

NEUROSCIENCE FOR NEUROLOGISTS

Molecular and cellular pathways of neurodegeneration in motor neurone disease

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The process of neuronal degeneration in motor neurone disease is complex. Several genetic alterations may be involved in motor neurone injury in familial amyotrophic lateral sclerosis, less is known about the genetic and environmental factors involved in the commoner sporadic form of the disease. Most is known about the mechanisms of motor neurone degeneration in the subtype of disease caused by SOD1 mutations, but even here there appears to be a complex interplay between multiple pathogenic processes including oxidative stress, protein aggregation, mitochondrial dysfunction excitotoxicity, and impaired axonal transport. There is new evidence that non-neuronal cells in the vicinity of motor neurones may contribute to neuronal injury. The final demise of motor neurones is likely to involve a programmed cell death pathway resembling apoptosis.

during the disease process are the presence of ubiquitinated proteinaceous inclusions within motor neurone cell bodies, and neurofilament accumulations within motor neurone axons.⁴

The person afflicted by MND typically develops a combination of upper and lower motor neurone signs, with progressive muscle weakness and wasting, usually accompanied by pathologically brisk reflexes, eventually involving the limb and bulbar muscles. Clinical variants of the disease may initially affect only the spinal lower motor neurones (progressive muscular atrophy variant); only the upper motor neurones (primary lateral sclerosis variant); or only the bulbar musculature (progressive bulbar palsy variant). With disease progression, the majority of patients will develop features of ALS. Certain motor neurone groups are less vulnerable to the pathological process, including those in upper brain stem nuclei controlling eye movements, and those in Onuf's nucleus within the sacral spinal cord controlling the pelvic floor musculature.⁵ The rate of disease progression varies between individuals, but the average survival is only of the order of three years from symptom onset.⁶ However, approximately 10% of patients will have a slower disease course with survival beyond 10 years.⁷ Death in most patients results from neuromuscular respiratory failure. The World Federation of Neurology diagnostic criteria for ALS/MND require the presence of signs of upper and lower motor neurone degeneration, with evidence of progression, in the absence of evidence of other disease processes.⁸

The selectivity of the pathological process for the motor system is now recognised to be relative rather than absolute. Detailed investigation has revealed involvement of extramotor parts of the CNS, including changes in other long tract systems—for example, sensory and spinocerebellar pathways—and cellular injury of neuronal groups including the substantia nigra neurones and dentate granule cells within the hippocampus.⁹ Overt dementia is found in approximately 2–3% of MND patients,¹⁰ and detailed neuropsychological testing shows more subtle neurophysiological changes, particularly affecting frontal function, in approximately 30% of patients.¹¹ Thus MND is now regarded as a multisystem disease in which motor neurones tend to be affected earliest and most prominently.⁴

Abbreviations: ALS, amyotrophic lateral sclerosis; CNTF, ciliary neurotrophic factor; GEF, guanine nucleotide exchange factor; HRE, hypoxia response element; MND, motor neurone disease; VAPB, vesicle associated membrane protein/synaptobrevin associated membrane protein; VEGF, vascular endothelial growth factor

Motor neurone disease (MND), known in many countries as amyotrophic lateral sclerosis (ALS), is the commonest adult onset disorder of motor neurones, and among the most common of adult onset neurodegenerative diseases. The incidence of the disease is 1–2 per 100 000 and is fairly uniform throughout the world, with the exception of several high incidence foci, for example on the island of Guam in the Western Pacific and on the Kii peninsula of Japan. The lifetime risk of developing ALS/MND is approximately 1 in 2000. At any one time there are approximately 5000 individuals suffering from MND in the United Kingdom. Some epidemiological studies indicate that the incidence of MND may be increasing, though the contribution to this of population aging and better developed neurological diagnostic services is currently unknown.¹ The disease is predominantly a condition of middle age and elderly life, with an average age of onset between 50 and 60 years, though rare juvenile onset forms of the condition also exist. For reasons that are not currently understood, MND affects men more commonly than women, with a male to female ratio of approximately 1.6/1.²

The pathology accompanying the clinical phenotype was first described by Jean-Martin Charcot in 1869.³ The salient pathological feature is progressive injury and cell death of lower motor neurone groups in the spinal cord and brain stem and of upper motor neurones in the motor cortex. Characteristic cytopathological features in motor neurones not yet eliminated

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WHAT DO WE KNOW ABOUT THE CAUSES OF MOTOR NEURONE DEGENERATION?

The primary pathogenic processes underlying MND are likely to be multifactorial, and the precise mechanisms underlying selective cell death in the disease are at present unknown. Current understanding of the neurodegenerative process in MND suggests that there may be a complex interplay between multiple mechanisms including genetic factors, oxidative stress, excitotoxicity, protein aggregation, and damage to critical cellular processes, including axonal transport and organelles such as mitochondria (table 1). Recently there has been growing interest in the role played by non-neuronal neighbourhood cells in the pathogenesis of motor neurone injury and in dysfunction of particular molecular signalling pathways (fig 1). The relative importance of these different pathways may well vary in different subgroups of patients, and a very important task for clinicians and scientists in the future is to further delineate the subcategories of MND. Evidence has also accumulated that the final process of motor neurone death is likely to occur through a caspase dependent programmed cell death pathway resembling apoptosis.

GENETICS OF ALS/MND

MND is sporadic in 90–95% of cases and familial in approximately 5–10%. Inheritance in familial MND is usually autosomal dominant, though autosomal recessive and X linked inheritance may be seen in some pedigrees. It is apparent that multiple abnormal gene products can set the scene for motor neurone degeneration. There are at least six dominantly inherited adult onset ALS genes (table 2), of which only three have so far been identified.

Copper-zinc superoxide dismutase (SOD1)

A major research breakthrough 11 years ago came from the finding that 20% of families with autosomal dominant MND showed mutations in the gene on chromosome 21q22.1 which encodes the free radical scavenging enzyme superoxide dismutase 1 (SOD1).¹² More than 100 different mutations have been identified throughout the SOD1 gene.^{13–14} The majority of mutations in SOD1 are missense mutations, with a small number of deletion and insertion mutations resulting in truncated SOD1 polypeptides. SOD1 is a metalloenzyme of 153 amino acids which functions as a homodimer whose major function is to convert intracellular superoxide free radicals—a toxic waste product of mitochondrial oxidative phosphorylation—to hydrogen peroxide which is in turn removed by the action of other free radical scavenging enzymes. The SOD1 enzyme contains an essential copper atom at the active site which is alternately reduced and oxidised by superoxide. The presence of zinc is thought to stabilise the protein structure. SOD1 is an abundant protein in the CNS, accounting for about 1% of brain protein,

but it is also ubiquitously expressed in all other tissues. SOD1 was initially thought to be confined to the cytosolic compartment of cells but it is now recognised that a small proportion of the protein is located in the intermembrane space of the mitochondria.¹⁵ The reasons why motor neurones are especially vulnerable to injury in the presence of SOD1 mutations are not yet clear.

Despite 11 years of intensive research effort, the pathways leading to the cell death of motor neurones in the presence of SOD1 mutations have not yet been fully identified, though there is a convincing body of evidence that the mutant SOD1 protein exerts its detrimental effects through a toxic gain of function rather than a loss of function. Most of our current level of understanding of disease mechanisms in ALS/MND has come from the study of the effects of SOD1 mutations, but even in this defined genetic subgroup of disease the pathways to neurodegeneration appear to be complex and multifactorial.¹⁶

There is considerable variation in disease phenotype in terms of age of onset and rate of disease progression in human SOD1 related MND. It is apparent that the clinical phenotype must be modified by other genetic or environmental factors, or both. There has been much interest in the D90A SOD1 mutation, which has a dominant inheritance in some genetic backgrounds but is recessively inherited, with two mutated copies of the gene required to cause disease, in Scandinavian populations, implying a co-inherited protective factor.¹⁷ Some intensively studied SOD1 mutations such as the A4V mutation do not show 100% penetrance¹⁸ and the disease phenotype in mice can vary significantly according to the background strain of mouse employed.¹⁹

Mutant SOD1 transgenic mice that develop a disease which clinically and pathologically resembles human MND have been developed. The most extensively studied are SOD1 G93A, SOD1 G37R, and SOD1 G85R.^{20–22} Transgenic rats, carrying G93A or H46R SOD1, also develop an MND phenotype.^{23–24} In addition, cellular models of SOD1 related MND have been generated which have helped to elucidate cellular mechanisms of disease.^{25–27}

The toxic gain of function of mutant SOD1 has not yet been fully defined, but several pathophysiological processes may be involved, including oxidative stress, mitochondrial dysfunction, excitotoxicity, protein aggregation, and inflammation. These mechanisms are not mutually exclusive and it is possible that all of them play a role in the development of motor neurone injury. These potential mechanisms will be discussed in subsequent sections.

The genetic alterations underlying the remaining 80% of cases of autosomal dominant MND at present remain unknown. However, three other genes have recently been identified as causative in rare cases of familial MND.

ALS 2: alsin

In 2001, two groups identified alsin as the causative gene for an autosomal recessive form of juvenile ALS linked to chromosome 2q33.^{28–29} Mutations in alsin can also cause a motor neurone degenerative disorder with a predominant upper motor neurone phenotype, infantile onset ascending hereditary spastic paralysis,^{30–31} and one family has been described with autosomal recessive complicated hereditary spastic paraplegia.³² Thus, ALS2 mutations account for several juvenile onset autosomal recessive neurodegenerative disorders of motor neurones.

ALS2 encodes a 184 kDa protein which contains three putative guanine nucleotide exchange factor (GEF) domains. GEFs are known to activate small GTPase proteins by stimulating the release of guanosine diphosphate (GDP) in exchange for guanosine triphosphate (GTP).³³ Given the conserved GEF domains of ALS2, it is predicted to function as

Table 1 Pathogenic mechanisms which may contribute to motor neurone injury and cell death in motor neurone disease

- Genetic factors
 - Oxidative stress
 - Protein aggregation
 - Glutamatergic toxicity
 - Mitochondrial dysfunction
 - Impairment of axonal transport
 - Inflammatory cascades/contribution of non-neuronal cells
 - Dysfunctional signalling pathways, eg through VEGF, Nrf2
- The above factors alone or in combination may lead to a programmed cell death mechanism similar to apoptosis

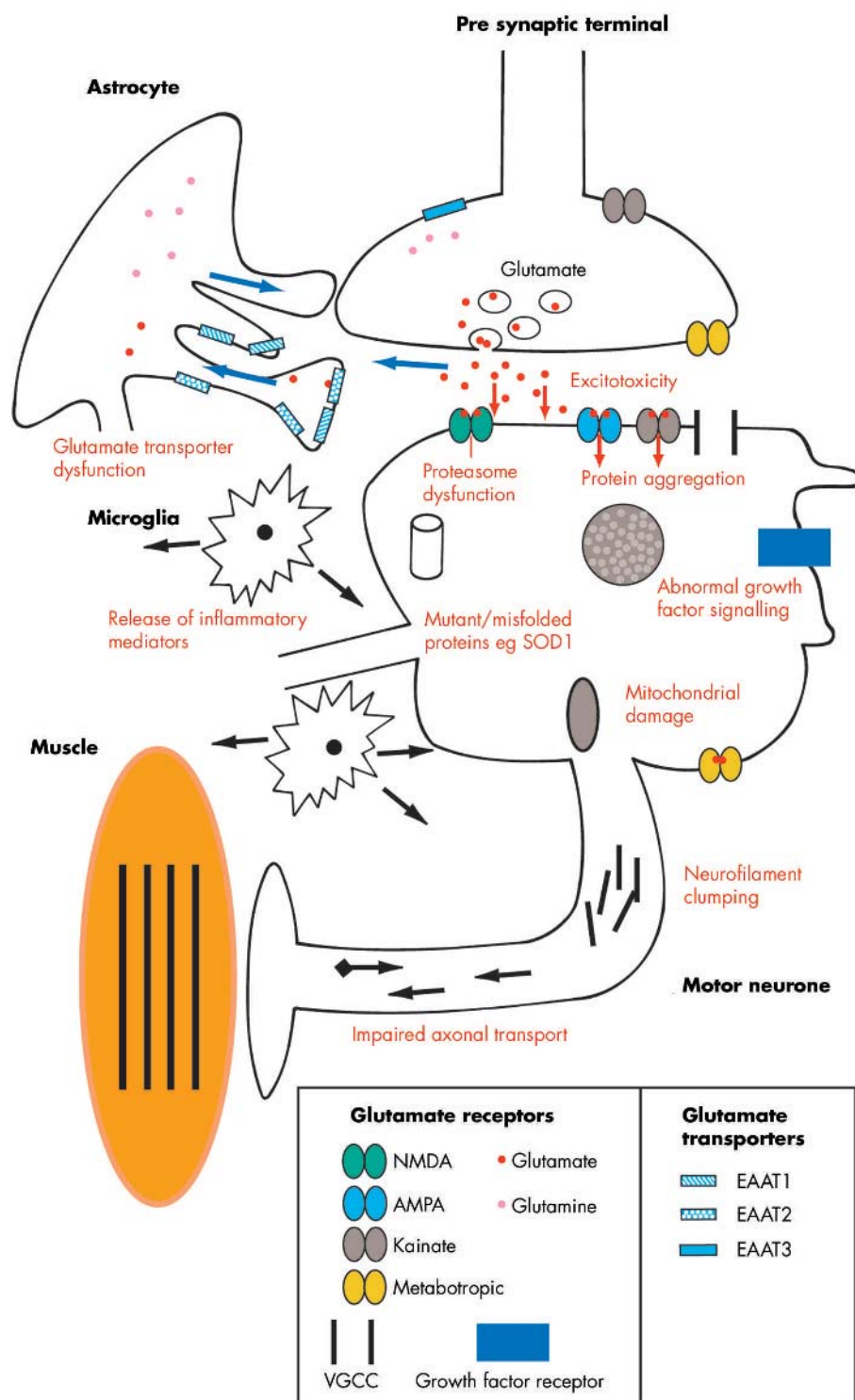


Figure 1 Molecular mechanisms that may contribute to motor neurone injury in motor neurone disease.

an activator of particular small GTPases. The small GTPases control a range of important cellular processes including nuclear transport, cytoskeletal reorganisation, transcription, cell migration, and membrane trafficking. They function as binary switches—alternating between inactive GDP bound and active GTP bound states. The alsin protein is widely expressed, but enriched within the CNS, where it is localised

to the cytoplasmic face of endosomal membranes.³⁴ The functions of alsin are still being worked out, but to date it has been shown to bind specifically to the small GTPase, Rab5, and to function as a guanine nucleotide exchange factor or activator of Rab5.^{35–36} This implies that alsin is important in endosomal dynamics, and the working hypothesis is that it normally regulates trafficking of signalling molecules

Table 2 Genetic subtypes of ALS/MND

Subtype of ALS/MND	Gene	Inheritance, chromosome
ALS1	<i>SOD1</i>	Dominant-adult onset, 21q22.1
ALS2	<i>ALSIN</i>	Recessive-juvenile onset, 2q33
ALS3	–	Dominant-adult onset, 18q21
ALS4	<i>Senataxin (SETX)</i>	Dominant-juvenile onset, 9q34
ALS5	–	Recessive-juvenile onset, 15q15.1–q21.1
ALS6	–	Dominant-adult onset, 16q12
ALS7	–	Dominant-adult onset, 20p11–p13
ALS8	<i>Vesicle associated membrane protein (VAPB)</i>	Dominant-adult onset, 20q13.33
ALS-FTD	–	Dominant-adult onset, 9q21–22
ALS-X	–	Dominant-adult onset, Xp11–q12
ALS with parkinsonism and dementia	<i>Microtubule associated protein tau (MAPT)</i>	Dominant-adult onset, 17q21
Progressive LMN disease	<i>Dynactin p150 subunit (DCTN1)</i>	Dominant-adult onset, 2p13

ALS, amyotrophic lateral sclerosis; LMN, lower motor neurone; MND, motor neurone disease.

important for proper development or maintenance of the health of motor neurones. ALS2 knockout mice have been generated but no major motor system disease phenotype has yet been reported.^{37, 38}

Alsln/ALS2 is alternatively spliced to generate a short and a long transcript. It has recently been reported that the long isoform of alsin specifically binds, through its RhoGEF domain, to mutant SOD1 and protects cultured motor neurones from mutant SOD1 mediated toxicity.³⁹ Further examination of this interaction may help elucidate motor neurone specific pathways of neurodegeneration.

ALS4: senataxin

The ALS4 locus linked to chromosome 9q34 was originally identified in a single large pedigree with juvenile onset, autosomal dominant ALS/MND. The disease course in this family was indolent and did not reduce life expectancy. Chen and colleagues identified three different missense mutations (L309S, R2136H, and T31I) in three families with this subtype of ALS/MND.⁴⁰ The SETX gene encodes senataxin, a large 302.8 kDa protein of unknown function. Much of the protein has no homology with other known proteins, but there is one DNA/RNA helicase domain. DNA/RNA helicase proteins are known to have roles in processes such as repair, replication, recombination or transcription of DNA and RNA processing, RNA transcript stability, and the initiation of translation. Recessive loss of function mutations in SETX are associated with ataxia-oculomotor apraxia type 2.⁴¹ It is predicted that the different phenotype of dominantly inherited ALS4 is likely to be caused by a toxic gain of function of the mutated senataxin protein.

ALS8: VAPB (vesicle associated membrane protein/synaptobrevin associated membrane protein)

Very recently Nishimura and coworkers described a novel missense mutation (P56S) in the VAPB gene at chromosome 20q 13.3 in a Brazilian family with ALS8, an autosomal dominant slowly progressive disorder characterised by fasciculation, cramps, and postural tremor.⁴² They subsequently found the same mutation in six further families with different clinical phenotypes, including late onset spinal muscular atrophy and classical rapidly progressive ALS/MND. Thus, modifier genes or environmental factors are likely to play an important role in modulating the clinical course of disease in individuals carrying the same mutation. Vesicle associated proteins are intracellular membrane proteins that can associate with microtubules and have been shown to function in membrane transport. The VAPB protein has three identifiable structural domains. The first 150 residues form an MSP domain conserved between all members of this protein family; the central region contains an amphipathic

helical structure predicted to form a coiled/coil protein–protein interaction motif and at the carboxy terminus is a hydrophobic region that acts as a membrane anchor. Preliminary cell biological studies have indicated that the wild-type VAPB protein localises predominantly to the endoplasmic reticulum. The P56S mutation dramatically disrupts the subcellular distribution and induces the formation of intracellular protein aggregates.⁴²

Dynactin mutation

Puls and colleagues identified a mutation (G59S) substitution in the gene encoding the P150 subunit of dynactin (DCTN1) in a single family with a slowly progressive lower motor neurone degenerative disorder.⁴³ The described family had a highly unusual and characteristic phenotype, presenting in early adulthood with respiratory difficulties from vocal cord paralysis, progressive facial weakness, weakness and atrophy of the hands, and the later development of lower motor neurone signs distally in the lower limbs. The amino acid change caused by the mutation would be predicted to distort the folding of the microtubule binding domain of dynactin.

The dynactin–protein complex is required for dynein mediated retrograde axonal transport of vesicles and organelles along the microtubule system. It provides the link between the specific cargo, the microtubule, and cytoplasmic dynein during vesicle transport. Interestingly, it has also been shown that overexpression of the P50 subunit of dynactin also disrupts the function of this protein complex and causes late onset progressive motor neurone degeneration in genetically engineered mice.⁴⁴

Other ALS/MND loci

The genes for several other subtypes of ALS remain to be identified, as indicated in table 2. Three separate families have shown linkage to chromosome 16, allowing significant refinement of the region of interest. ALS/MND with fronto-temporal dementia has been mapped to a 17-cM interval chromosome 9q21,⁴⁵ and one Swedish family with a similar phenotype without linkage to the chromosome 9 locus has recently been identified, suggesting genetic heterogeneity for this subtype of disease.⁴⁶ Motor neurone degeneration may sometimes occur in patients with fronto-temporal dementia and Parkinson's disease, associated with mutations in the microtubule associated protein tau.^{47–48} The mutant tau protein forms filamentous inclusions and insoluble aggregates that are associated with neurodegeneration. Some patients with familial fronto-temporal dementia, parkinsonism, and ALS/MND do not have identified mutations in tau,^{49–50} suggesting that further genes causing this triad of features remain to be identified.

Possible genetic risk factors in sporadic ALS/MND

There have been reports of genetic variants found in individuals with apparently sporadic MND (table 3). Deletions or insertions have been described in the KSP repeat region of the gene encoding the neurofilament heavy protein, which is a major component of the neuronal cytoskeleton.^{51–52} The apolipoprotein E $\Sigma 4$ genotype, which correlates with an earlier age of onset in Alzheimer's disease, has been examined in ALS/MND and the results obtained are not clear. Some studies have indicated that the apolipoprotein $\Sigma 4$ genotype may be a risk factor for the development of bulbar onset ALS.⁵³ Another study provided evidence that this genotype correlates with a shortened survival in patients with ALS, though it did not obviously influence age of onset.⁵⁴ However, several other groups have been unable to confirm these findings. A single patient with an ALS/MND phenotype has been described with a mutation in the mitochondrially encoded subunit 1 of cytochrome C oxidase, which is an important component of the mitochondrial respiratory chain.⁵⁵ Decreased expression of the glial glutamate transporter EAAT₂ is observed in the spinal cord of human ALS/MND patients and in mouse and rat SOD1 transgenic murine models.^{24–56–58} Initially it was suggested that aberrant splicing of EAAT₂ RNA might underlie this alteration in transporter expression,⁵⁹ but subsequent studies have indicated that EAAT₂ splice variants are observed as frequently in the CNS of controls as in patients with ALS/MND.^{60–61} A single individual with sporadic ALS/MND has been found to have a mutation (N2065) within a putative glycosylation site of EAAT₂, which appeared functionally significant in causing aberrant targeting to the cell membrane and reduced glutamate transport.⁶² A further component of the glutamate neurotransmitter system has been found to be altered in ALS/MND. Kawahara and colleagues reported that the editing of the GluR2 AMPA receptor subunit appeared defective in motor neurones isolated from ALS spinal cord.⁶³ The normal RNA editing process of this receptor subunit changes a glutamine to an arginine residue in almost 100% of transcripts, with the important functional consequence that the encoded AMPA receptor channel is impermeable to calcium.

Homozygous deletions of the survival motor neurone gene (SMN1) located on chromosome 5 cause autosomal recessive proximal spinal muscular atrophy, usually of childhood onset. A second adjacent gene, SMN2, has five nucleotide differences between intron 6 and exon 8 which distinguish it from SMN1. One of these polymorphic variants causes frequent skipping of exon 7, which in turn results in low expression of full length SMN2 protein. Two early studies failed to provide evidence for homozygous deletion of SMN1 in sporadic ALS.^{64–65} Several studies have been conducted to determine whether alterations of SMN1 or SMN2 are associated as genetic risk factors for ALS. The results are conflicting. Veldink and coworkers found that SMN2 gene deletions were overrepresented in patients with sporadic ALS

(16% ALS patients *v* 4% of controls).⁶⁶ Corcia *et al* reported no difference in SMN2 copy number, but that patients with ALS were significantly more likely to have an abnormal copy number of SMN1 (one or three copies).⁶⁷ Further work is necessary to determine whether alterations in SMN copy number are true risk factors for the development of ALS.

Ciliary neurotrophic factor (CNTF) is a potent survival factor for motor neurones. Inactivation of the CNTF gene causes mild progressive motor neurone loss in adult mice but does not result in an ALS/MND phenotype. A splice site acceptor mutation in the CNTF gene causes a null mutant allele lacking biological activity, and 1–2% of the normal human population are homozygous for this null allele. A study of SOD1 related familial ALS/MND with a V148G mutation and marked intrafamilial variation in the clinical phenotype indicated that a homozygous null mutation in CNTF might correlate with early onset and rapid disease progression.⁶⁸ In addition, G93A SOD1 mice crossed with CNTF knockout mice developed disease at a significantly earlier age.

Vascular endothelial growth factor (VEGF) is an angiogenic factor essential for the formation of new blood vessels during embryogenesis and in many pathological conditions, and has also recently been found to have a significant role as a neurotrophic factor.⁶⁹ The expression of VEGF is normally upregulated as a response to tissue hypoxia. Lambrechts *et al* have recently shown that VEGF is a modifier of ALS/MND in both humans and mice.⁷⁰ Deletion of the hypoxia response element (HRE) from the VEGF promoter in mice unexpectedly caused a late onset motor neurone disease which resembled ALS.⁷¹ Cross breeding of these VEGF mice with G93A SOD1 transgenic mice resulted in a worsening of the phenotype with earlier onset of disease. No mutations of the HRE of VEGF have been identified in human ALS/MND.⁷⁰ In a meta-analysis of a pooled population of 1900 individuals from four different European populations, two “at risk” haplotypes of promoter (5') polymorphisms were identified which conferred an overall 1.8-fold increased risk of ALS/MND. These haplotypes were associated with lower serum VEGF levels in both control and ALS/MND populations and with reduced *in vitro* transcription of a luciferase reporter gene.⁶⁸ There was substantial heterogeneity in terms of the risk of ALS/MND associated with VEGF promoter polymorphisms between the different geographical groups of patients included in this study, and some studies with smaller numbers of patients and controls have shown that these polymorphisms do not confer an increased risk of ALS/MND.^{72–73} Thus further exploration of the role of VEGF in the pathogenesis of human ALS/MND is required.

OXIDATIVE STRESS

The effects of oxidative stress within non-dividing cells such as neurones may be cumulative, and cellular injury by free radical species is a major potential cause of the age related deterioration in neuronal function that occurs in

Table 3 Potential genetic risk factors in sporadic ALS/MND and genetic modifiers in familial ALS/MND

Gene	Chromosome	Genetic variant	ALS association
Neurofilament heavy (NEFH)	22q12.1–q13.1	KSP deletion/insertion	Sporadic
Apolipoprotein E (Apo E $\Sigma 4$)	19q13.2	$\Sigma 4$ genotype	Sporadic
Cytochrome c oxidase subunit 1	Mt	Microdeletion	Sporadic
Excitatory amino acid transporter 2 (EAAT ₂)	11p13–p12	Decreased expression	Familial/sporadic
AMPA receptor subunit (GluR2)	4q32–q33	Altered RNA editing	Sporadic
? Survival motor neurone 1 (SMN1)	5q12.2–q13.3	Copy number	Sporadic
? Survival motor neurone 2 (SMN2)	5q12.2–q13.3	Copy number	Sporadic
Ciliary neurotrophic factor (CNTF)	11q 12.2	Null allele	Familial
Vascular endothelial growth factor (VEGF)	6p12	Promotor polymorphisms	Sporadic

ALS, amyotrophic lateral sclerosis; MND, motor neurone disease.

neurodegenerative diseases. There has been particular interest in the role of oxidative stress in ALS/MND, given that mutations in SOD1—which encodes a key cellular antioxidant defence protein—underlie around 20% of familial ALS/MND cases. The close clinical and pathological similarity between sporadic and SOD1 related familial subtypes of MND suggest that common pathophysiological mechanisms may be operating. Studies of CSF and human postmortem CNS tissue have shown the presence of biochemical changes which represent the effects of free radical damage or abnormal free radical metabolism, and these changes are more pronounced in ALS/MND cases than in controls.^{74–77} Fibroblasts cultured from the skin of patients with both familial and sporadic MND show increased sensitivity to oxidative insults compared with those from control cases.⁷⁸

In relation to the toxic gain of function of the mutant SOD1 protein, oxidative damage or metal mishandling, or both, have been strongly implicated. The main hypotheses have been that mutations alter the structure of the SOD1 protein, allowing greater access of abnormal substrates to the active copper site of the dimeric enzyme, resulting in the production of damaging free radical species including peroxynitrite and hydroxyl radicals. Nitration of tyrosine residues on cellular proteins by peroxynitrite can have damaging consequences.⁷⁹ Some mutations in SOD1 render the protein more likely to form a zinc deficient variant,^{80–81} which in turn makes the copper site more accessible to abnormal substrates. In vitro studies have shown that zinc deficient SOD1 causes peroxynitrite dependent cell death.⁸¹ However, several experiments have raised questions as to whether the toxicity of mutant SOD1 can be explained by copper dependent oxidative mechanisms. Thus SOD1 that has been engineered not to bind copper by mutating the four histidine residues for copper binding still causes ALS/MND in transgenic mice.⁸² Also, knock out of the gene encoding the copper chaperone protein normally required for insertion of copper into SOD1 has no effect on the disease phenotype in SOD1 transgenic mice.⁸³ Finally, reduction in nitric oxide (NO) synthesis by pharmacological inhibition of neuronal nitric oxide synthase (nNOS) or genetic manipulation of nNOS would be expected to ameliorate the disease phenotype in mutant SOD1 transgenic mice if peroxynitrite is indeed a key contributor to motor neurone injury. However, these interventions have not been shown to have a significant effect on the murine disease,^{84–85} nor did deletion of inducible NOS, which is normally expressed within astrocytes and microglia.⁸⁶

EXCITOTOXICITY

Glutamate is the major excitatory transmitter in the human CNS, and tremendous complexity has been uncovered in the molecular structure of the repertoire of receptors for this neurotransmitter system. Excitotoxicity is the term coined for neuronal injury induced by excessive stimulation of glutamate receptors, by mechanisms which include derangement of intracellular calcium homeostasis and excessive free radical production. Motor neurones are particularly susceptible to toxicity through activation of cell surface AMPA receptors.⁸⁷ A body of evidence, which is still circumstantial, has implicated glutamatergic toxicity as a contributory factor to motor neurone injury (reviewed by Heath and Shaw⁸⁸). The key findings are that the expression and function of the major glial glutamate reuptake transporter protein EAAT₂ may be impaired in the CNS of MND patients and that CSF (and therefore CNS extracellular fluid) levels of glutamate appear to be abnormally raised at least in a proportion of MND patients.^{56–58–89–90} The balance of evidence does not favour RNA mis-splicing as the cause of reduced EAAT₂ expression as discussed above.

Excitotoxicity has provided one of the few examples of a mechanistic link between mutant SOD1 mediated MND and the sporadic form of the disease. The presence of mutant SOD1 increases the sensitivity of motor neurones to glutamate toxicity,^{27–91} causes alteration in AMPA receptor subunit expression,⁹² and causes reduced expression of the major glutamate reuptake transporter EAAT₂.^{24–93}

Whether as a primary or a propagating process, it appears that glutamate toxicity plays a contributory role to the injury of motor neurones in ALS/MND. This is supported by the finding that anti-glutamate treatment with riluzole has some effect, albeit modest, in prolonging survival in human ALS patients and in mutant SOD1 mouse models.^{94–95}

MITOCHONDRIAL DYSFUNCTION

Important properties of mitochondria include the generation of intracellular ATP, the buffering of intracellular calcium, the generation of intracellular free radicals, and involvement in the initiation of apoptotic cell death. Age related deterioration in mitochondrial function is considered a potentially important factor contributing to late onset neurodegenerative diseases.

There is a body of evidence emerging from investigation of human material and cellular and animal models indicating that mitochondrial dysfunction may contribute to motor neurone injury in ALS/MND, and this has been reviewed.^{96–97} The key evidence for mitochondrial dysfunction in human ALS/MND includes the following:

- alteration in the morphology of mitochondria in hepatocytes, muscle, and motor neurones;
- increased mitochondrial volume and calcium levels within motor axon terminals in muscle biopsies from sporadic ALS/MND cases⁹⁸;
- reduced complex IV activity in spinal motor neurones in sporadic ALS⁹⁹;
- high frequency of mitochondrial DNA mutations in motor cortex tissue in sporadic ALS¹⁰⁰;
- multiple mutations and decreased mitochondrial DNA in muscle and spinal cord in sporadic ALS¹⁰¹;
- ALS-like phenotype in one patient with a deletion in the cytochrome oxidase c subunit/gene.⁵⁵

Further evidence for the role of mitochondrial dysfunction as a contributory factor to motor neurone injury has come from the examination of cellular models of SOD1 related ALS/MND. Expression of mutant (G93A) SOD1 in the NSC34 motor neurone cell line results in the development of morphologically swollen mitochondria, impaired activity of complexes II and IV of the mitochondrial respiratory chain, impaired cellular bioenergetic status, and alteration in the mitochondrial proteome.^{102–103} Takeuchi and colleagues showed that molecular targeting of mutant SOD1 to the mitochondria but not to the nucleus or endoplasmic reticulum leads to activation of the apoptosis cascade and cell death.¹⁰⁴

Mitochondrial dysfunction has also been studied in mutant SOD1 transgenic mice. At least in some strains (for example, G93A) mitochondrial vacuolation within motor neurones is an early feature of the pathology.²⁰ Whereas SOD1 was previously considered to be an exclusively cytosolic protein, it is now recognised also to reside in the intermembrane space of mitochondria.¹⁰⁵ SOD1 has been shown to accumulate in vacuolated mitochondria in mutant SOD1 mice.¹⁰⁶ It has been shown that the activities of several complexes of the mitochondrial respiratory chain are reduced before disease onset and that these changes increase with age.¹⁰⁷ Mattiazzi and colleagues reported the presence of oxidative damage to mitochondrial protein and lipids and decreased ATP synthesis

at the onset of the murine disease.¹⁰⁸ Several groups have shown translocation of cytochrome C, an initiator of apoptosis, from the mitochondria to the cytosol during disease progression in the mouse.^{109–110} Partial deficiency of the mitochondrial form of SOD (MnSOD) exacerbates disease in transgenic SOD1 mice.¹¹¹ Recently Lui *et al* reported that mutant SOD1 is selectively and aberrantly recruited to the cytoplasmic face of mitochondria in spinal cord tissue from mutant SOD1 transgenic mice. Covalently damaged adducts of mutant SOD accumulated on the cytoplasmic face of mitochondria in the spinal cord.¹¹² This tissue specific recruitment raises the possibility that mitochondrial abnormalities may be involved in the initiation of motor neurone injury. Pasinelli *et al* also recently showed that the anti-apoptotic protein Bcl2 may be entrapped within large protein aggregates of SOD1 in spinal cord tissue, which may result in reduction of the availability of this protein to regulate apoptosis.¹¹³

Therapeutic effects of compounds which modulate mitochondrial function have begun to be investigated in SOD1 transgenic mouse models. Creatine buffers energy levels within the cell, maintains ATP levels, and stabilises mitochondrial creatine kinase, which inhibits opening of the mitochondrial permeability transition pore. Administration of creatine to G93A transgenic mice improved motor function and extended survival in a dose dependent manner, as well as causing a reduction in biochemical indices of oxidative damage in the spinal cord.¹¹⁴ Minocycline, a tetracycline derivative which inhibits microglial activation and blocks release of cytochrome c from mitochondria, also slows disease in mutant SOD1 mice.¹¹⁰

CYTOSKELETAL ELEMENTS AND AXONAL TRANSPORT

Neurofilament proteins form a major component of the cytoskeleton of neurones, and important functions include maintenance of cell shape and axonal calibre, as well as axonal transport. Neurofilaments are the most abundant structural proteins in large cells with long axons such as motor neurones. Neurofilament subunits are assembled in the motor neurone cell body, and transported down the axon by slow axonal transport, with progressive phosphorylation during movement down the axon.

Neurofilament proteins are potential subcellular targets for injury in ALS/MND and other forms of motor neurone degeneration. Accumulation and abnormal assembly of neurofilaments are common pathological hallmarks of ALS/MND. Ubiquitinated inclusions with compact or Lewy body-like morphology within surviving motor neurones in ALS/MND may show immunoreactivity for neurofilament epitopes. In some cases of SOD1 related ALS, large argyrophilic hyaline conglomerate inclusions expressing both phosphorylated and non-phosphorylated neurofilament epitopes have been observed in the cell bodies and axons of motor neurones.⁴ The importance of neurofilaments in the normal functioning of motor neurones is demonstrated by the finding that approximately 1% of sporadic ALS/MND cases have deletions of insertions in the KSP repeat region of the neurofilament heavy (NFH) gene.^{51–52} In addition, pathological changes within motor neurones develop in mice over-expressing NF-light or NF-heavy subunits, or in mice expressing mutations in the NF-light gene.^{115–117} Transgenic mice which carry mutations in SOD1 also show alterations in neurofilament organisation, with the development of neurofilament spheroids, as well as reduced neurofilament protein and decreased transport rate in the ventral root axons.^{118–119} Genetic manipulations to alter the expression of neurofilament proteins have been shown to alter the disease course in SOD1 transgenic mice. Increased expression of NF-heavy,

which traps most neurofilaments within the cell body, robustly improves the disease course—by as much as six months in mutant SOD1 mice.¹²⁰ The reasons for this somewhat counterintuitive effect are not understood, though it has been suggested that excess neurofilaments within the cell body may function as a buffer for some other deleterious process, for example offering phosphorylation sites for dysregulated intracellular kinases, or reducing the burden of axonal transport.

Another intermediate filament protein, peripherin, may play a role in motor neurone degeneration. Genetically engineered mice which overexpress the major peripherin isoform (peripherin 58) develop late onset motor neurone degeneration accompanied by disruption of neurofilament assembly.¹²¹ Another isoform, peripherin 61, is toxic when expressed in primary motor neurones and this toxic isoform is detectable in the spinal cord of sporadic ALS/MND cases.¹²² However, manipulating the level of expression of peripherin in SOD1 transgenic mice does not appear to have any effect on the disease phenotype.¹²³

Motor neurones, which in the human nervous system may have axons up to one metre in length, are highly reliant on an efficient intracellular transport system with anterograde and retrograde components. It is interesting that in SOD1 mutant mice, axonal transport is demonstrably impaired several months before clinical disease onset.¹²⁴ The kinesin complex of proteins are important molecular motors for anterograde axonal transport on the microtubule system. Mutations of genes encoding several kinesin proteins have been shown to cause various types of motor neurone degeneration including hereditary spastic paraplegia (SPG10) and type 2A Charcot-Marie-Tooth disease,^{125–126} though they have not yet been associated with ALS/MND. The dynein–dynactin complex is the important motor for retrograde transport on the microtubule system, returning components (for example, multi-vesicular bodies and neurotrophic factors) back to the cell body. Mutations in dynein and the dynactin complex, which is an activator of cytoplasmic dynein, cause progressive motor neurone disease in mice.^{44–47} As discussed in the genetics section, a dominant point mutation is the P150 subunit of dynactin, which causes a lower motor neurone disorder with vocal cord paresis in human subjects.⁴³

PROTEIN AGGREGATION

A recurring theme highlighted in research into neurodegenerative diseases has been the misfolding of mutant proteins with the formation of intracellular aggregates. There is continuing debate as to whether such aggregated proteins play a key role in disease pathogenesis, whether they represent harmless bystanders, or whether they could be beneficial to the cell by sequestering potentially toxic abnormal proteins. In the SOD1 transgenic mouse model of familial ALS, the mutant SOD1 protein forms conspicuous cytoplasmic inclusions in motor neurones and sometimes in astrocytes, which develop before the onset of motor dysfunction. Several hypotheses have been put forward to explain how mutant SOD1 aggregates could produce cellular toxicity. First, there might be sequestration of other proteins required for normal motor neurone function. Several additional proteins have been found present in SOD1 aggregates including CCS (copper chaperone for SOD1), ubiquitin neurofilaments, glial fibrillary acidic protein, two neuronal glutamate transporters, Bcl2, and proteins involved in chaperone and proteasome functions.^{113–128} Second, by repeatedly misfolding, the SOD1 aggregates may reduce the availability of chaperone proteins required for the folding and function of other essential intracellular proteins.¹²⁹ Third, the SOD1 mutant protein aggregates may reduce proteasome activity needed for normal protein turnover.^{130–131} Fourth,

there could be inhibition of the function of specific organelles (for example, mitochondria) by aggregation on or within these organelles. Overexpression of chaperone proteins can reduce mutant SOD1 aggregation and enhances the survival and function of motor neurones in culture.¹³² In addition, arimoclamol—a drug which enhances the expression of heat shock proteins—increases the life span of G93A SOD1 mice by 22%.¹³³ Clearly protein aggregates, which can be identified by ubiquitin immunostaining, are a feature of sporadic as well as familial SOD1 related ALS. SOD1 containing aggregates are not a characteristic feature of sporadic ALS, and determining the nature of the protein inclusions in sporadic ALS is a key research goal.

INFLAMMATORY CASCADES AND THE ROLE OF NON-NEURONAL CELLS

Recently there has been increasing interest in the possibility that non-neuronal cells, including activated microglia and astrocytes, may contribute to the pathogenesis or propagation of the disease process in ALS/MND. Several studies in genetically engineered mouse models have indicated that expression of mutant SOD1 in neurones alone is insufficient to cause motor neurone degeneration and that participation of non-neuronal cells may be required.^{134–135} More recently Clement and colleagues produced several sets of chimeric mice which have both normal and mutant SOD1 expressing cells.¹³⁶ Motor neurones expressing mutant SOD1 could escape disease if surrounded by a sufficient number of normal non-neuronal cells. Conversely normal motor neurones surrounded by mutant SOD1 containing non-neuronal cells developed signs of abnormality, with the development of ubiquitinated intraneuronal deposits. Thus mutant SOD1 may cause neurotoxicity indirectly by disturbing the function of non-neuronal cells, for example microglia. Microglia play a critical role as resident immunocompetent and phagocytic cells within the CNS. Activation is associated with transformation to phagocytic cells capable of releasing potentially cytotoxic molecules including reactive oxygen species, nitric oxide, proteases, and proinflammatory cytokines such as interleukin-1 β , tumour necrosis factor α (TNF α), and interleukin 6 (IL-6).¹³⁷ Given this, there is little doubt that activated microglia can inflict significant damage on neurones, but their role is complex and they are capable of stimulating neuroprotective as well as neurotoxic effects.

Proliferation of activated microglia is a prominent histological feature in the spinal ventral horn both in mutant SOD1 transgenic mice and in human ALS/MND.^{138–139} In the mice, microglial activation is present before the onset of significant motor neurone loss or motor weakness. Various inflammatory cytokines or enzymes are upregulated in the spinal cord or CSF of ALS/MND patients (IL-6, IL-1 β , cyclo-oxygenase 2 (COX2), and prostaglandin E2 (PGE2)) or, in the spinal cord of mutant SOD1 mice (IL-1 β , TNF α , COX2, PGE2).^{140–143} Microglia appear to mediate the toxicity to neurones in culture of CSF from patients with ALS/MND by releasing factors that enhance glutamate toxicity.¹⁴⁴ Minocycline, which inhibits microglial activation, ameliorates disease progression in mutant SOD1 mice.^{110–145}

There is a tendency in ALS/MND for the disease to start focally and to spread “like a bush fire” to contiguous groups of motor neurones.¹⁴⁶ It would be very relevant to identify molecules that contribute to this propagation and those released from activated microglia would clearly be plausible candidates.

APOPTOSIS

Apoptosis describes the controlled removal of cells by an energy dependent cell death programme. Key molecular players contributing to the control of apoptosis include: the

caspase family of proteolytic enzymes which orchestrate cell destruction by destroying several intracellular targets including structural and regulatory proteins; the Bcl2 family of oncoproteins, where the balance and subcellular distribution between pro- and anti-apoptotic members is important in regulating cell survival or destruction; and the apoptosis inhibitor family of proteins which suppress apoptosis by preventing proteolytic activation of specific caspases. Three main pathways triggering caspase activation have been identified including: release of proapoptotic factors (for example cytochrome c) from mitochondria; activation of cell surface ligand receptor systems of the tumour necrosis factor family including Fas-Fas ligand, with subsequent recruitment of cytosolic adaptor proteins; and stress to the endoplasmic reticulum with activation of caspase 12.

The evidence that motor neurones may die in ALS/MND by a programmed cell death pathway has been reviewed.^{147–148} Key evidence from investigation of human necropsy material includes the following:

- evidence of structural morphology of degenerating motor neurones compatible with the apoptosis as well as internucleosomal DNA fragmentation detected by TUNEL staining¹⁴⁹;
- increased expression of specific apoptosis related molecules, for example Le^y antigen and prostate apoptosis response-4 protein in spinal cord;
- alteration in the balance of expression and subcellular compartmental localisation of pro- and anti-apoptotic members of the Bcl2 family in a direction favouring apoptosis^{149–150};
- significant increases in the activities of caspases 1 and 3 in the spinal cord.¹⁴⁹

Study of cellular models of SOD1 related ALS/MND has indicated that motor neuronal cells expressing mutant SOD1 are more likely to die by apoptosis when oxidatively stressed.²⁵ In addition, under unstressed basal culture conditions, these mutant SOD1 containing cells appear to be “primed” for apoptosis by expressing increased amounts compared with control cells of phosphatidyl serine on the cell surface and increased cleavage/activation of the initiator caspase 9.¹⁵¹ In the mutant SOD1 mouse model, there is evidence of DNA laddering, increased expression and activation of caspase 1 and caspase 3 in the spinal cord of symptomatic mice, and alterations in the balance of key members of the Bcl2 protein family in a direction favouring apoptosis.^{152–153} Cross breeding experiments between G93A SOD1 transgenic mice and mice genetically engineered to overexpress antiapoptotic molecules results in amelioration of the murine disease. The administration of caspase inhibitors has a partial neuroprotective effect in cellular models,¹⁵¹ and intraventricular administration of a broad spectrum caspase inhibitor to mutant SOD1 mice prolongs life span by approximately 20%.¹⁵²

CELL SPECIFIC FEATURES OF MOTOR NEURONES WHICH MAY PREDISPOSE TO NEURODEGENERATION

One of the unsolved enigmas in neurodegenerative diseases in general, and in motor neurone degeneration in particular, is the selective vulnerability of certain neuronal groups to the neurodegenerative process. This vulnerability is relative rather than absolute. SOD1 is a ubiquitously distributed antioxidant defence protein, yet when the protein is mutated, it is motor neurones that are most susceptible to injury. The cell specific features of motor neurones that may predispose to age related degeneration have been reviewed^{154–155} and are outlined in table 4. Key features are likely to include the cell

Table 4 Cell specific features of motor neurones predisposing to neurodegeneration

- Cell size and axonal length
- High metabolic rate
- ? Specific features of motor neurone mitochondria
- Cytoskeleton, neurofilament content and reliance on efficient intracellular transport system
- Characteristic profile of cell surface glutamate receptors, with high relative expression of calcium permeable AMPA receptors, lacking the GluR2 subunit
- High expression of glutamate transporters in the vicinity of vulnerable motor neurone groups
- Low expression of specific calcium binding proteins
- High expression of SOD1 protein
- High threshold for mounting a heat shock response/upregulation of chaperone proteins

size of motor neurones, which has consequences for intracellular transport, energy metabolism, and neurofilament content. The neurones vulnerable to degeneration in ALS have a particular sensitivity to glutamatergic toxicity through AMPA receptor activation and differ from most other neuronal groups in expressing a high preponderance of calcium permeable AMPA receptors, which lack the GluR2 subunit.¹⁵⁶ Motor neurones also have a relative lack of expression of calcium buffering proteins¹⁵⁷ and appear to have a high threshold for mounting a protective heat shock response.¹⁵⁸ Recent studies suggest that the properties of mitochondria from the spinal cord may differ from those of mitochondria from other tissues.^{112–159}

CONCLUSIONS

The process of motor neurone degeneration in ALS/MND is complex and multifactorial. Several genetic alterations can set the scene for motor neurone injury in familial ALS, but much remains to be learned about the genetic and environmental factors predisposing to the commoner sporadic form of the disease. Most has been learned about the mechanisms of motor neurone degeneration in the subtype of disease caused by SOD1 mutations, but even here there appears to be a complex interplay between multiple pathogenic processes including oxidative stress, protein aggregation, mitochondrial dysfunction excitotoxicity, and impaired axonal transport. New evidence is emerging that non-neuronal cells in the vicinity of motor neurones may contribute to neuronal injury. Evidence has accumulated that the final demise of motor neurones is likely to occur by a programmed cell death pathway resembling apoptosis.

To date only the antilutamate agent riluzole has been shown reproducibly to prolong the survival of patients with ALS/MND, and this is a modest effect. In the recent past, robust cellular and animal models of motor neurone degeneration have emerged which are being used to evaluate new potential therapeutic strategies. New technologies including gene expression profiling using microarray platforms,^{160–161} analysis of the repertoire of cellular proteins using proteomic approaches,^{103–130} and the ability to sub-dissect motor neurones from complex tissues using laser capture microdissection¹⁶² are likely to lead to clarification of our knowledge of the cellular mechanisms of disease in ALS/MND over the next few years. Important priorities for future research include the search for other genes associated with familial MND and for genetic and environmental factors predisposing to the sporadic form of the disease. In addition, further probing for insights into the cell specific biochemistry and physiology of motor neurones and the cellular pathways deranged during motor neurone degeneration are likely to lead to the development of more effective neuroprotective treatments for patients. The space constraints of this article have not permitted a detailed discussion of potential therapies targeted to the outlined molecular mechanisms of motor neurone injury. This topic has recently been reviewed

in relation both to experimental models and to human MND.^{16–163} Future treatment of ALS/MND is likely to involve a cocktail of neuroprotective compounds akin to chemotherapeutic combinations for malignant disease, which interfere with several molecular pathways that lead to neuronal injury.

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