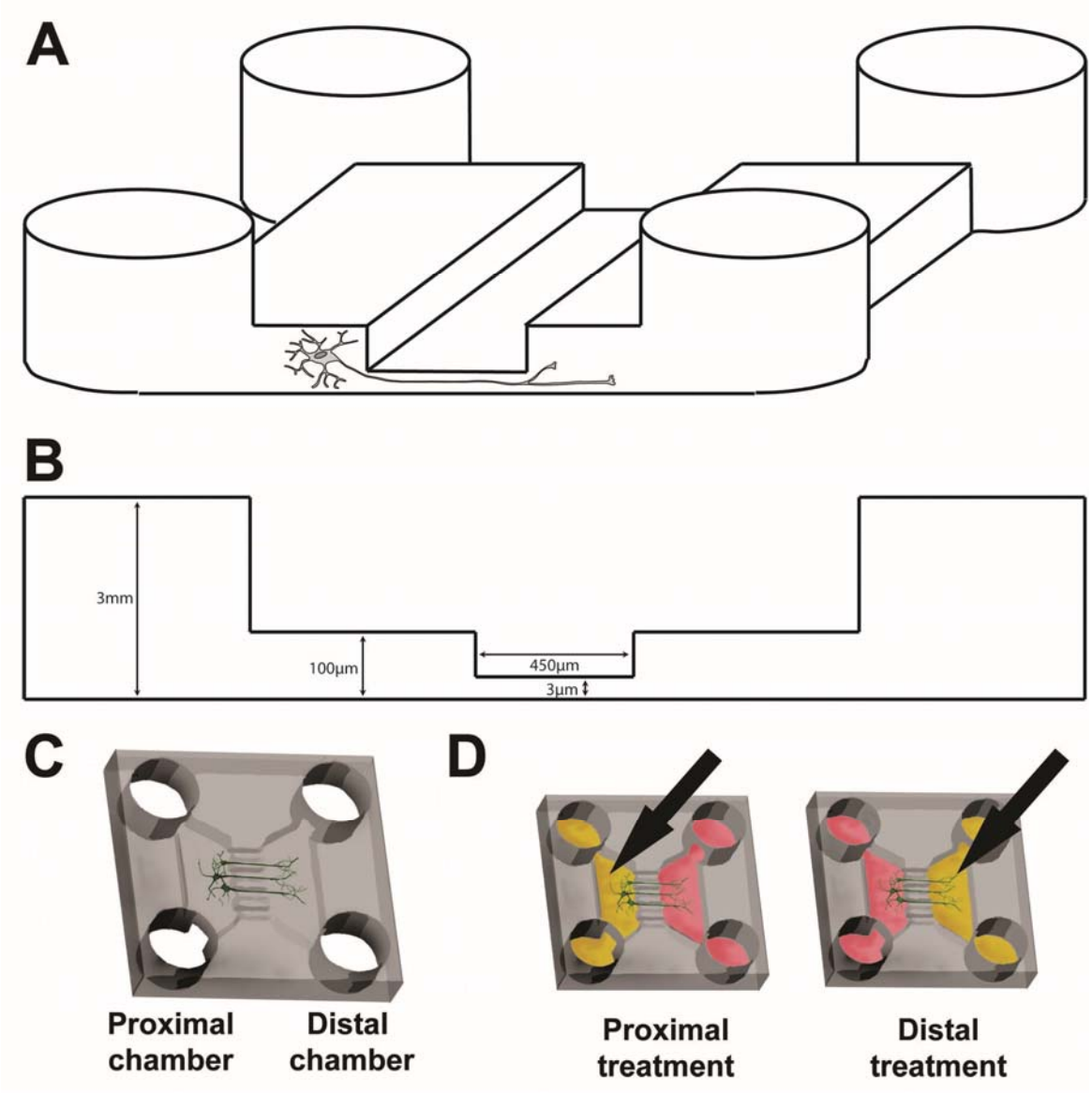

A
B
3mm
100µm
450µm
3µm
C
D
Proximal chamber
Distal chamber
Proximal treatment
Distal treatment

Figure 3.1 – Schematic of neuronal growth within the microfluidic culture device.

(**A**) Quantitation of axon degeneration following excitotoxicity was achieved by analysing axonal segments in each square of a superimposed grid. (**B**) Axonal morphology was scored as whole, beaded or fragmented.

Scale A grid = 50µm, B = 5µm.

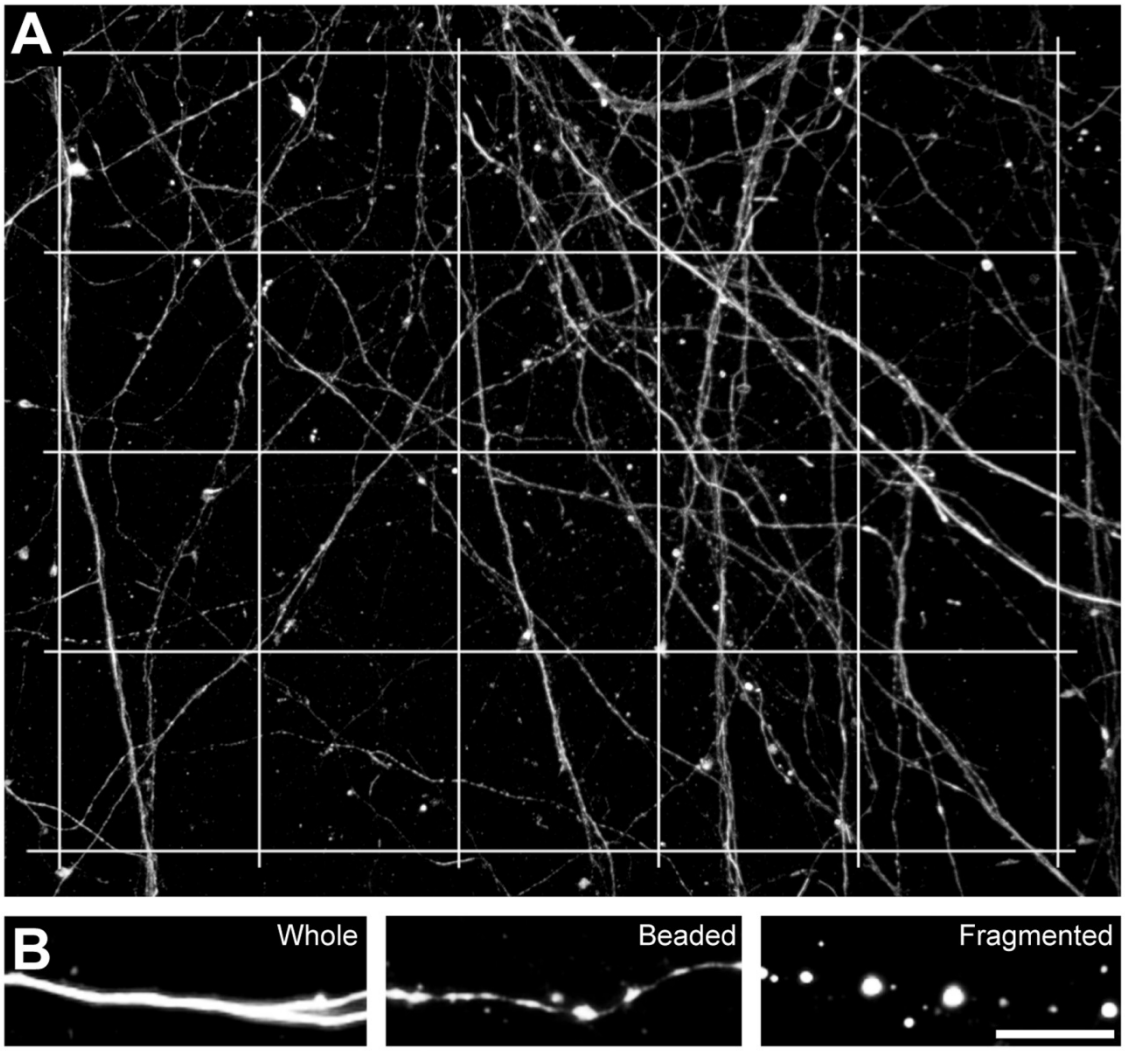

A
B
Whole
Beaded
Fragmented

Figure 3.2 – Neuronal growth in compartmented chambers.

Initial neuronal growth in compartmented culture (2 DIV) (**A**) is restricted to the somal chamber, with axons extending into the distal chamber to form a branched network at 11 DIV (**B**). At 5DIV neuronal growth is characterized by branched neurites within the somal chamber (**C**) and neurites visible within the microchannels (arrow) (**D**). At 11DIV, neurites within somal chamber (**E**) were densely branched, and axons within the distal axon chamber (**F**) had formed extensive branched networks. Neurons retained normal healthy morphology within the somal chamber at 15DIV (**G**) despite severe deterioration of isolated axons (**H**).

Scale A,B = 100μm, C-H = 50μm.

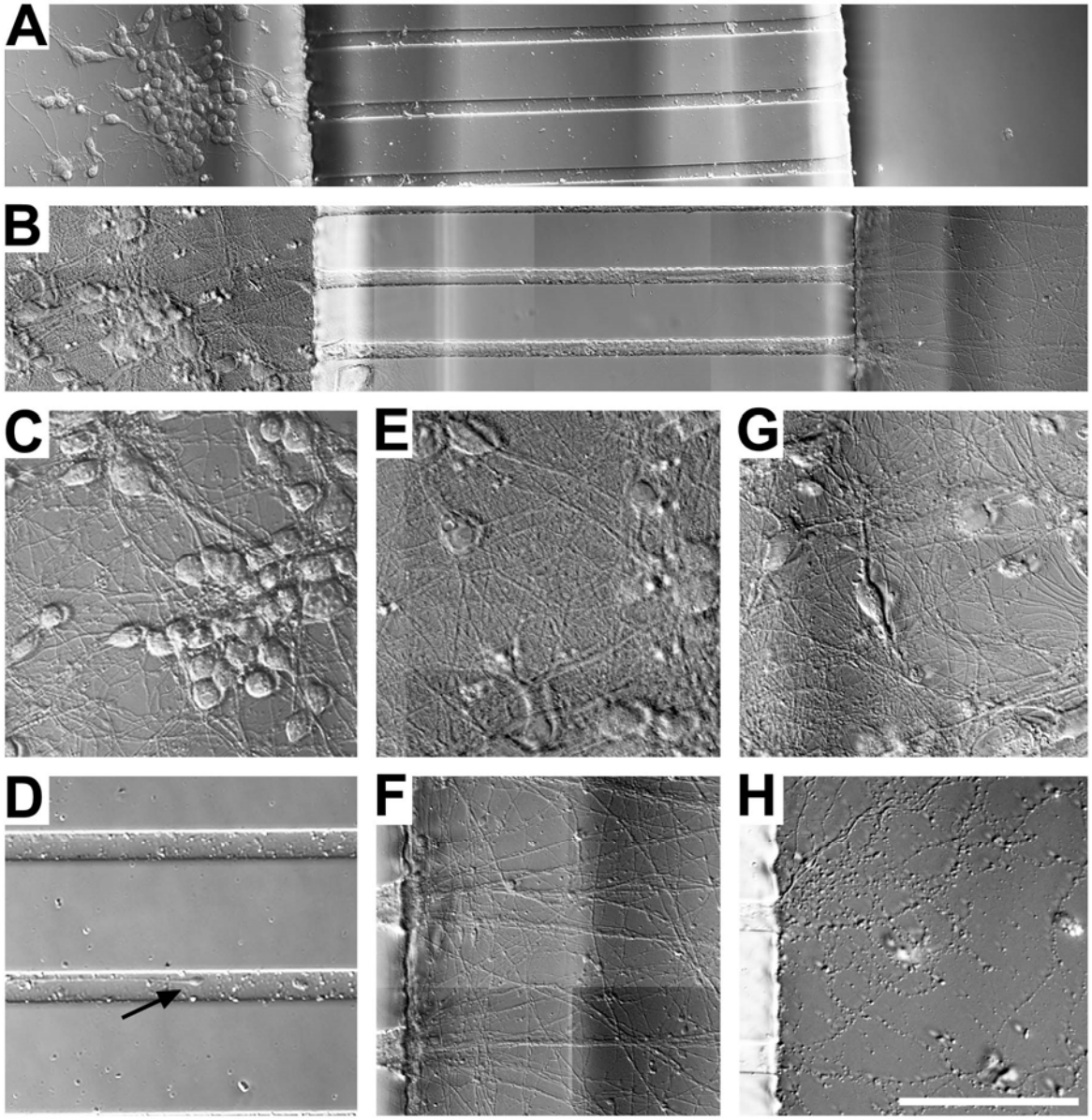

A
B
C
E
G
D
F
H

Figure 3.3 – Compartmentalisation of neuronal cultures.

Immunolabelling of 11DIV neurons demonstrates cell bodies (MAP2, green) restricted to the somal chamber and axons (NF-M, red) present within the somal chamber and extending to the distal axon chamber (**A**). Neurons (Nuclear Yellow, blue) with isolated axons were identified by CM-DiI (red) retrograde labelling from the distal axon chamber (**B**). Arrowhead indicates a neuron positive for CM-DiI uptake, arrow indicates a neuron negative for CM-DiI.

Scale A = 100μm, B = 2μm.

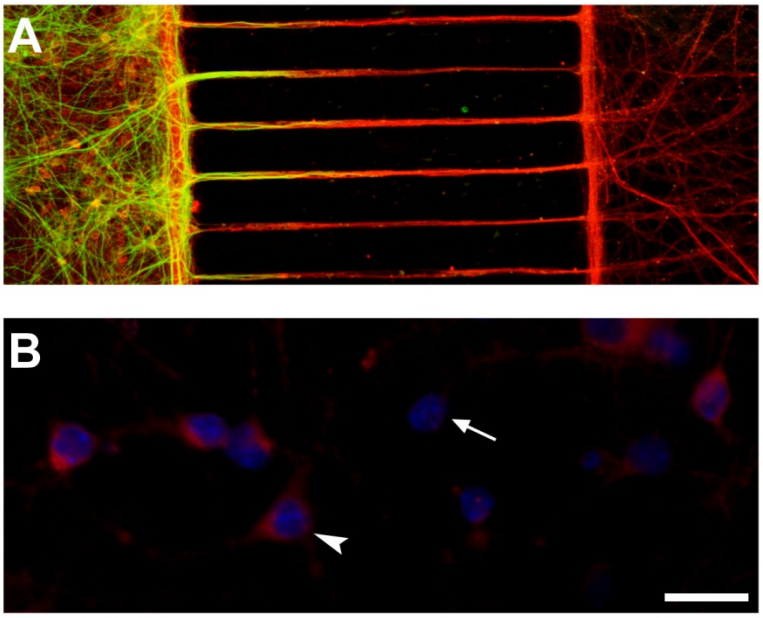

A
B

Figure 3.4 – Markers of neuronal maturity in compartmented culture.

(**A**) At 11 DIV, synaptophysin expression is localised to puncta (arrows) along MAP2 immunoreactive dendrites within the somal chamber. (**B**) GFP expression indicates short, mushroom-shaped spines (arrows) along dendrites (**C**) in addition to long filopodial spines (arrows). (**D**) Axons within the distal axon chamber retain growth cones, immunoreactive for filamentous actin, (**E**) shows higher power of growth cone morphology.

Scale A = 25µm, B-C = 4µm, D = 20µm, E = 7µm.

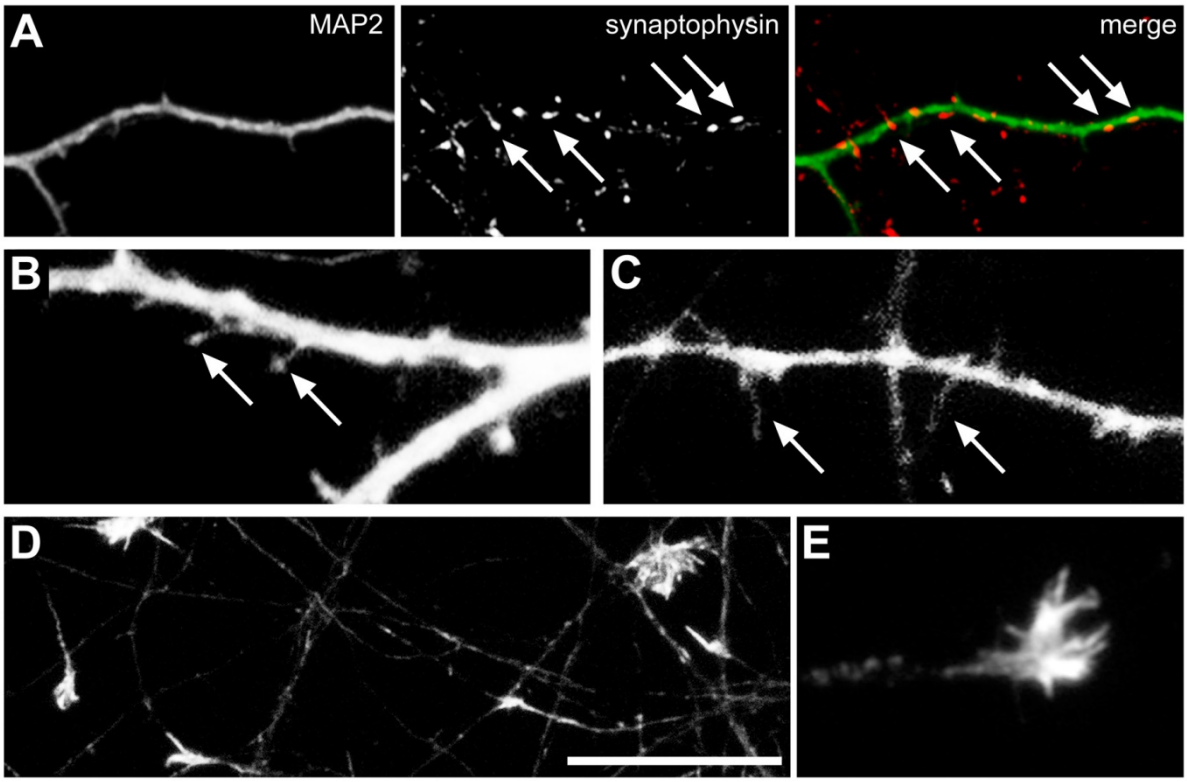

A
MAP2
synaptophysin
merge
B
C
D
E

Figure 3.5 – Expression of AMPA and NMDA glutamate receptor subunits in compartmented culture.

(A) AMPA (GluR1) and (**B**) NMDA (NR1) immunoreactivity were both present throughout the soma, with punctate expression along the dendrites. (**C**) Distal axon AMPA (GluR4) immunoreactivity was frequently present, along distal axons and within the growth cone. (**D)** Expression of NMDA receptors (NR1) was occasionally present within distal axon growth cones. (**E**) Western blot analysis of somal and distal axonal expression indicates AMPA subunits in both chambers, however NMDA receptors were not detected in the distal axon chamber (**F**).

Scale A-B = 20μm, C-D = 50μm.

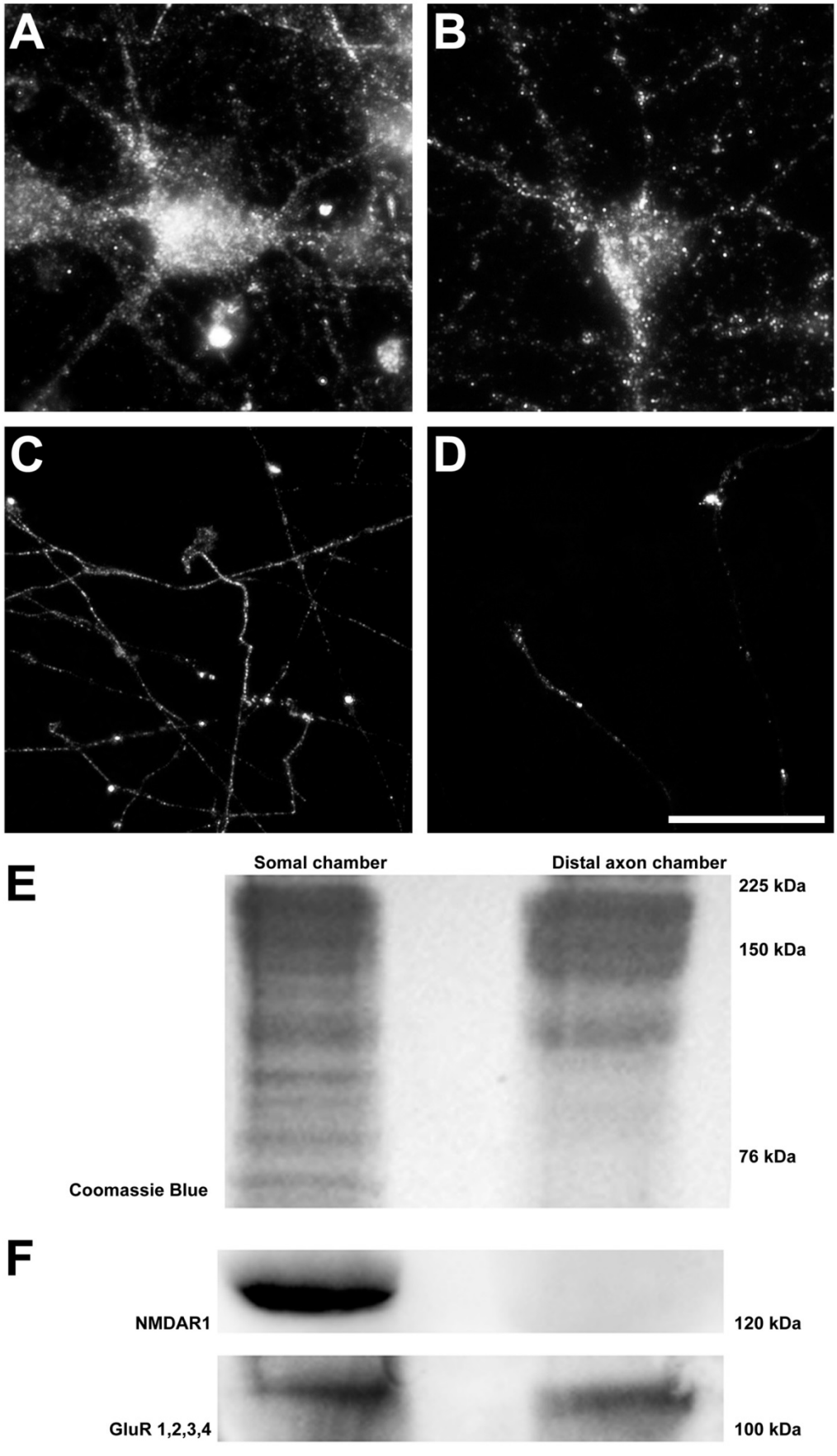

A
B
C
D
E
Somal chamber
Distal axon chamber
225 kDa
150 kDa
76 kDa
Coomassie Blue
F
NMDAR1
120 kDa
GluR 1,2,3,4
100 kDa

Figure 3.6 – Axon degeneration following excitotoxicity.

Somatodendritic excitotoxicity resulted in a significant ($P<0.01$) three-fold increase in distal axon degeneration (**A**). Distal axonal excitotoxicity resulted in a significant ($P<0.01$) 1.5 fold increase in distal axon degeneration (**A**). Distal axons demonstrated increased beading and fragmentation between control (**B**), somal treated (**C**) and distal axon treated (**D**) cultures, visualised with NF-M immunoreactivity. Arrows indicate whole axons, arrowheads indicate fragmented and beaded axons. Within the somal chamber, MAP2 immunoreactive dendrites demonstrated increased beading between control (**E**) and somal treated (**F**) cultures. Distal axon treated cultures (**G**) did not demonstrate changes to dendritic morphology. Arrows indicate soma, arrowheads indicate dendrites. Similarly, nuclear health (Nuclear Yellow) declined between control (**H**) and somal treated cultures (**I**) as demonstrated by an increase in apoptotic nuclei (arrowhead) versus normal nuclei (arrow). Distal axon treated cultures (**J**) showed no change in nuclear morphology compared with controls.

One-way ANOVA with Tukey post-test comparison, $*P<0.05 \pm$ SEM, n = 5.
Scale = 50μm.

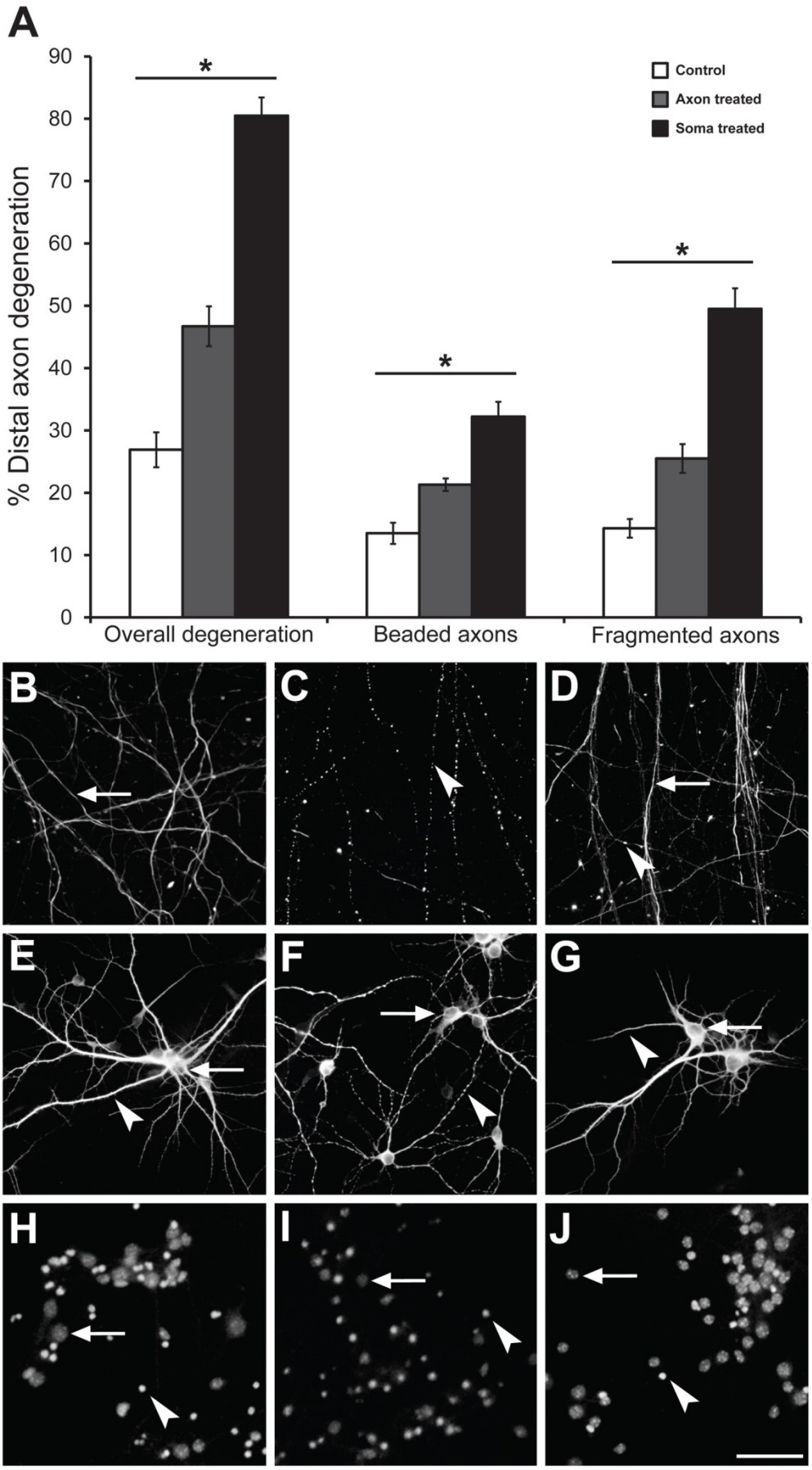

A
% Distal axon degeneration
90
80
70
60
50
40
30
20
10
0
*
*
*
Control
Axon treated
Soma treated
Overall degeneration
Beaded axons
Fragmented axons
B
C
D
E
F
G
H
I
J

Figure 3.7 – Effects of long term distal axonal excitotoxicity.

Application of glutamate to the distal axon compartment for 72 hours significantly ($P<0.01$) increased the percentage of axon degeneration compared with untreated controls, however did not significantly ($P>0.05$) alter the percentage of axon degeneration from 24 hour treated cultures (**A**). Application of glutamate to the distal axon chamber for 72 hours did not significantly ($P>0.05$) alter the percentage of apoptotic nuclei between untreated, 24 hour and 72 hour treated cultures (**B**).

(A) Students unpaired t-Test, $*P<0.05 \pm$ SEM, n = 3.
(B) One-way ANOVA with Tukey's post-test comparison, $*P<0.05 \pm$ SEM, n = 3.

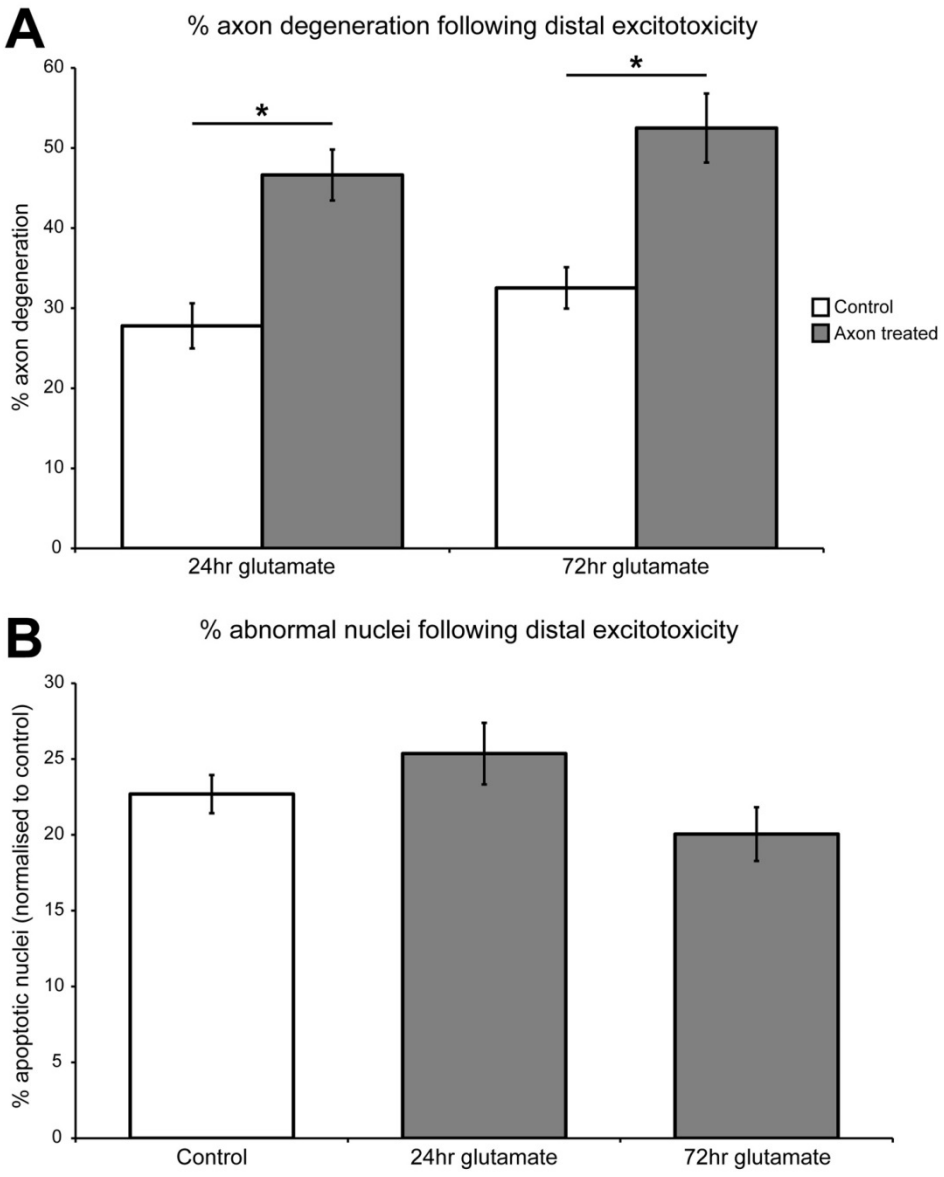

A
% axon degeneration following distal excitotoxicity
% axon degeneration
60
50
40
30
20
10
0
*
*
24hr glutamate
72hr glutamate
Control
Axon treated
B
% abnormal nuclei following distal excitotoxicity
% apoptotic nuclei (normalised to control)
30
25
20
15
10
5
0
Control
24hr glutamate
72hr glutamate

Figure 3.8 – Both NMDA and non-NMDA receptors may mediate the process of distal axon excitotoxicity.

Excitoxicity induced by proximal exposure of cortical neurons to glutamate, kainic acid and NMDA resulted in a significant ($P<0.05$) increase in distal axon degeneration (A). Distal application of KA did not result in a significant ($P>0.05$) change in distal axon degeneration, however distal NMDA did result in significant ($P<0.05$) increase in distal axon degeneration. Preliminary evidence (n = 2) suggests that pharmacological blockade of either proximal or distal receptors using CNQX, MK801 and subsequent excitotoxicity with glutamate did not result in a trend to reduction of distal axon degeneration (B). Application of both CNQX and MK801 together with glutamate excitotoxicity appears to have reduced distal axon degeneration to near-control levels.

(A) One-way ANOVA with Tukey's post-test comparisons, $*P<0.05 \pm$ SEM, n = 5.

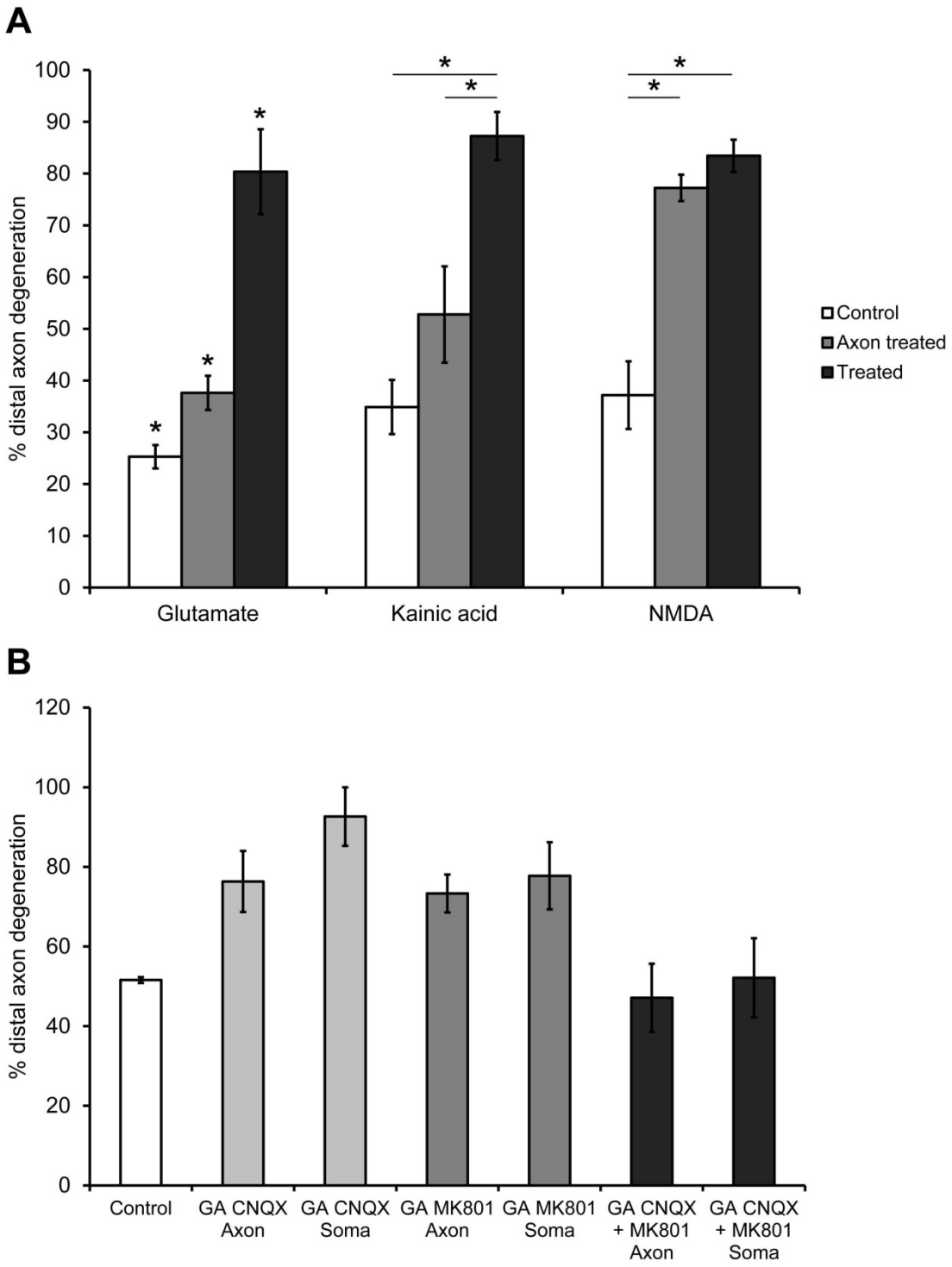

A
100
90
80
70
60
50
40
30
20
10
0
% distal axon degeneration
*
*
*
*
*
*
Glutamate
Kainic acid
NMDA
Control
Axon treated
Treated
B
120
100
80
60
40
20
0
% distal axon degeneration
Control
GA CNQX Axon
GA CNQX Soma
GA MK801 Axon
GA MK801 Soma
GA CNQX + MK801 Axon
GA CNQX + MK801 Soma

Figure 3.9 – Mechanisms of axon degeneration following focal excitotoxicity.

Inhibition of caspase activity, in either the somal or the axonal chamber significantly ($P<0.01$) decreased axon degeneration following somal excitotoxicity (**A**), and following axonal excitotoxicity (**B**). Results expressed relative to untreated controls.

One-way ANOVA with Tukey's post-test comparisons, $*P<0.05$ ± SEM, n = 3.

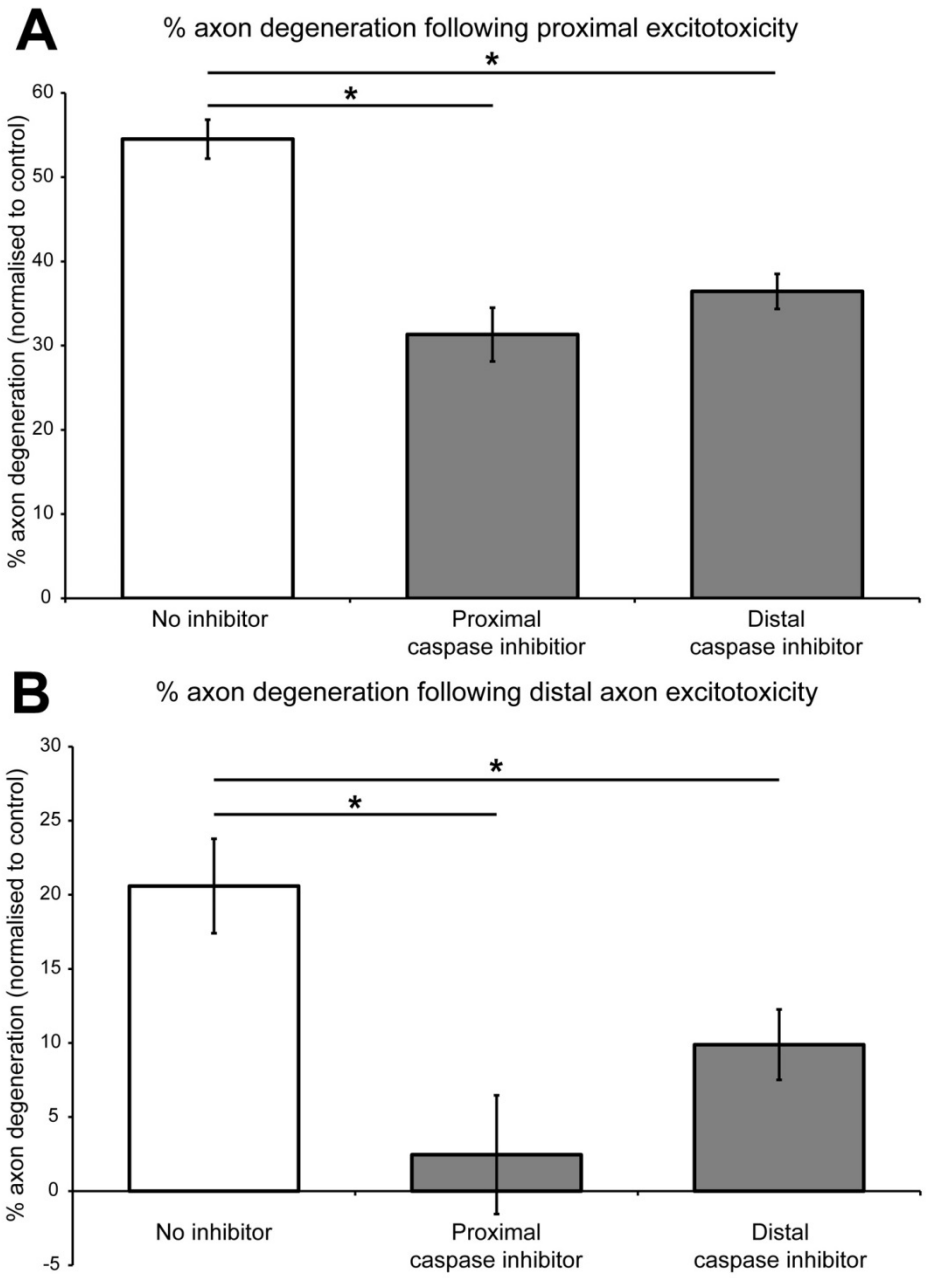
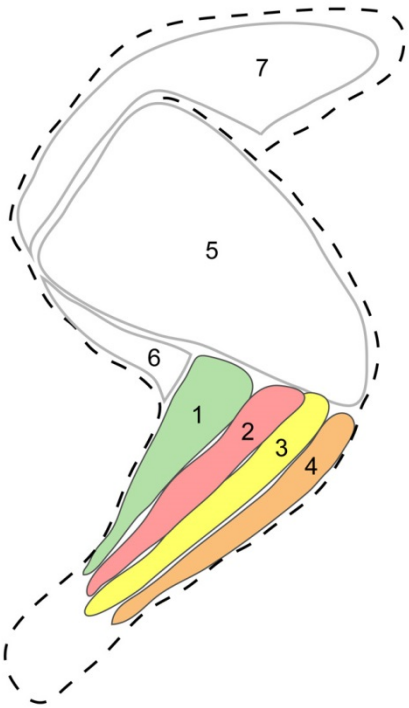

A
% axon degeneration following proximal excitotoxicity
*
*
% axon degeneration (normalised to control)
60
50
40
30
20
10
0
No inhibitor
Proximal caspase inhibitior
Distal caspase inhibitor
B
% axon degeneration following distal axon excitotoxicity
*
*
% axon degeneration (normalised to control)
30
25
20
15
10
5
0
-5
No inhibitor
Proximal caspase inhibitor
Distal caspase inhibitor

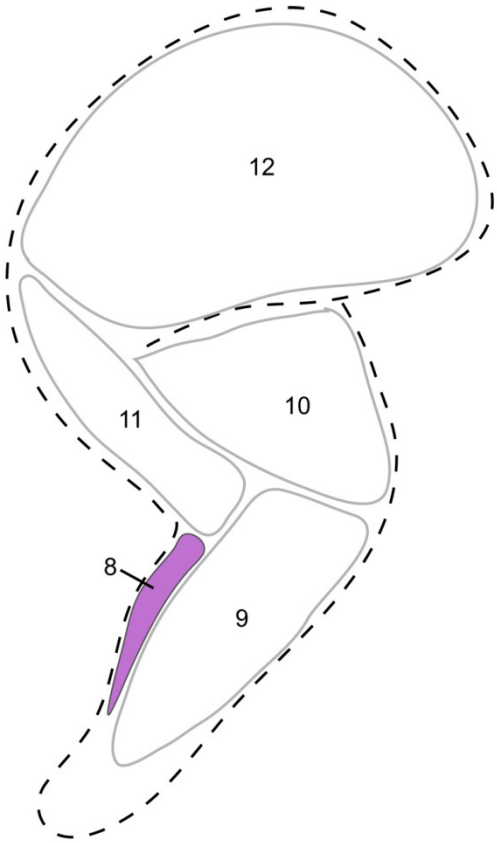
Figure 4.1 – Forelimb skeletal muscles of the mouse.

Diagrams showing the name and anatomical position for the muscles used in this study.

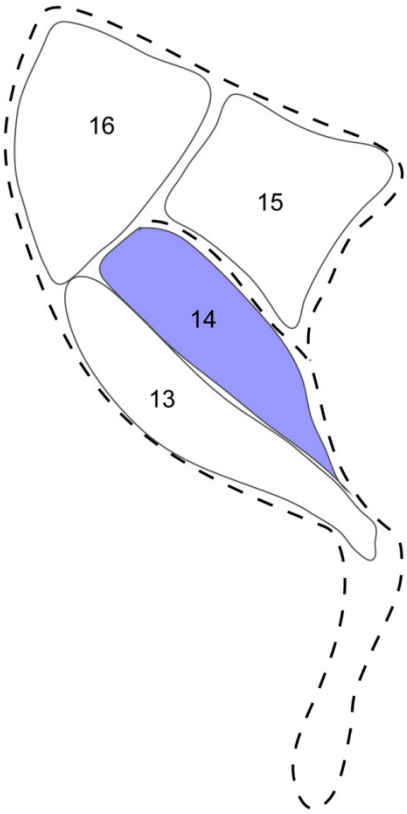
1 Extensor carpi radialis longus (green)
2 Extensor digitorum communis (red)
3 Extensor digitorum lateralis (yellow)
4 Extensor carpi ulnaris (orange)
5 Triceps brachii
6 Biceps brachii
7 Deltoideus, pars scapularis and infraspinatus
8 Extensor caroi radialis (purple)
9 Flexor carpi radialis and flexor digitorum profundus
10 Triceps brachii
11 Biceps brachii
12 Subscapularis and teres major
13 Extensor digitorum longus and tibialis cranialis
14 Gastrocnemius (blue)
15 Vastus lateralis, biceps feboris, adductor, semimebranosus, semitendinosus
16 Rectus femoris
17 Flexor digitorum longus et tibia
18 Tensor fasciae latae, rectus femoris, pectineus, vastus medialis, adductor, gracialis, semimembranosus, semitendinosus



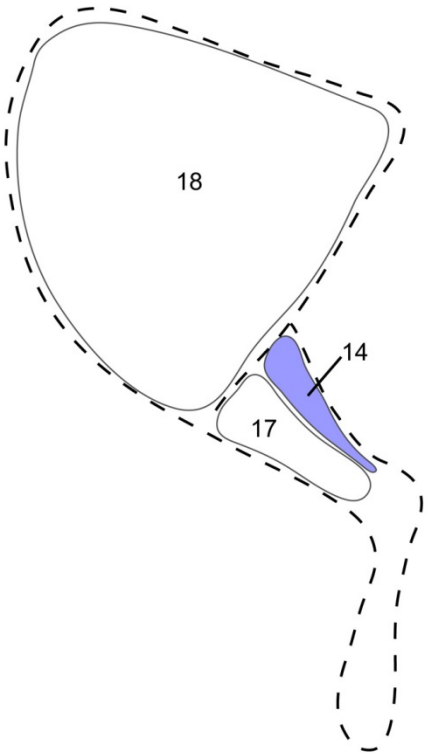
Forelimb lateral view



Forelimb medial view



Hindlimb lateral view



Hindlimb medial view

Figure 4.2 – Colocalisation of pre- and post-synaptic terminals at the NMJ

Quantitation of NMJ innervation was assessed by colocalisation of neurofilaments and synaptophysin (green) and α-BTx (red) using immunofluorescence. Full colocalisation between pre- and post-synaptic elements (A) and partial colocalisation (B) were scored as 'occupied'. No colocalisation between pre- and post-synaptic elements (C) was quantified as 'non-occupied'.

Scale = 20μm.

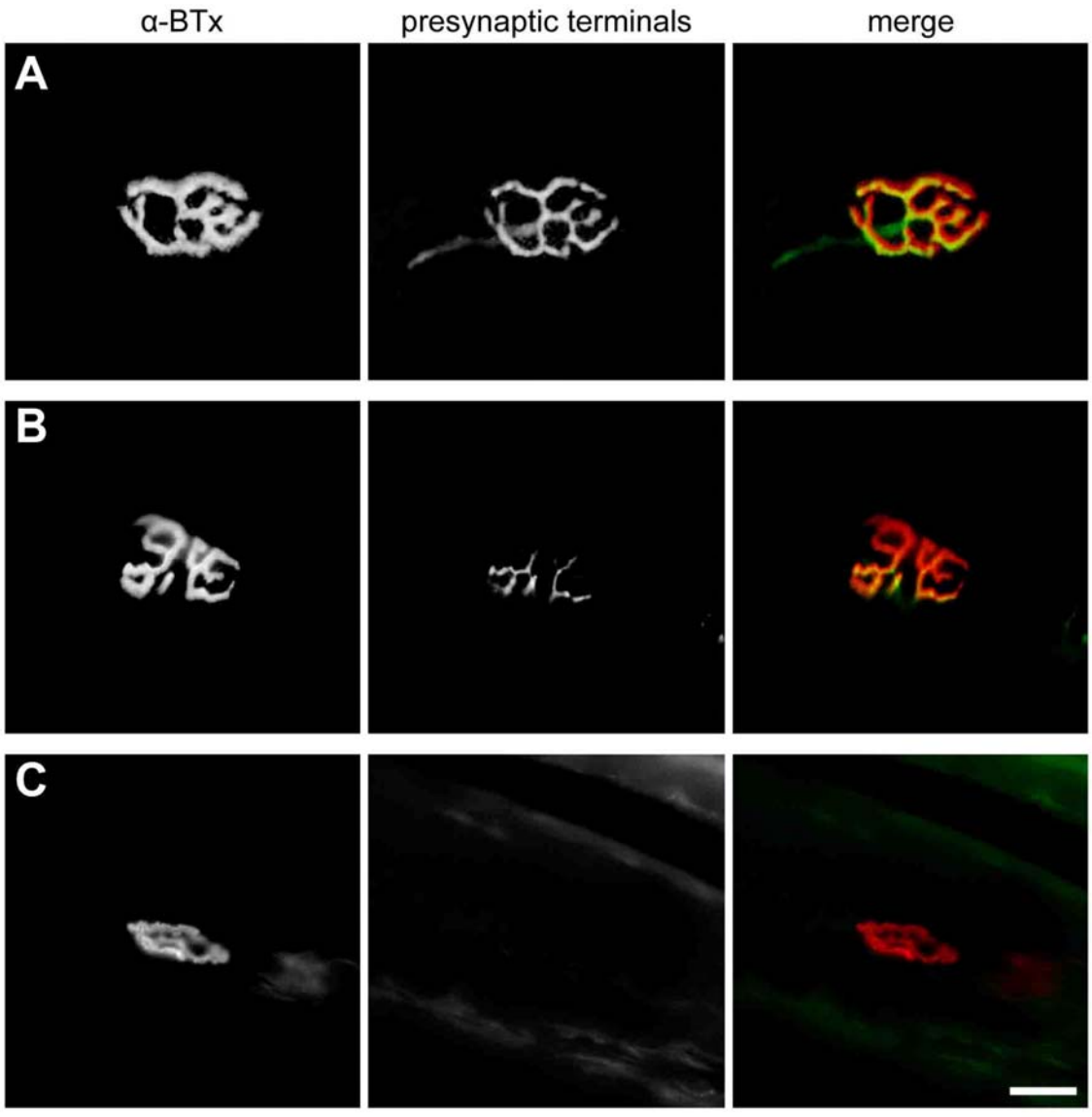

α-BTx
presynaptic terminals
merge
A
B
C

Figure 4.3 – Progressive decrease in the percentage of NMJs with colocalised a-BTx and selected markers

At 28 days of age there was no significant (P>0.05) difference in the proportion of NMJs with colocalised α-BTx and any of the markers examined (**A**). By 56 days, there was a significant (P<0.05) decrease in the percentage of NMJs with colocalised presynaptic terminals and S100 immnuoreactivity with α-BTx (**B**). At 84 days, there is a significant (P<0.05) decrease in the proportion of NMJs with colocalised nestin and rapsyn with α-BTx in addition to decreased presynaptic terminals and S100 (**C**). At 112 days there was a significant (P<0.05) decrease in the percentage of NMJs with colocalised dystrophin and α-BTx (**D**). At 140 days, there was a significant (P<0.05) decrease in the percentage of NMJs colocalised with all the markers examined with α-BTx (**E**).

Two-way ANOVA with Tukey's post-hoc analysis ± SEM.

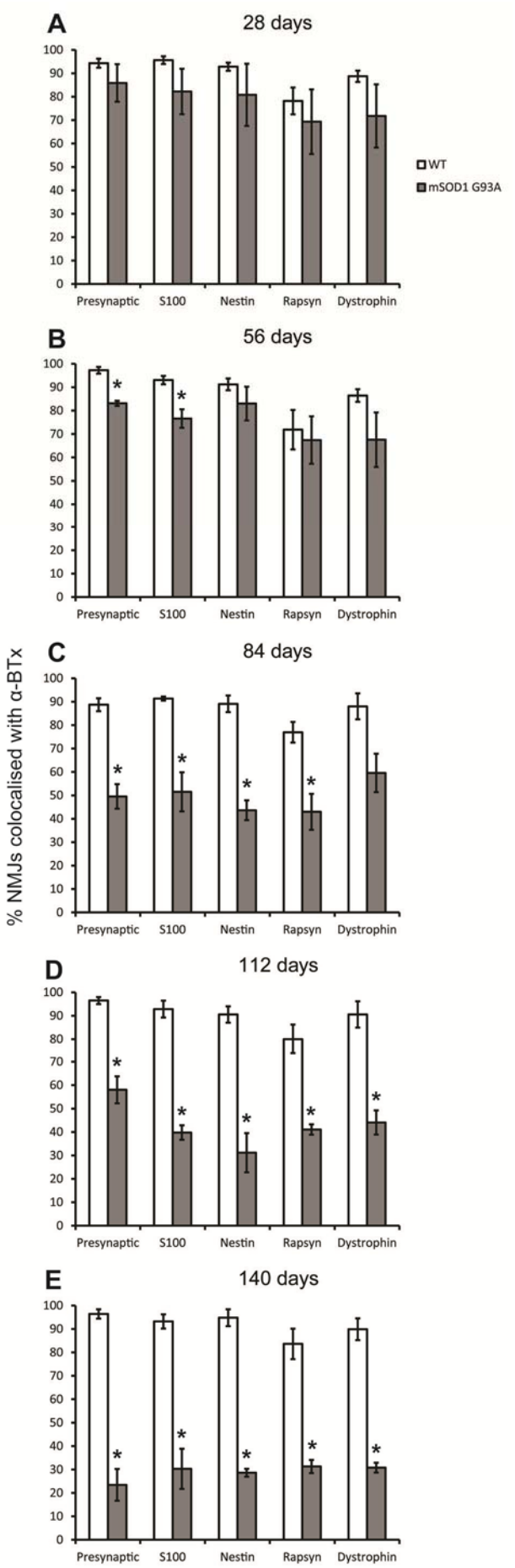

A
28 days
B
56 days
C
84 days
D
112 days
E
140 days
% NMJs colocalised with α-BTx
WT
mSOD1 G93A
Presynaptic
S100
Nestin
Rapsyn
Dystrophin
0
10
20
30
40
50
60
70
80
90
100
*

Figure 4.4 – Normal localisation between rapsyn, dystrophin and nestin compared with α-BTx staining.

(**A**) rapsyn immunoreactivity follows the pretzel organisation of α-BTx staining. (**B**) dystrophin immunoreactivity is localised primarily to α-BTx staining, with some immunoreactivity also present around distal nerves (arrow). (**C**) nestin immunoreactivity represent a 'halo' or negative image of α-BTx staining. Isotype (**D**) and no-primary (**E**) controls indicated no non-specific immunoreactivity.

Scale = 50μm.

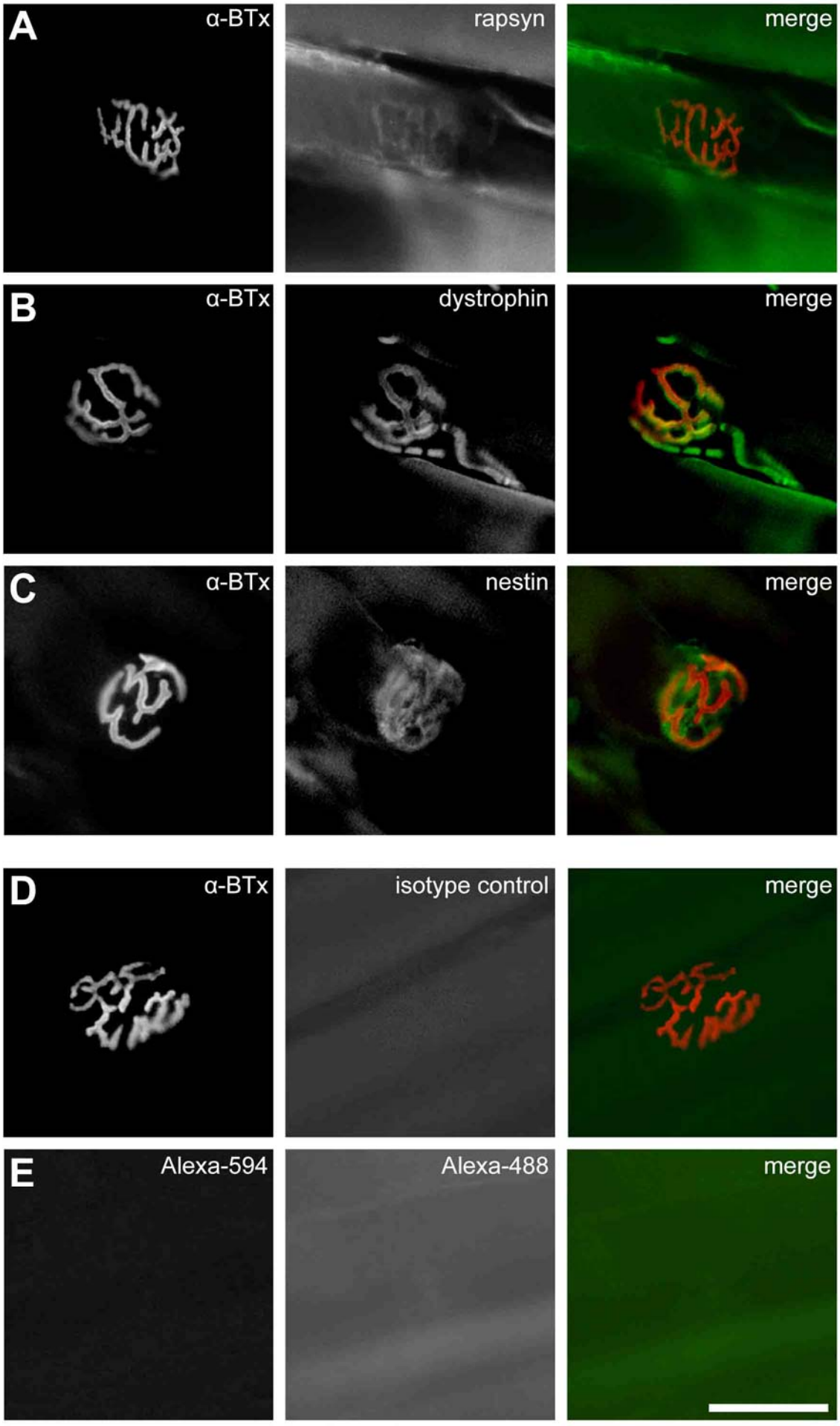

A
α-BTx
rapsyn
merge
B
α-BTx
dystrophin
merge
C
α-BTx
nestin
merge
D
α-BTx
isotype control
merge
E
Alexa-594
Alexa-488
merge

Figure 4.5 – Loss of S100 immunoreactivity in aging mSOD1 G93A mice does not equate to loss of Schwann cells

Analysis of wild-type (**A**) and mSOD1 G93A (**B**) tissue indicated that the progressive loss of S100 immunoreactivity relative to α-BTx staining was not likely to occur from immediate loss of cells, rather from a loss of protein. DAPI staining to visualise nuclei (arrows) indicates presence of nuclei clustered around α-BTx staining when S100 was localised to α-BTx and when it was not.

Sale = 50μm.

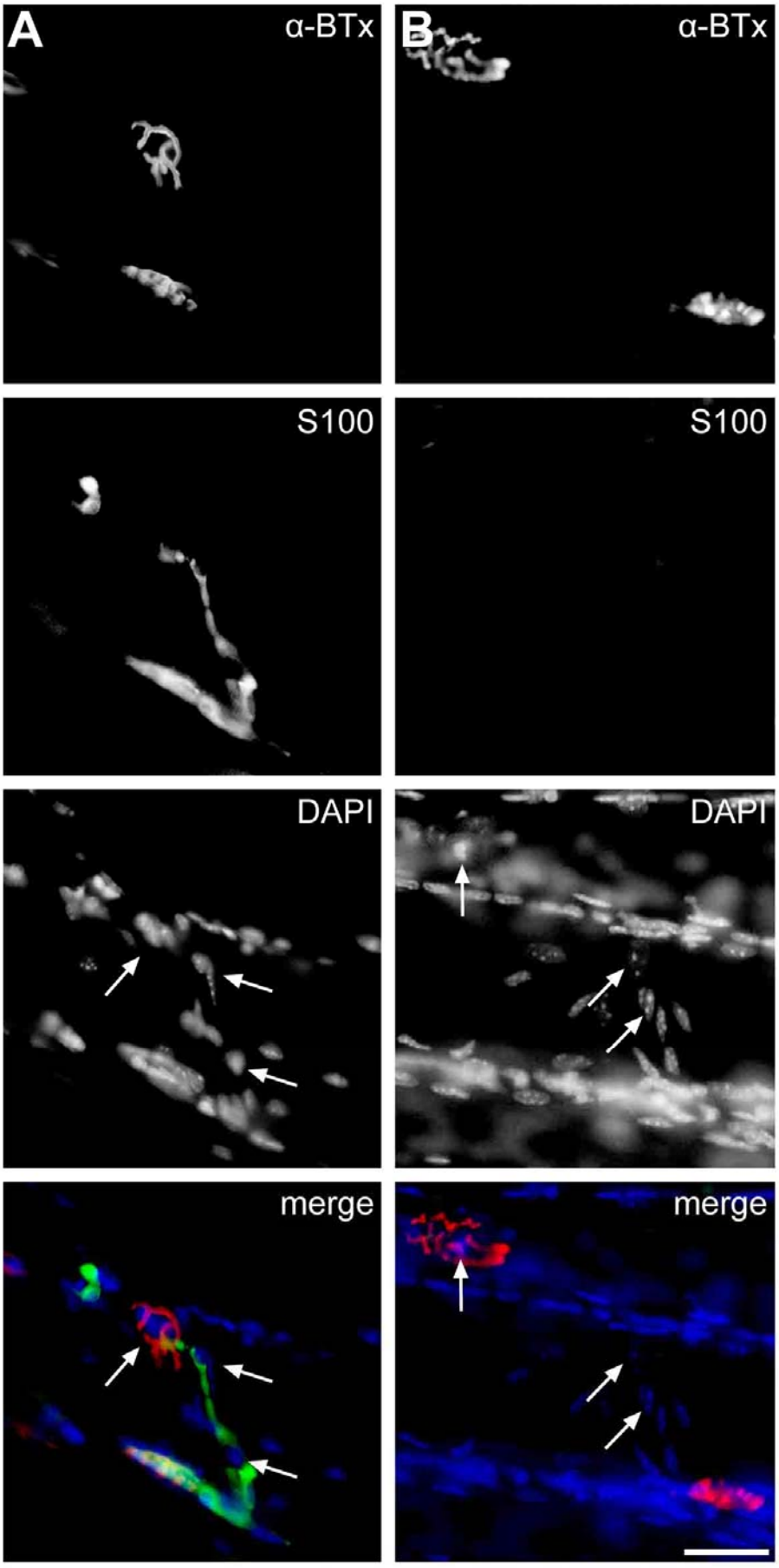

A
α-BTx
B
α-BTx
S100
S100
DAPI
DAPI
merge
merge

Figure 4.6 – Delayed loss of colocalised structures in the forelimb muscles of aging mSOD1 G93A mice.

Quantitative analysis of markers colocalised with α-BTx in aging mSOD1 G93A mice relative to wild-type controls indicated a significant ($P<0.05$) decrease in colocalisation of presynaptic terminals with α-BTx at 140 days only. There was no significant ($P>0.05$) change in colocalisation between α-BTx and the other markers examined.

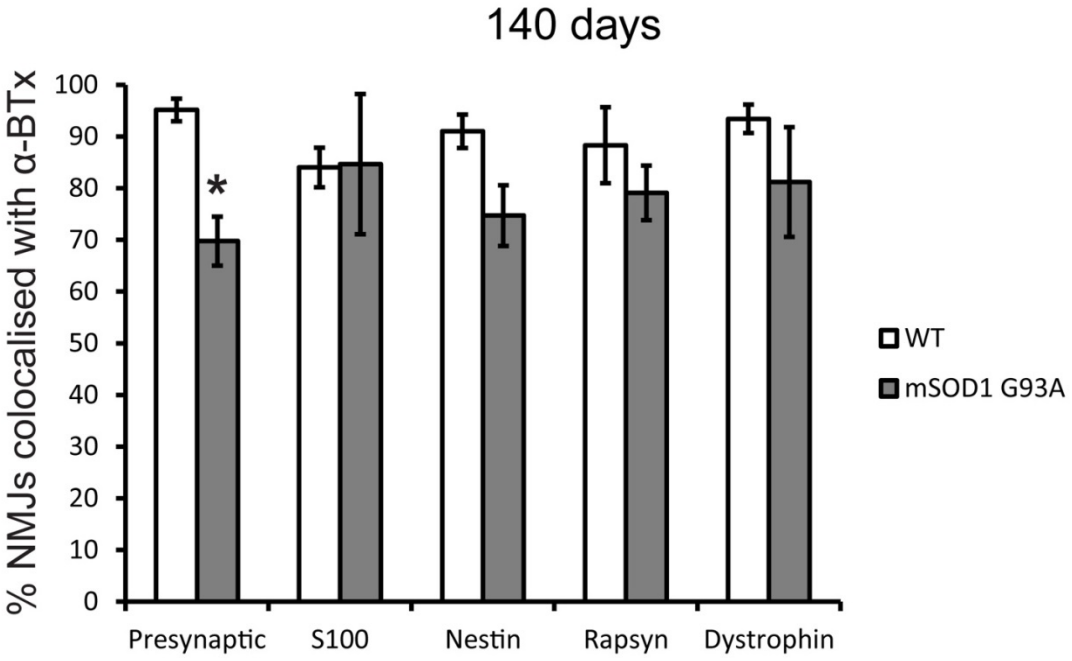

140 days
% NMJs colocalised with α-BTx
100
90
80
70
60
50
40
30
20
10
0
*
Presynaptic
S100
Nestin
Rapsyn
Dystrophin
WT
mSOD1 G93A

Figure 5.1 – Schematic of culture development.

Motor neurons (red) are isolated from the spinal cords of embryonic rats and placed within the proximal chamber of microfluidic chambers, coated with poly-L-laminin. Spinal glial cells (green) are obtained from the spinal cords of neonatal rats and grown to confluency in poly-L-lysine coated P25 flasks. Glial cells are added to motor neuron cell bodies when the motor neurons have reached 2DIV. Skeletal myocytes (blue) are obtained from the hind limb muscle of neonatal rats and enriched for myoblasts in collagen-coated P25 flasks. Myoblasts are added to the distal chamber of the microfluidic chambers at motor neuron age 14DIV.

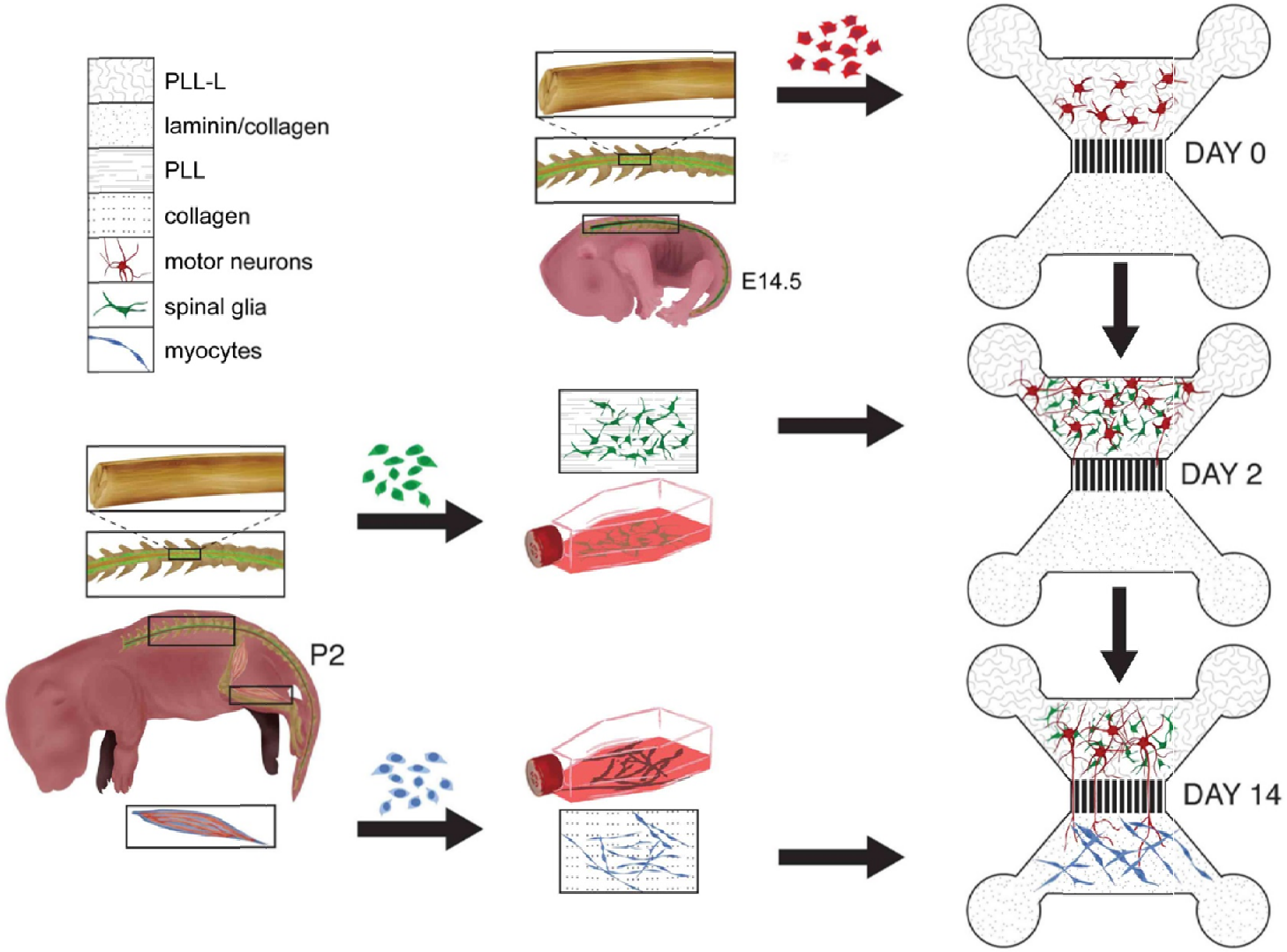

PLL-L
laminin/collagen
PLL
collagen
motor neurons
spinal glia
myocytes
E14.5
P2
DAY 0
DAY 2
DAY 14

Figure 5.2 – Timeline for development of compartmented cultures.

(**A**) Timeline for assembling compartmented motor neuron-glia-muscle cultures derived from primary rat cells. Days are expressed relative to motor neuron age in culture. (**B**) Timeline for assembling compartmented motor neuron-glia-muscle cultures derived from primary mouse cells and C2C12 myoblasts.

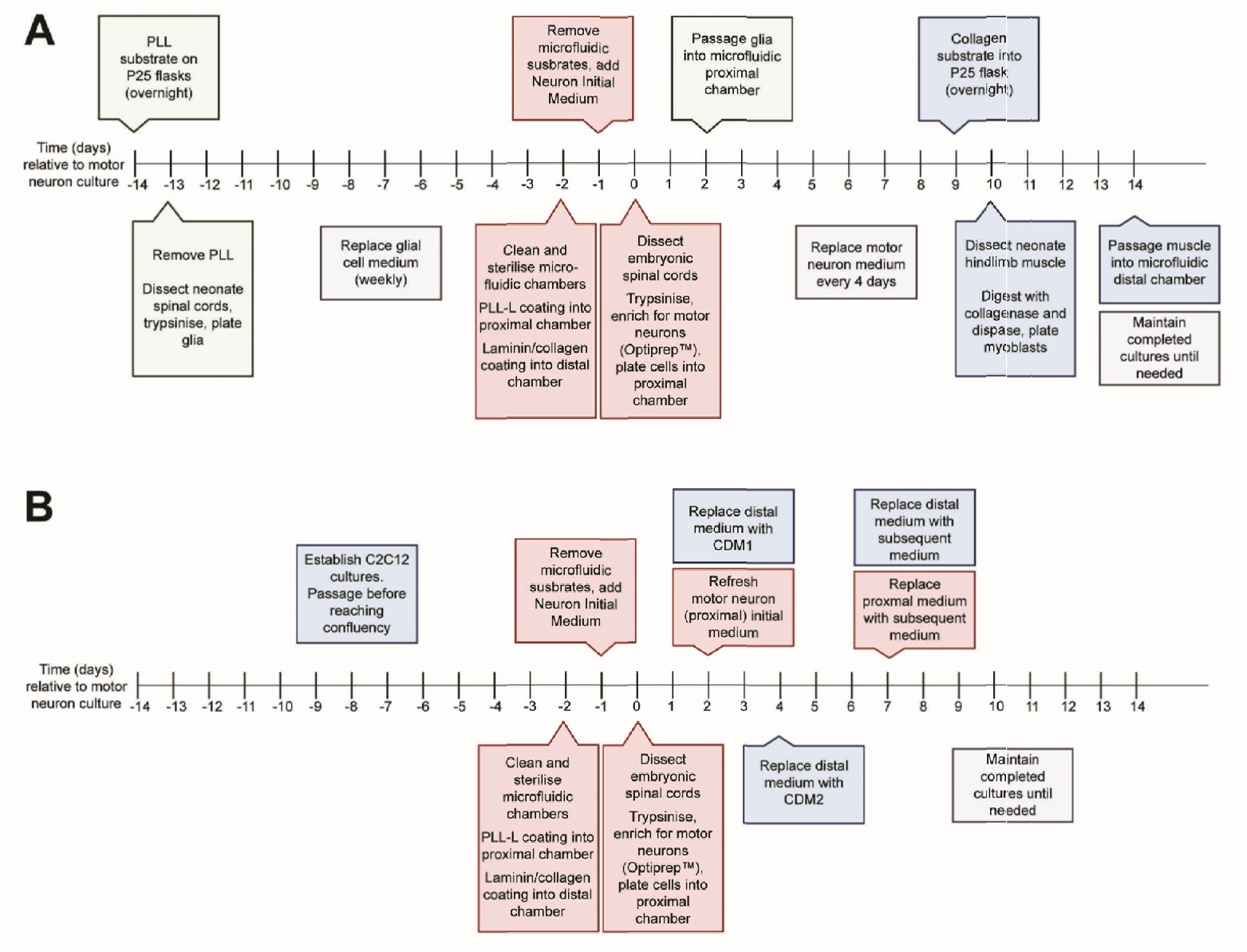

A
PLL substrate on P25 flasks (overnight)
Remove microfluidic susbrates, add Neuron Initial Medium
Passage glia into microfluidic proximal chamber
Collagen substrate into P25 flask (overnight)
Time (days) relative to motor neuron culture
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Remove PLL
Dissect neonate spinal cords, trypsinise, plate glia
Replace glial cell medium (weekly)
Clean and sterilise micro-fluidic chambers
PLL-L coating into proximal chamber
Laminin/collagen coating into distal chamber
Dissect embryonic spinal cords
Trypsinise, enrich for motor neurons (Optiprep™), plate cells into proximal chamber
Replace motor neuron medium every 4 days
Dissect neonate hindlimb muscle
Digest with collagenase and dispase, plate myoblasts
Passage muscle into microfluidic distal chamber
Maintain completed cultures until needed
B
Establish C2C12 cultures. Passage before reaching confluency
Remove microfluidic susbrates, add Neuron Initial Medium
Replace distal medium with CDM1
Refresh motor neuron (proximal) initial medium
Replace distal medium with subsequent medium
Replace proxmal medium with subsequent medium
Time (days) relative to motor neuron culture
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Clean and sterilise microfluidic chambers
PLL-L coating into proximal chamber
Laminin/collagen coating into distal chamber
Dissect embryonic spinal cords
Trypsinise, enrich for motor neurons (Optiprep™), plate cells into proximal chamber
Replace distal medium with CDM2
Maintain completed cultures until needed

Figure 5.3 – MNs grow differently on different substrates.

The growth characteristic of cultured rat spinal motor neurons was shown to vary greatly depending on the culture substrate they were grown on. (**A**) Motor neurons extended a significantly ($P<0.05$) longer axon at 3DIV when co-cultured with a skeletal muscle feeder layer compared with motor neurons cultured on glial cells or on a poly-L-lysine + laminin (PLL-L) substrate. (**B**) Motor neurons developed significantly ($P<0.05$) more neurites when co-cultured with glial cells compared with those grown on skeletal muscle feeder layers or on a PLL-L substrate.

One-way ANOVA with Tukey's post-test comparisons, $*P<0.05 \pm$ SEM, n = 3.

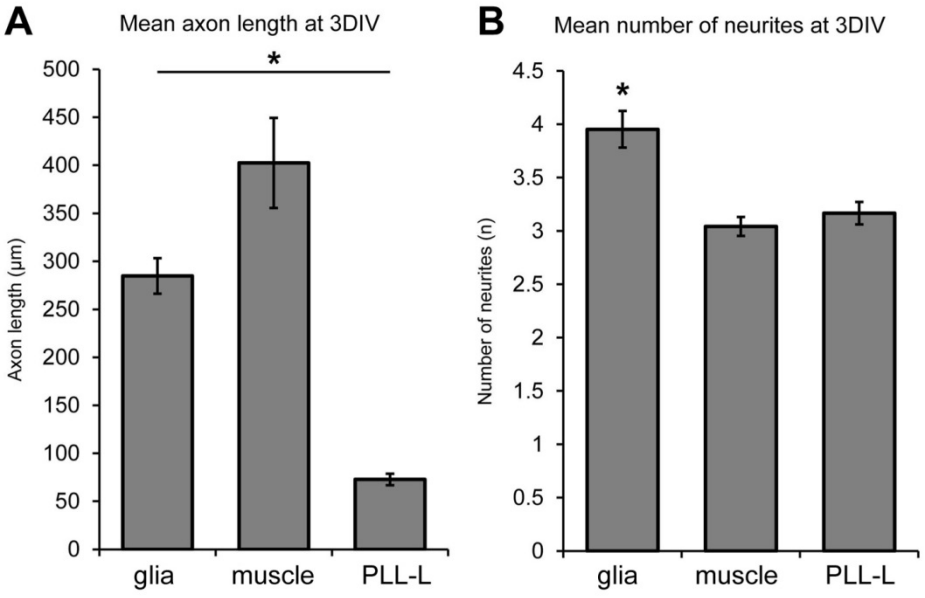

A
Mean axon length at 3DIV
*
Axon length (µm)
500
450
400
350
300
250
200
150
100
50
0
glia
muscle
PLL-L
B
Mean number of neurites at 3DIV
*
Number of neurites (n)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
glia
muscle
PLL-L

Figure 5.4 – Motor neurons develop within microfluidic chambers with the addition of glial and muscle cells, forming unique cellular compartments.

Motor neuron cell bodies (**A**, arrows) extend neurites (arrowheads), differentiating to extend axons into the microgrooves separating the two chambers (**B**, arrows). Axons extend into the distal chamber (**C**) from the microgrooves to form a dense network by 14DIV (**D**), with recently added myoblasts (arrows). By 18DIV (**E**), axons (arrows) extend towards differentiated myotubes (arrowheads).

Scale = 20μm.

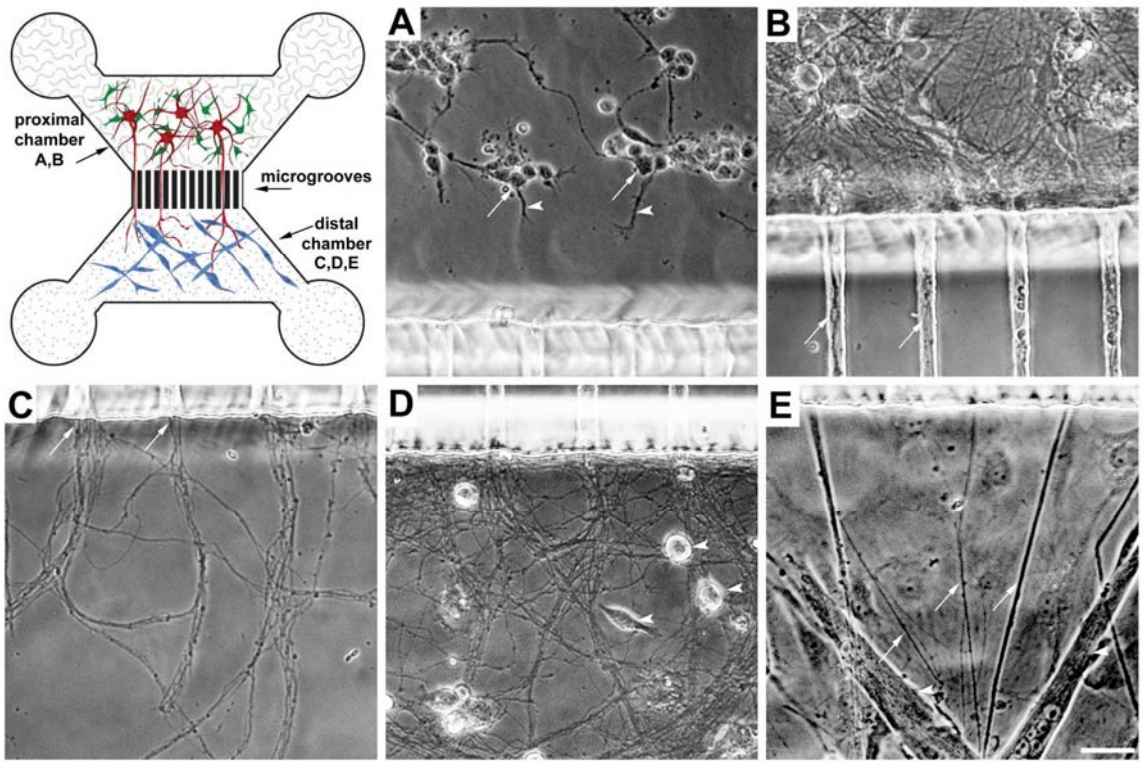

proximal
chamber
A,B
microgrooves
distal
chamber
C,D,E
A
B
C
D
E

Figure 5.5 – Motor neurons interact with spinal glial and skeletal muscle cells in distinct compartments.

Within the proximal compartment, axons are immunoreactive for NFM (red), and dendrites immunoreactive for MAP2 (green) (**A**). Neurofilament positive axons (NFM; red) are supported by GFAP immunoreactive glial cells (green) (**B**). Neurofilament positive axons (NFM; red) are present within the distal chamber by 14 DIV (**C**). Skeletal myocytes (desmin; blue) are present within the distal chamber with axons (red) at 14DIV (**D**). By 18DIV, axons (red) interact with differentiated myotubes (desmin; blue) (**E**). Nuclear staining indicates differentiated myotubes contain multiple nuclei (**F**).

Scale = 50μm.

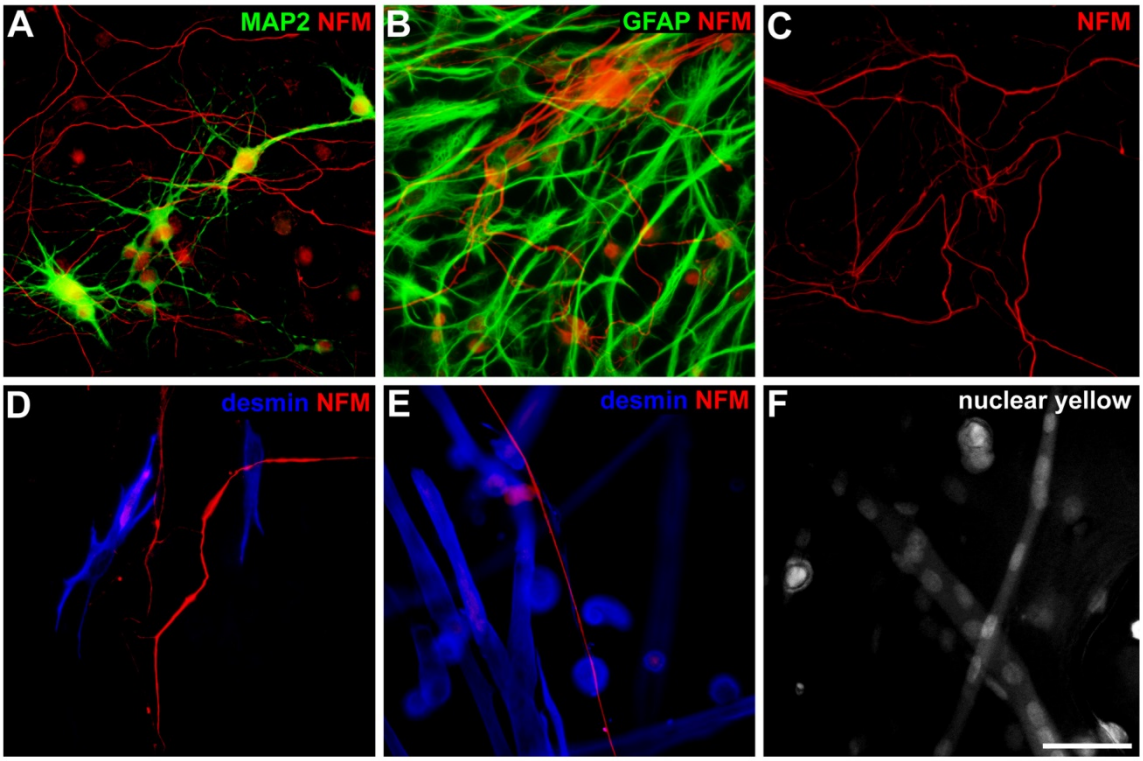

A
MAP2 NFM
B
GFAP NFM
C
NFM
D
desmin NFM
E
desmin NFM
F
nuclear yellow

Figure 5.6 – Neuromuscular junctions form between isolated distal axons and differentiated skeletal myocytes in the distal chamber.

SEM indicates motor neuron axons grew in close contact with cultured myotubes (**A**), pseudocoloured to visualise motor axons (red), myotubes (blue) and fibroblasts (green) which formed a confluent layer within the distal chamber (**B**). Synapse formation is indicated by differential α-BTx clustering on myotubes in contact with motor axons (**C**), as distinct from α-BTx pre-patterning (inset). Arrows indicate co-localisation between α-BTx (green) and NFM (red) (**D**). α-BTx staining (red) is closely associated with synaptophysin positive vesicles (arrows) in motor axons (**E**). Alexafluor 594 non-specific fluorescence is visible in myotube nuclei surrounding neuromuscular junctions (star).

Scale: A,B 5µm; C 20µm; D,E 10µm.

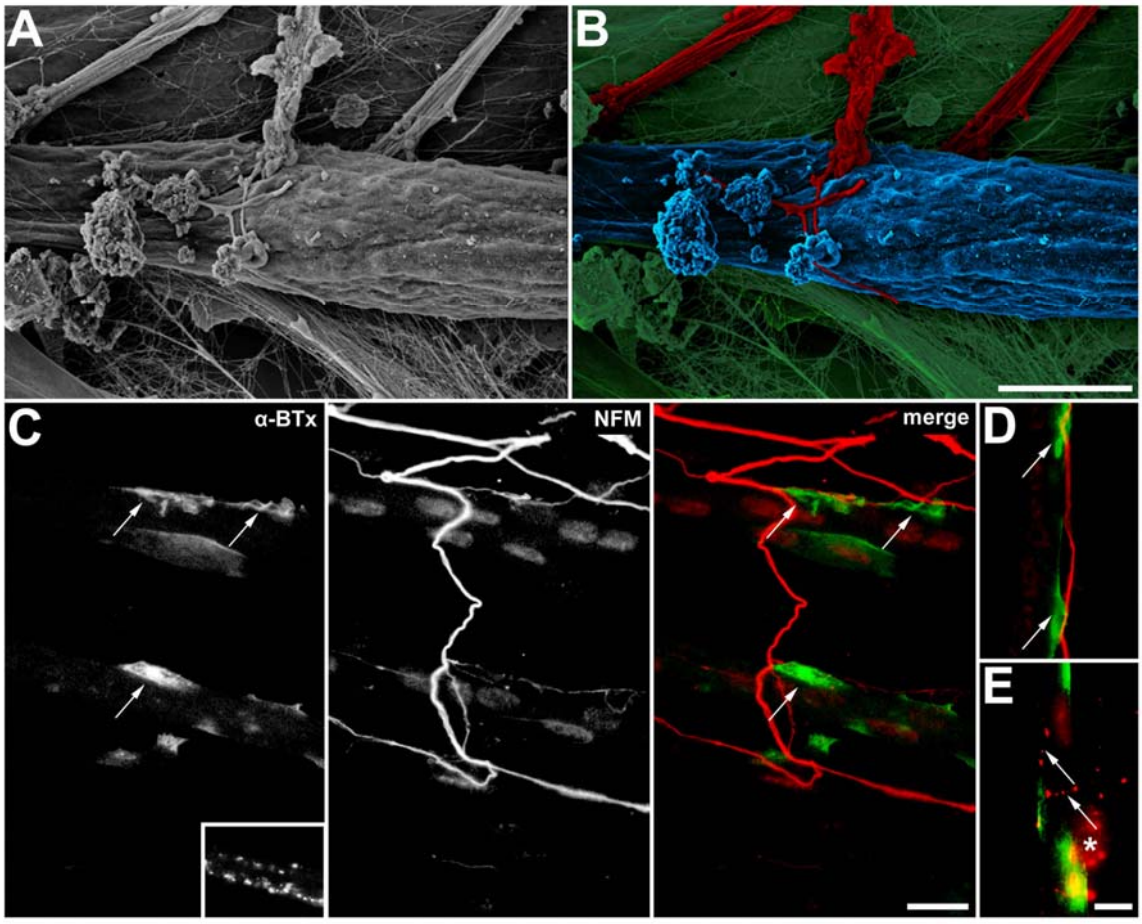

A
B
C
α-BTx
NFM
merge
D
E
*

Figure 5.7 – Motor neuron axons induce synaptic-like clustering of AChRs on the skeletal muscle membrane.

The mean particle area of AChR clusters in the presence of a motor axon terminal is significantly ($P<0.05$) smaller than clusters in the absence of an axon (**A**). The original α-BTx staining (above) and a masked image (below) indicate the characteristic synaptic-like clustering of AChRs in the vicinity of an axon terminal (**B**). Note the absence of α-BTx staining outside of the synaptic-like clusters. The formation of more than one synaptic site is common during development of the neuromuscular junction. In the absence of a motor neuron axon, AChR clusters are less uniform in size and are distributed over a larger percentage of the muscle fibre (**C**). α-BTx staining (above) and masked image (below) demonstrate that AChRs cover a much larger proportion of the muscle fibre membrane when neuron terminals are not present.

Students unpaired t-Test, $*P<0.05 \pm$ SEM. Scale = 10μm.

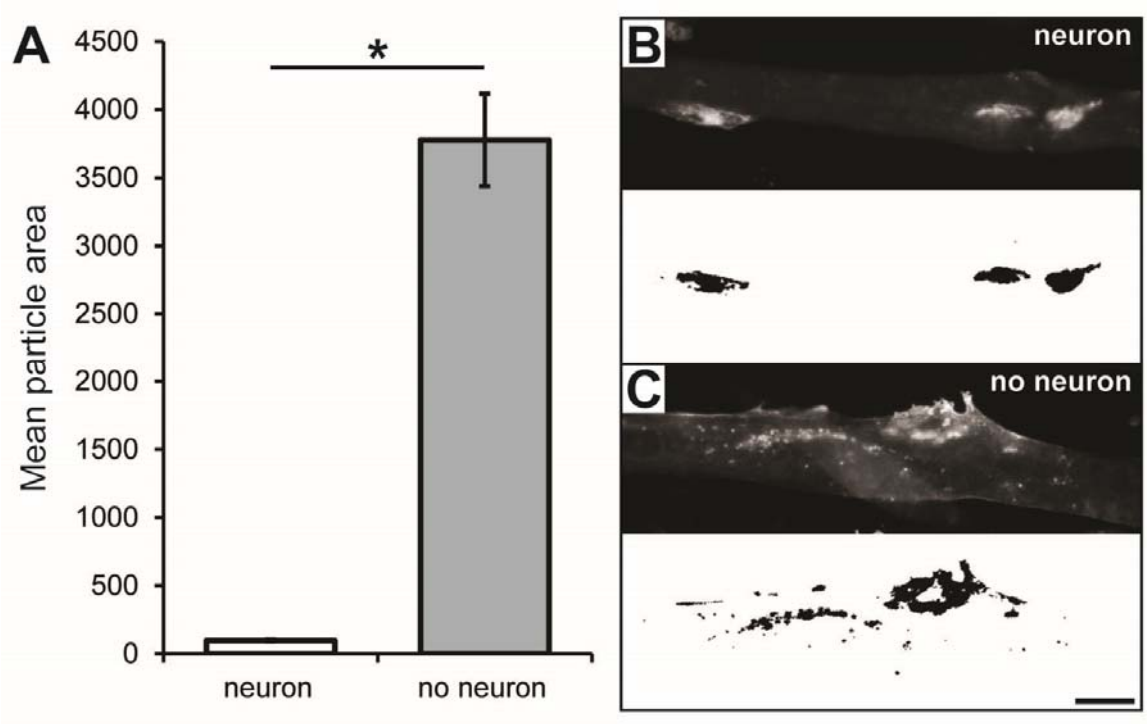

A
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Mean particle area
*
neuron
no neuron
B
neuron
C
no neuron

Figure 5.8 – Rapsyn colocalises to synaptic-like a-BTx staining in the presence of spinal motor neurons.

Spinal mouse motor neurons (blue) induced plaque-like colocalisation of rapsyn (green) with α-BTx (red) (arrows) (**A**) Rapsyn immunoreactivity was not detected on C2C12 myotubes without co-cultured spinal motor neuron (**B**).

Scale = 40μm.

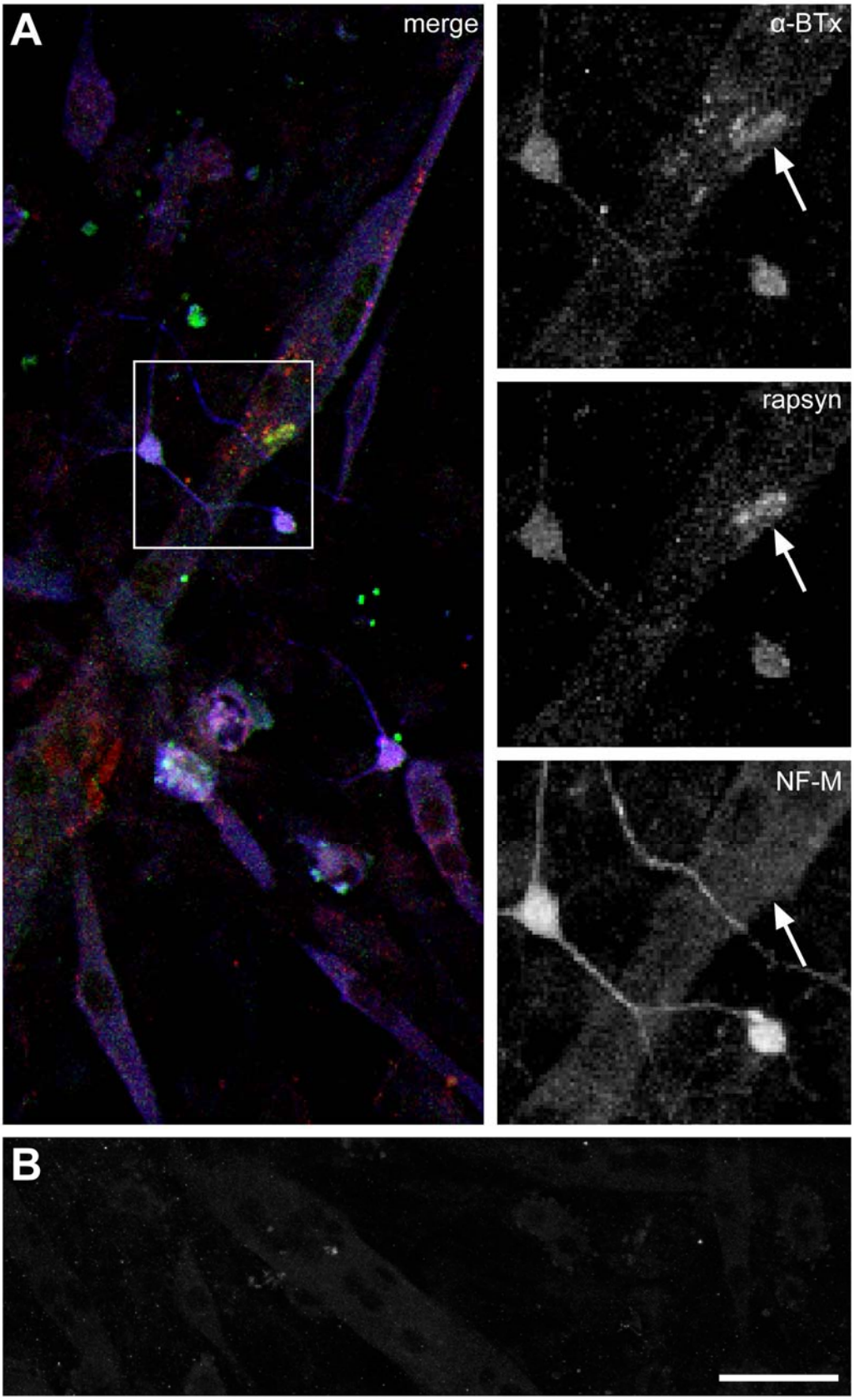

A
merge
α-BTx
rapsyn
NF-M
B

Figure 5.9 – Nestin alters distribution in C2C12 myofibres in co-culture with spinal motor neurons.

Spinal mouse motor neurons (blue) induced a subtle reorganisation of nestin (green) immunoreactivity in C2C12 myofibres, resulting in a immunoreactive patch in the presence of a motor neuron axon and α-BTx staining (red) and a void on either side (**A**). Nestin immunoreactivity in C2C12s cultured alone was relatively uniform throughout the myocytes (**B**).

Scale = 40μm.

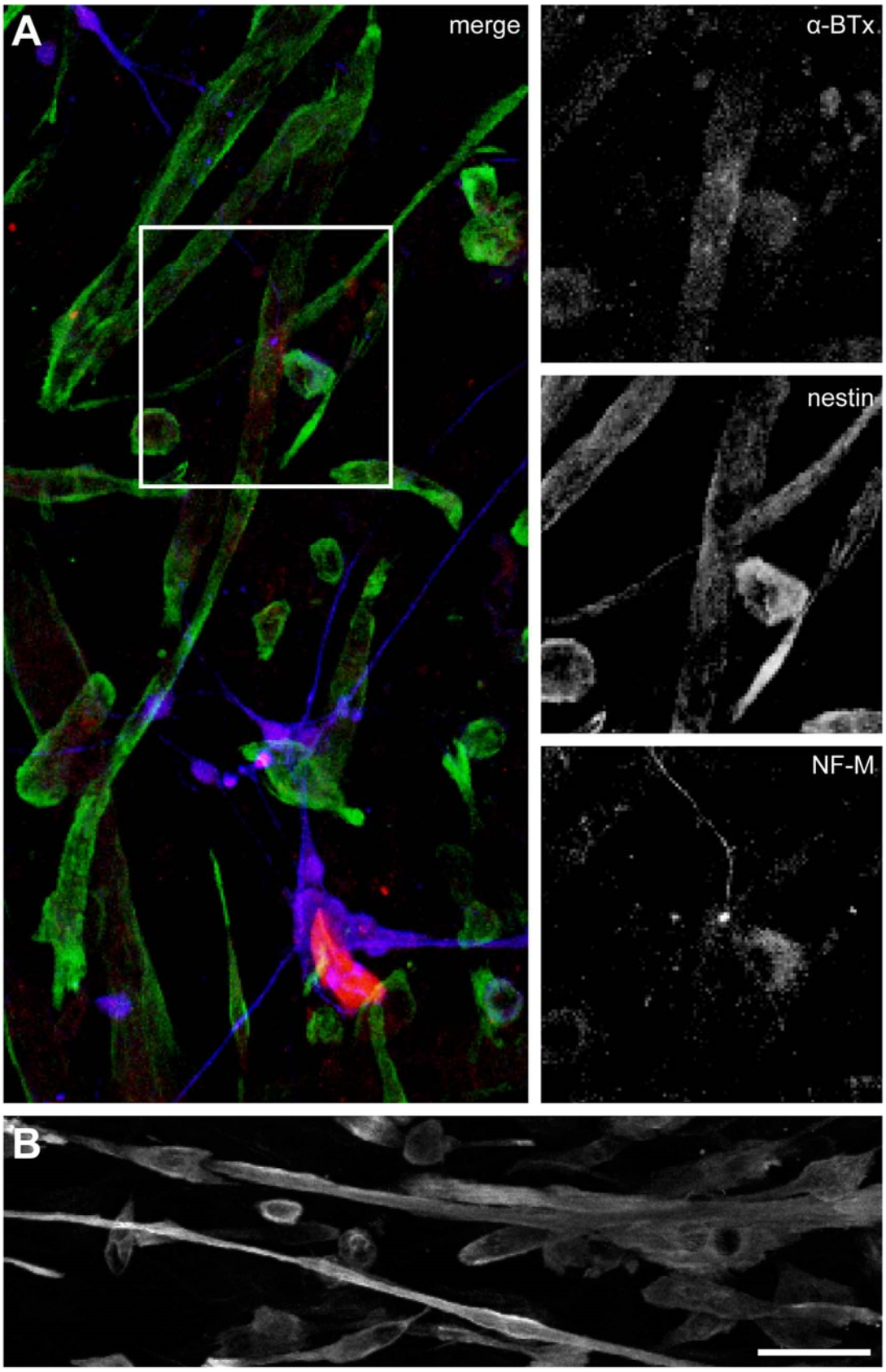

A
merge
α-BTx
nestin
NF-M
B

Figure 5.10 – Differential organisation of dystrophin staining is present adjacent to spinal motor neuron axons.

Dystrophin (green) immunoreactivity was localised to the myofibre periphery and a number of puncta in the vicinity of spinal motor neuron axons (blue) and α-BTx (red) (arrows) (**A**). In contrast, dystrophin immunoreactivity was dispersed throughout the myofibres cultured without spinal motor neurons (**B**).

Scale = 40μm.

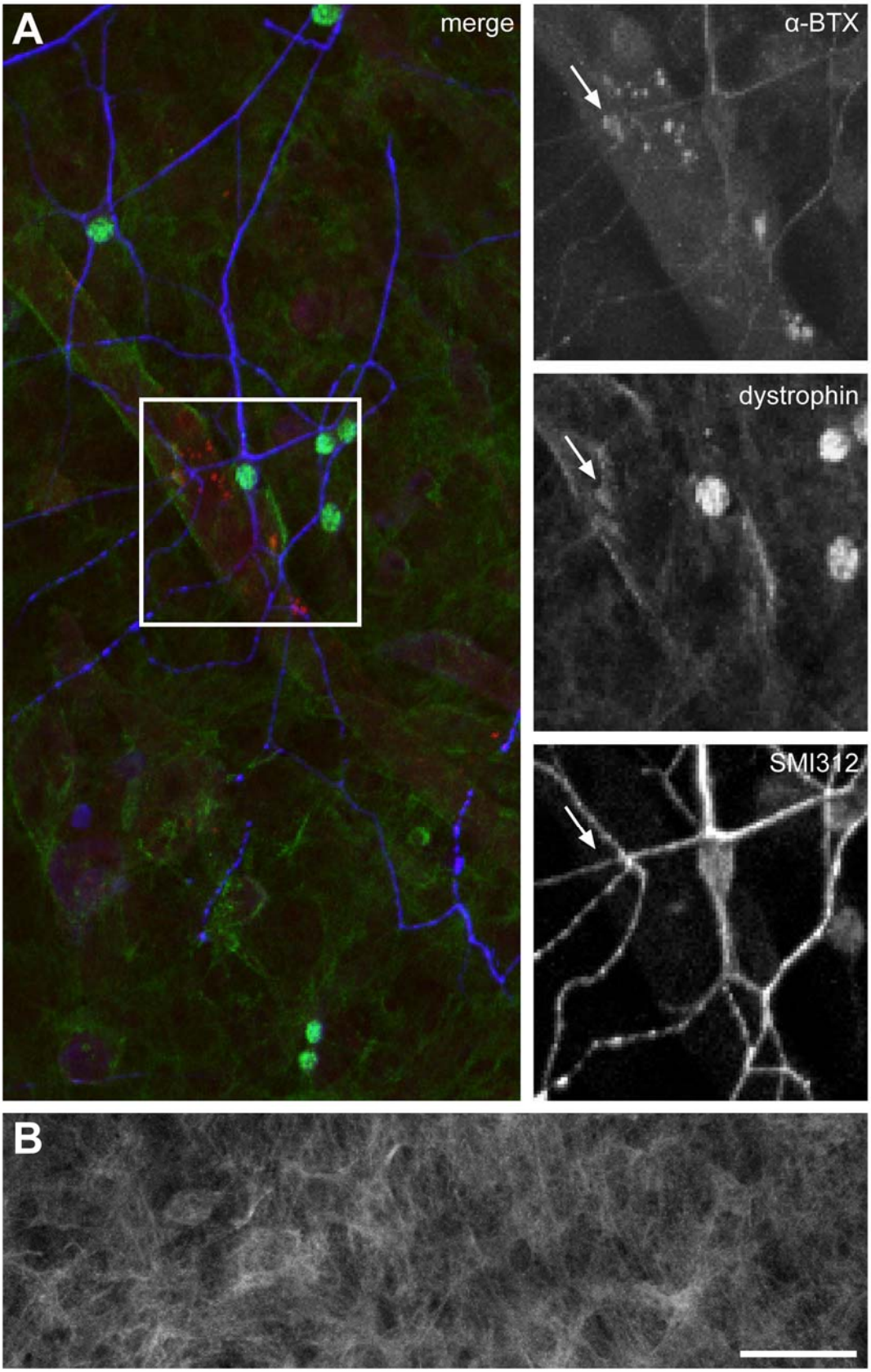

A
merge
α-BTX
dystrophin
SMI312
B

Figure 5.11 – Spinal mouse motor neurons degenerate in response to proximally applied excitotoxicity.

Control untreated motor neurons demonstrate smooth neurofilament (SMI312) and MAP2 immunoreactivity within the proximal chamber (**A**), and even neurofilament (SMI312) immunoreactivity within the distal chamber (**B**). 24-hour kainic acid-induced excitotoxicity to the proximal compartment resulted in extensive degeneration (arrows) of neurofilaments and loss of MAP2 immunoreactivity (arrowheads) (**C**) and degeneration of distal axons (arrows) (**D**). Kainic acid excitotoxicity for four hours did not result in extensive degeneration within either the proximal (**E**) or distal compartments (**F**). Kainic acid applied to the distal chamber for 24 hours did not result in overt degenerative changes within the proximal (**G**) or the distal compartment (**H**).

Scale A, C, E, G = 100μm, B, D, F, H = 50μm

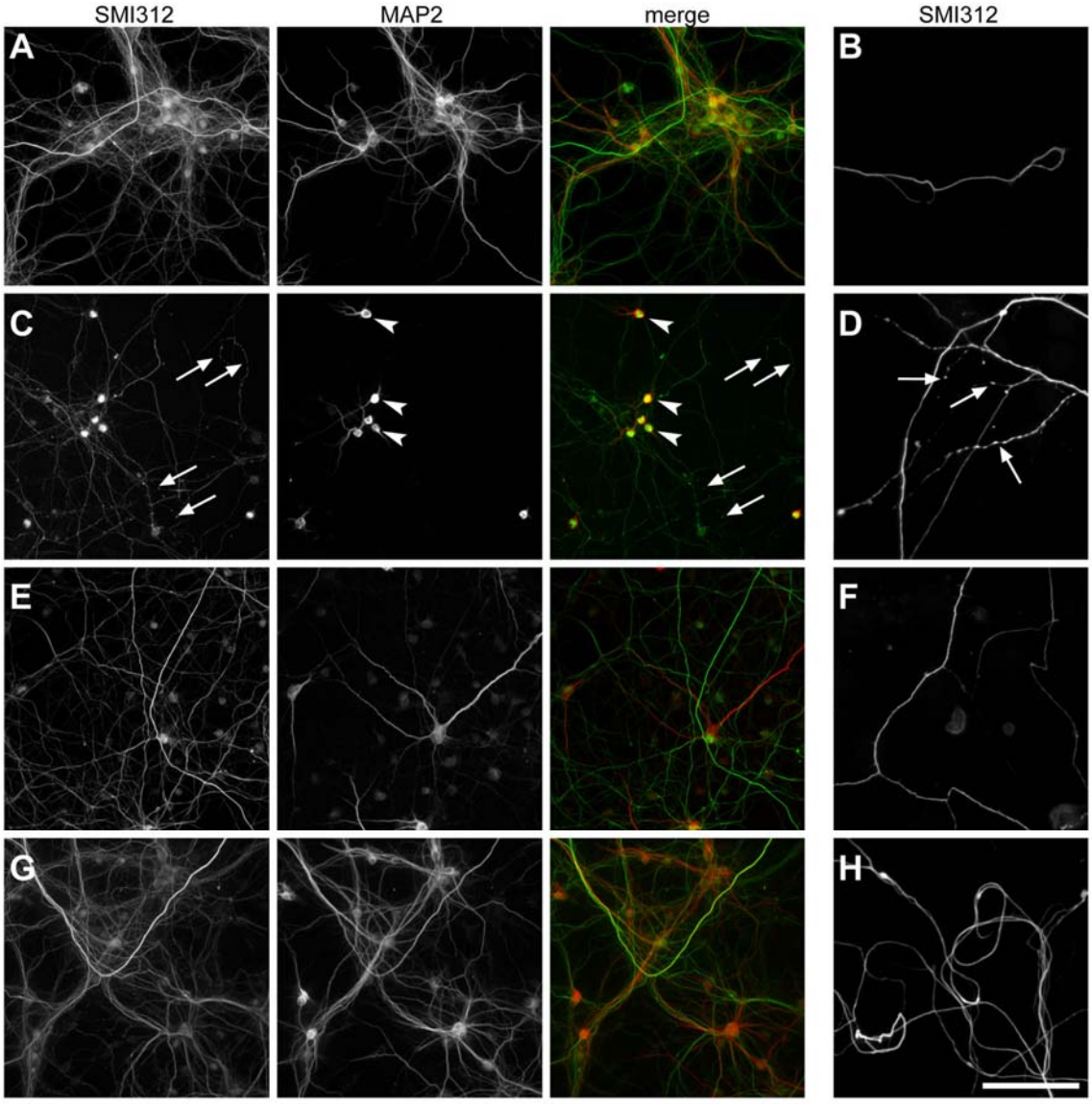

SMI312
MAP2
merge
SMI312
A
B
C
D
E
F
G
H

Figure 5.12 – Spinal mouse motor neuron distal axons degenerate in response to proximally applied excitotoxicity.

Kainic-acid (KA) induced excitotoxicity (100μM) to the proximal compartment for 24 hours resulted in a significant ($P<0.05$) increase in distal axon degeneration of mouse spinal motor neurons. This was largely due to a significant ($P<0.05$) increase in distal axon fragmentation. There was no significant ($P>0.05$) change in distal axon beading following proximal kainic acid excitotoxicity. Furthermore, there was no significant ($P>0.05$) change in distal axon degeneration following distal treatment with kainic acid.

One-way ANOVA with Tukey's post-test comparisons, *$P<0.05$ ± SEM, n = 5.

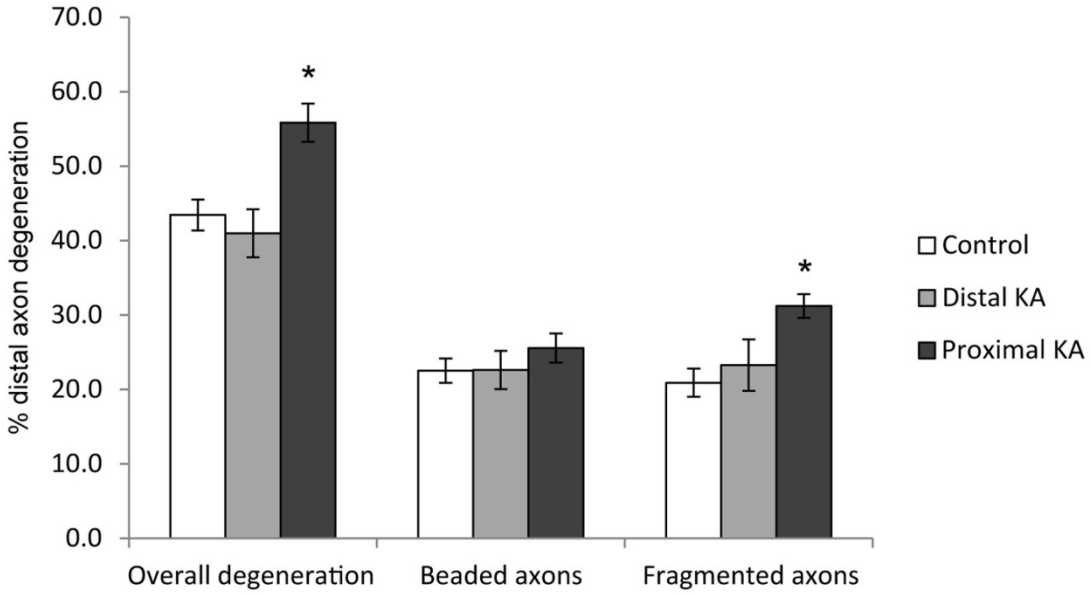

70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% distal axon degeneration
*
*
Overall degeneration
Beaded axons
Fragmented axons
Control
Distal KA
Proximal KA

Figure 5.13 – Kainic acid excitotoxicity alters caspase immunoreactivity.

Within the proximal compartment, healthy axons were immunoreactive for neurofilaments, and caspase-3indicating a small number of smoothly labelled caspase-3 positive axons (**A**). Kainic acid (100μM) applied to the proximal chamber resulted in extensive loss of neurofilaments and punctate-staining of activated caspase-3 (arrows) (**B**). Proximal kainic acid for 24 hours resulted in fragmentation of neurofilament-positive distal axons and activated caspase-3 positive puncta (arrows) (**C**). Kainic acid to the distal axon did not result in degenerative changes within the proximal compartment (**D**).

Within the distal compartment, there was a low level of smooth caspase-3 immunoreactivity (**E**). Kainic acid applied to the proximal compartment for 24 hours resulted in degeneration of the axons and formation of caspase-3 positive puncta (arrows) (**F**). Kainic acid applied to the proximal compartment for four hours resulted in the formation of axonal caspase-3 positive puncta (arrows) however there was no overt axon degeneration (**G**). Kainic acid to the distal compartment did not result in axon degeneration, however did induce localisation of caspase-3 to axonal swellings (arrows) (**H**).

Scale = 100μm.

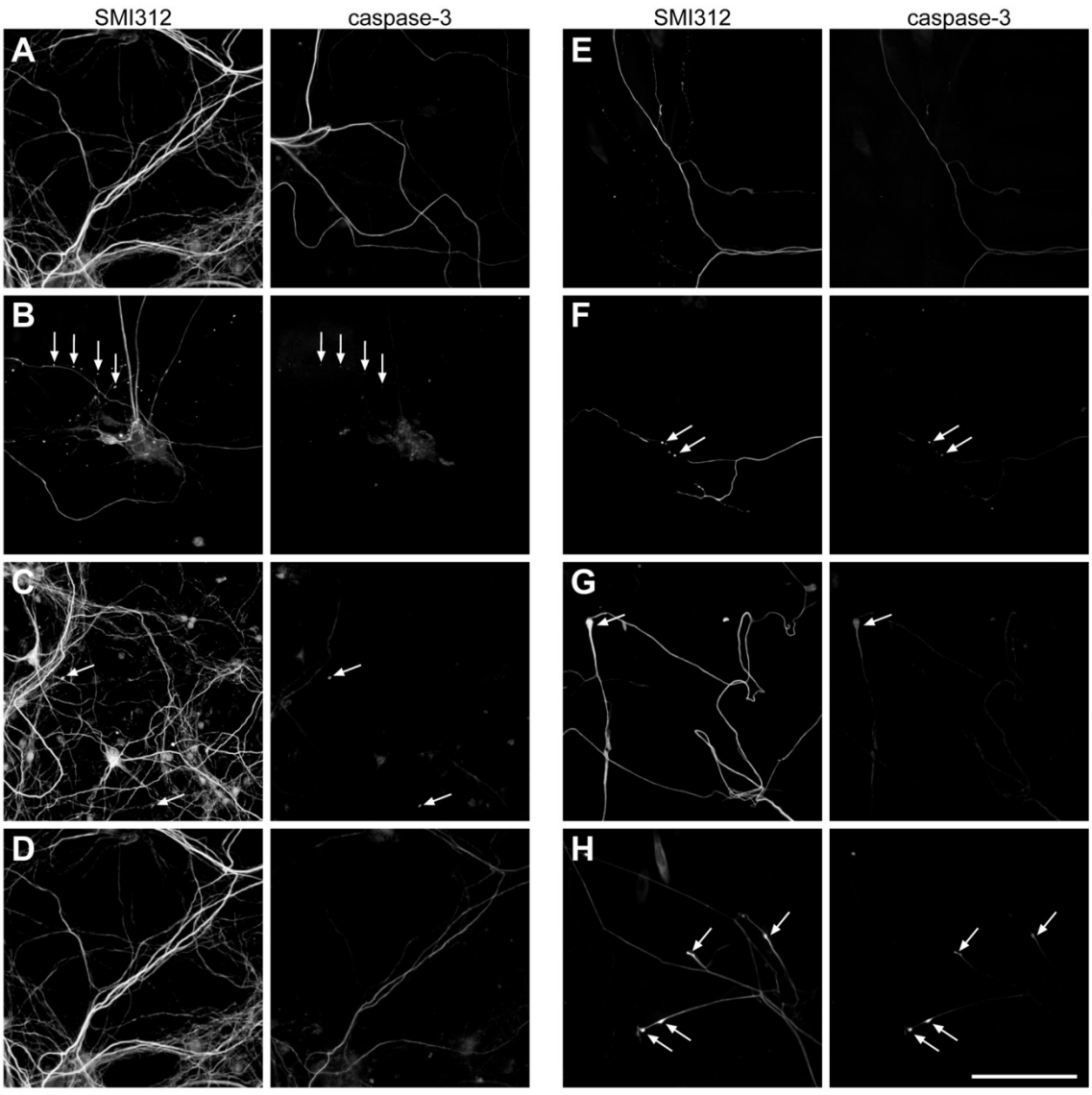

SMI312
caspase-3
SMI312
caspase-3
A
B
C
D
E
F
G
H

Figure 6.1 – Timeline of ALS pathology in mSOD1 G93A mice.

The mSOD1 G93A mouse develops progressive muscle weakness from 111 days of age (onset). The presymptomatic period before onset is characterised by a number of pathological processes, particularly focused on motor neurons. This study has highlighted a number of changes that occur in the skeletal muscle and distal axon terminal prior to disease onset.

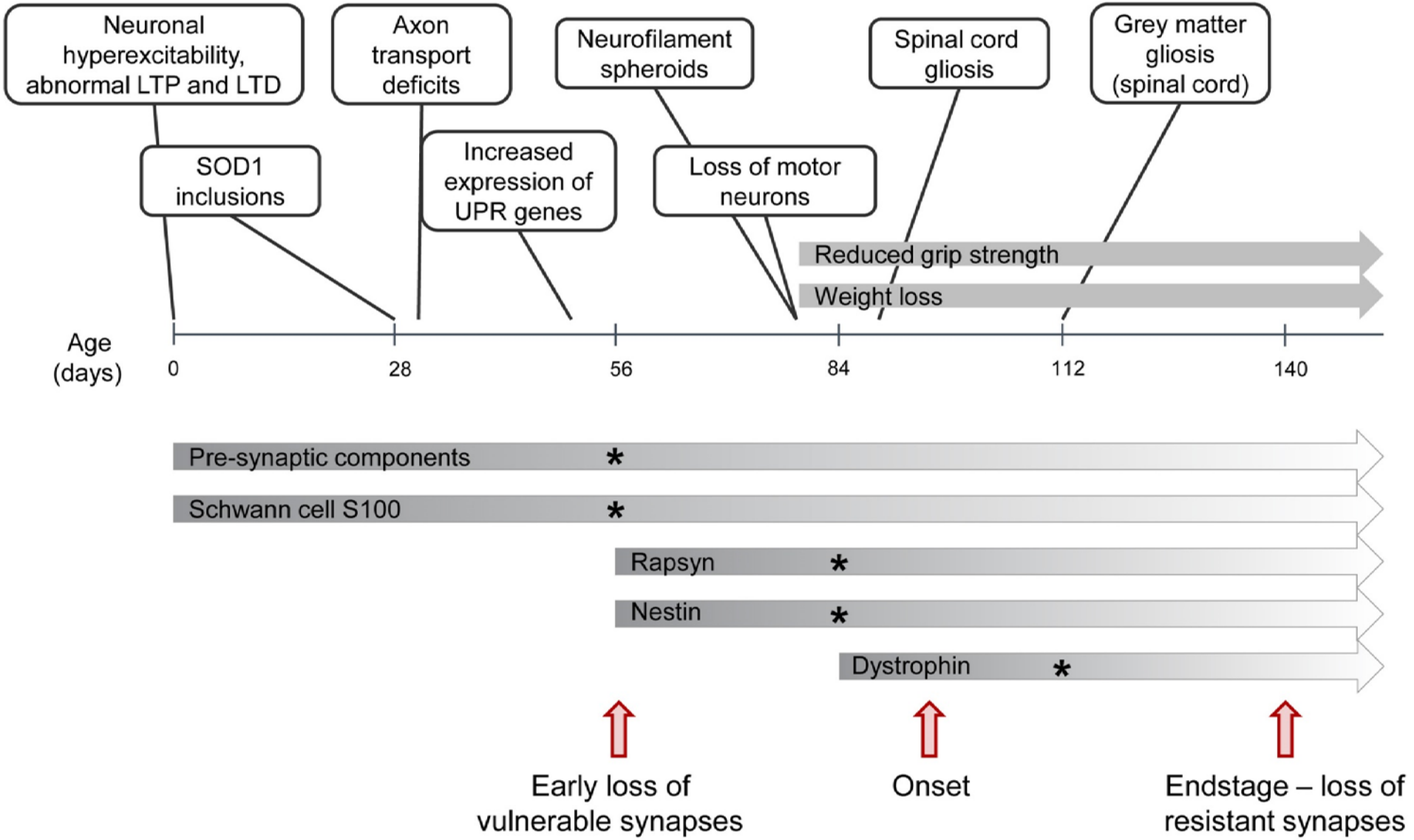
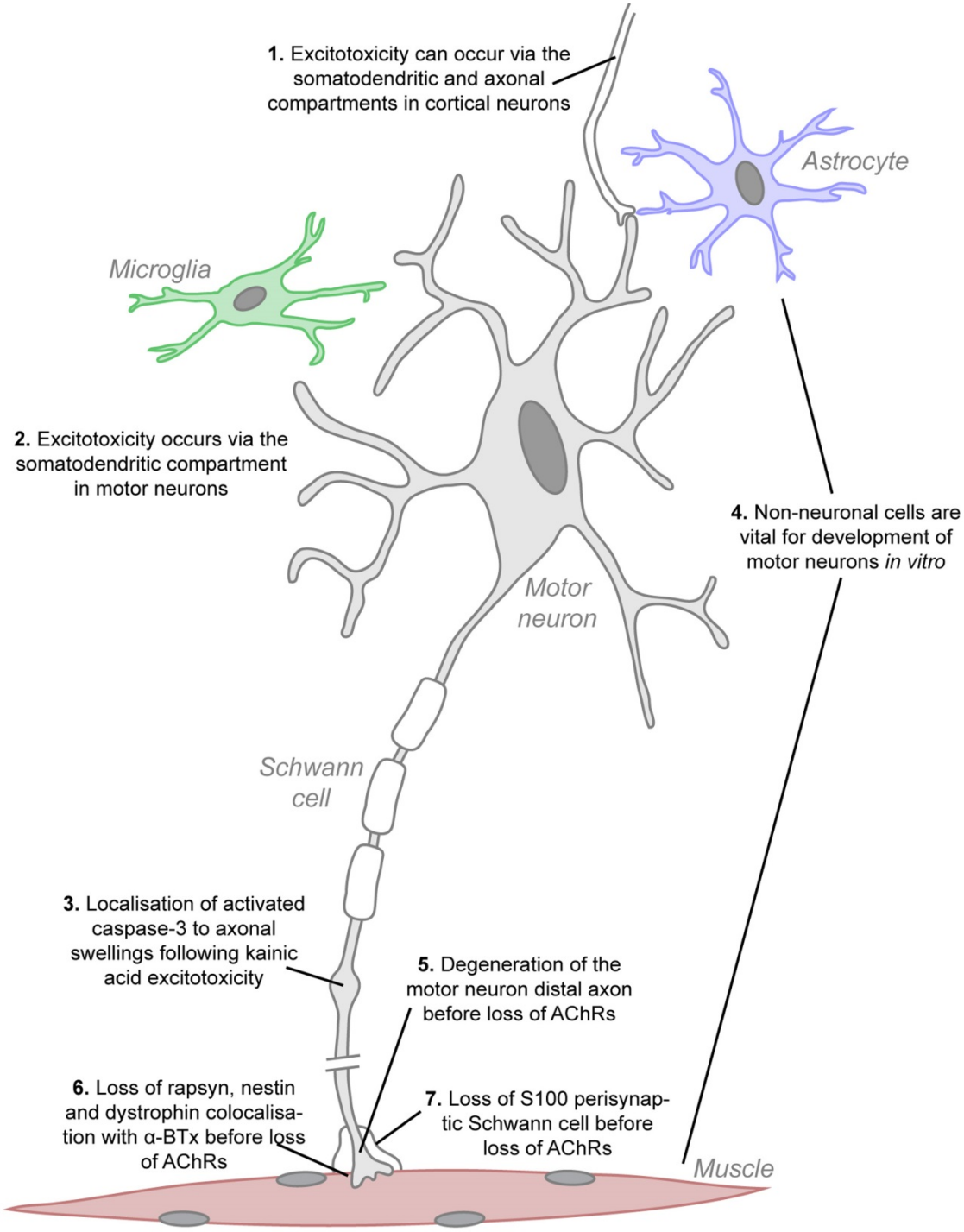

Neuronal hyperexcitability, abnormal LTP and LTD
Axon transport deficits
Neurofilament spheroids
Spinal cord gliosis
Grey matter gliosis (spinal cord)
SOD1 inclusions
Increased expression of UPR genes
Loss of motor neurons
Reduced grip strength
Weight loss
Age (days)
0
28
56
84
112
140
Pre-synaptic components
Schwann cell S100
Rapsyn
Nestin
Dystrophin
Early loss of vulnerable synapses
Onset
Endstage – loss of resistant synapses

Figure 6.2 – Results summary.

Summary of the key findings of this thesis.

1. Excitotoxicity can occur directly to the axons of cultured cortical neurons.
2. Excitotoxicity to cultured motor neurons occurs via the soma and dendrites.
3. Kainic acid excitotoxicity targeted to the spinal motor neuron axon induces caspase localisation to axonal swellings.
4. Different non-neuronal cell populations affect the growth of cultured spinal motor neurons.
5. Degeneration of the motor neuron distal axon occurs before loss of AChRs.
6. The NMJ structural proteins rapsyn, nestin and dystrophin are progressively lost from a proportion of NMJs during early disease.
7. Loss of S100 from the NMJ does not reflect a loss of cells.


1. Excitotoxicity can occur via the somatodendritic and axonal compartments in cortical neurons
Astrocyte
Microglia
2. Excitotoxicity occurs via the somatodendritic compartment in motor neurons
4. Non-neuronal cells are vital for development of motor neurons in vitro
Motor neuron
Schwann cell
3. Localisation of activated caspase-3 to axonal swellings following kainic acid excitotoxicity
5. Degeneration of the motor neuron distal axon before loss of AChRs
6. Loss of rapsyn, nestin and dystrophin colocalisation with α-BTx before loss of AChRs
7. Loss of S100 perisynaptic Schwann cell before loss of AChRs
Muscle