Mechanisms of neurodegeneration in amyotrophic lateral sclerosis

S Cluskey, D B Ramsden

Abstract
Amyotrophic lateral sclerosis (ALS) is the most common variant of motor neurone disease affecting adults that usually strikes during mid to late life. Its aetiology is still poorly understood, although a major breakthrough came with the discovery that mutations in the Cu/Zn superoxide dismutase (SOD1) gene affect approximately 20% of patients with familial ALS. Experiments using both transgenic mice and ALS tissues have been useful in delineating other genetic defects in ALS. However, because only a subset of cases can be attributed to one particular molecular defect (such as mutation of SOD1 or the gene encoding neurofilament H), the aetiology of ALS is likely to be multifactorial. This review discusses the major mechanisms of neurodegeneration in ALS, such as oxidative stress, glutamnergic excitotoxicity, damage to vital organelles, and aberrant protein aggregation.

(J Clin Pathol: Mol Pathol 2001;54:386–392)

Keywords: amyotrophic lateral sclerosis; motor neurone disease; neurodegeneration

Amyotrophic lateral sclerosis (ALS) is the most common variant of motor neurone disease affecting adults (incidence between 1 and 5/100 000 of the general population, with a slight male preponderance) that typically strikes during mid to late life. The aetiology of this lethal condition, for which no cure has been developed, is poorly understood.

ALS is characterised by degeneration of the large pyramidal neurones in the motor cortex and associated corticospinal tracts. Lower motor neurones originating in the brainstem nuclei and spinal cord anterior horn are also affected. However, oculomotor nuclei in the brainstem and Oun’s nucleus in the spinal cord remain relatively intact. The clinical manifestations of ALS reflect the involvement of both upper and lower motor neurones. Muscle biopsy reveals denervation atrophy. Weakness and muscle atrophy are a consequence of large α motor neurone degeneration in the brainstem and spinal cord. Spasticity, hyperreflexia, and extensor plantar reflexes are symptomatic of upper motor neurone involvement. Dementia is reported to occur in 3–5% of patients with ALS and subtle cognitive impairment may affect up to 25% of all cases.

The neuropathology may be summarised as follows:

• Neurofilamentous swelling of proximal axons.
• Neurofilament (NF) and peripherin accumulations in axons and neuronal cell bodies.
• Perikaryal inclusions with phosphorylated NF and ubiquitin immunoreactivity, and in some familial cases, Cu/Zn superoxide dismutase (SOD1) immunoreactivity.
• Lewy body-like cytoplasmic neuronal inclusions.
• Fragmentation of the Golgi apparatus.
• Reduced calibre of distal axons.
• Axonal wallerian degeneration.
• Attenuation of dendrites.

Mutations of the SOD1 gene
Between 5% and 10% of ALS is autosomally dominantly inherited, with almost complete penetrance. The major breakthrough in beginning to understand the molecular pathology of the disease has been the identification of SOD1 gene mutations in a significant minority of familial ALS (fALS), recently reviewed by Gaudette et al. Here, what are considered to be the major mechanisms of neurodegeneration in ALS are discussed, emphasising possible factors underlying the selective vulnerability of motor neurones to neurodegeneration.

Mutations in the SOD1 gene affect approximately 20% of patients with fALS. SOD1 is a copper and zinc dependent, cytoplasmic enzyme, existing as a homodimer. It accounts for approximately 1% of total brain protein. The structural gene is located on chromosome 21q22.1. To date, over 70 mutations (almost all single base changes) spanning all five coding exons of the SOD1 gene have been identified. Approximately 35% of these are the result of either G to A or A to G mutations on the sense strand, and a further 10% can be interpreted as arising from similar mutations on the antisense strand and consequent modification (C to T and T to C) of the sense strand by DNA repair enzymes. The age of onset and duration of disease are relatively constant for a particular mutation, whereas the clinical presentation may be highly variable, both between and within fALS kindreds.

The cause of fALS was first thought to be reduced enzyme dismutase activity as a consequence of the SOD1 gene mutations. However, the picture is more complex, and the evidence that has emerged from studies of more human kindreds and transgenic mice expressing mutant SOD1 is not wholly compatible with such a mechanism. Several mutations identified in human ALS leave SOD1 dismutase activity intact; others may enhance enzyme catalytic function. SOD1 knockout mice do not develop spontaneous motor neurone disease, whereas transgenic mice expressing mutant SOD1 develop paralysis,
Mechanisms of neurodegeneration in ALS

**Examples of mutations**

**Hu Gly 93→Ala (12)**  
**Hu Gly 37→Arg (13)**  
**Hu Gly 85→Arg (14)**  
**Ms Gly 86→Arg (15)**

**Neuropathological features**

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<th>Axonal changes</th>
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<th>Reduced axonal calibre</th>
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Despite possessing two normal mouse SOD1 alleles (fig 1).5 The offspring of transgenic mice expressing human G85R SOD1 and either SOD1 knockout mice or mice expressing wild-type human SOD1 have no wild-type SOD1 expression or raised concentrations of the enzyme, respectively, in addition to mutant enzyme, yet in neither case was the progression of disease influenced.5 Dimersation of mutant G41D or G85R SOD1 subunits, which have shorter half lives and reduced dismutase activity, with wild-type monomer did not affect wild-type subunit function.5 Thus, these data, and the fact that most patients with fALS and SOD1 mutations are heterozygotes, and so have one normal allele, suggest that the disease results from the gain of some cytotoxic function, rather than simply loss of dismutase activity. As a consequence, the search for the gain of function led to the investigation of pathways being explored in other neurodegenerative diseases—with oxidative damage, excitotoxicity, and impaired mitochondrial function being prime examples. However, the first mechanism to be considered is the result more of a loss of function (reduced ability to bind copper) than a gain of function.

**Reduced ability to bind to copper**

The cupric ion (Cu²⁺), when not complexed with protein, is cytotoxic, and organisms have evolved mechanisms that chaperone it until it can be incorporated into enzymes to take advantage of its electronic properties. One possible mechanism of neurotoxicity in ALS would be a reduced ability of mutant SOD1 to bind the ion, allowing either inappropriately high intracellular concentrations of free copper or excessive uptake on to other binding sites or on to sites that do not normally bind the metal. To explore this hypothesis, the effect of copper chelators on neuronal growth in the presence of mutant SOD1 was explored. Copper chelation was shown to improve spinal motor neuron survival in G93A SOD1 transgenic mice by more than 200% and to promote neurite outgrowth compared with that seen in untreated animals.20 21 Whether chelation is inhibiting the participation of free copper in deleterious oxidation reactions not directly involving SOD1, or via other pathways, is uncertain, but this seems a promising avenue for further research.

**Oxidative damage**

Mutant SOD1 may catalyse aberrant biochemical reactions, resulting in the production of potentially damaging reactive oxygen species (ROS), such as the superoxide anion (O₂⁻), the hydroxyl radical (OH•), hydrogen peroxide (H₂O₂), and peroxynitrite (ONOO⁻). Enhanced peroxidase activity of mutant SOD1 (generation of OH• from H₂O₂) has been reported in an in vitro system,22 although increased production of OH• radicals has not been detected consistently in the spinal cord and brain of mutant SOD1 transgenic mice.23 24 Peroxynitrite is generated non-enzymatically by the reaction between O₂⁻ and nitric oxide (NO) at a higher rate than that of superoxide dismutation catalysed by SOD1. NO is generated by calcium dependent nitric oxide synthase (NOS): its activity may be upregulated in conditions where intracellular calcium is raised, such as during glutamate excitotoxicity. SOD1 catalyses the reaction between ONOO⁻ and protein tyrosine residues, a reaction that is enhanced by the depletion of zinc from SOD1. Crow et al reported reduced affinity of mutant SOD1 for zinc and enhanced conversion of ONOO⁻ into nitronium ions compared with wild-type SOD1.25 The neurofilament light subunit has been implicated as a potential target of aberrant nitrosylation or as a high affinity binding partner for zinc ions that may deplete the SOD1 enzyme.26 Increased concentrations of 3-nitrotyrosine have been detected in the spinal cord, motor cortex, and cerebrospinal fluid (CSF) of patients with sporadic ALS and fALS.26 27 Nitrotyrosine may mimic phosphotyrosine and interfere with normal protein phosphorylation and phosphorylation mediated signalling. However, it should be noted that the 3-nitrotyrosine detected was not protein bound (free), raising doubts about its importance.25 26 These findings raise the question of the origin of the free nitrotyrosine in CSF. Is it formed directly from the free amino acid or from nitrated tyrosyl residues in proteins, such as low molecular weight neurofilament,28 that may be more rapidly catabolised because of induced abnormalities in structure? The latter could impose additional energy demands on the cell to maintain protein concentrations at normal steady state values, and both causes make nitrotyrosine available to exert its own effects. Increased concentrations
of neuronal and inducible NOS in specific disease related subregions of spinal cord from patients with ALS can be detected both immunologically and radiographically using tritiated L-arginine. The distribution of nitrotyrosine in brain tissue of both patients with ALS and transgenic animals expressing SOD1 mutants is becoming more clearly defined and this is present in cells that undergo degeneration in ALS. Most recently, the expression of NOS isoenzymes in reactive astrocytes emphasised the role of glial cells in the degenerative process. One important piece of evidence against a pathological role of ONOO− production is that G93A SOD1 mice on a null neuronal NOS background (which would presumably reduce the availability of NO to mutant SOD1) did not live significantly longer that transgenic mice expressing neuronal NOS. Nevertheless, these authors did report increased survival of transgenic animals treated with specific neuronal NOS inhibitor. Whether NOS inhibition will prove to be beneficial in the human condition remains to be determined.

Excitotoxicity

The detection of raised glutamate concentrations in the CSF of some patients with sporadic ALS was taken to imply that glutamate excitotoxicity might contribute to neurodegeneration in ALS. Glutamate receptor overactivity results in prolonged opening of associated ion channels, permitting sodium and water to enter postsynaptic cells. The resulting membrane depolarisation opens the N-methyl-D-aspartate (NMDA) receptor linked Na+/Ca++ channel, facilitating calcium influx, which initiates a cascade of events eventually causing cell death. Glutamate activity at the synaptic cleft is regulated by receptor inactivation and sodium/potassium coupled glutamate reuptake by transporter proteins (excitatory amino acid transporters; EAATs) in neurones and astrocytes. Glutamate transporter knockout mice have raised glutamate concentrations in the synaptic cleft and suffer seizures and impaired motor coordination, [39] illustrating the point that a reduction in transport could lead to motor neurone degeneration. Reduced glutamate concentrations in spinal cord tissue homogenates could be interpreted as the result of the reduction in intracellular glutamate, as a consequence of impaired glutamate uptake. [40] Reductions of the EAAT2 protein in the order of 30–90% have been described in most sporadic ALS postmortem specimens compared with controls. [41] Transporter loss did not extend to other transporter subtypes and was confined to the motor cortex and spinal cord in most cases. However, in the early stage of anterior horn cell dysfunction, both EAAT1 and EAAT2 expression is increased in the astrocytic foot attached to degenerating cells. [42] The specific loss of EAAT2 could not be attributed simply to cell death, because no significant astroglial loss was observed. [43] Furthermore, antisense knockdown of EAAT2 protein synthesis caused neuronal degeneration and was sufficient to induce a motor neurone disease phenotype in mice. [44] The absence of EAAT2 protein expression in ALS material was not accompanied, by a corresponding reduction in EAAT2 mRNA, and no mutations within the coding regions or in the vicinity of splice site sequences of the EAAT2 gene were found, [45] opening up the possibility that abnormalities of translation or posttranslational processing are causal. [46] In vitro expression studies indicated that the proteins translated from such mRNA were unstable and would undergo rapid degradation, a scenario that is consistent with the failure to detect EAAT2 protein. Although certain aberrant mRNA species have also been detected in normal brain, they were claimed to be far more abundant in ALS and usually only detected in the affected areas. Lin et al detected multiple aberrant mRNA species arising from exon skipping and intron retention in sporadic ALS brain specimens and in the CSF of living patients. [47] However, mutations causing abnormal EAAT2 properties, such as those described by Trotti et al, [48] are relatively infrequent, [49] and the point mutations that do occur are found with similar frequencies in patients and controls. Furthermore, other reports indicated that splice variants were not associated with ALS and that other reasons for increased glutamatergic activity must be found. [50]

The peripheral motor neurone system is densely populated with NMDA and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) glutamate receptors. The AMPA subtype of the glutamate receptor, which is responsible for most fast excitatory transmission in the mammalian central nervous system (CNS), comprises four subunits, termed GluR1 to GluR4. Most AMPA receptors in the CNS incorporate the GluR2 subunit, which renders them relatively impermeable to calcium. In contrast, the low or absent expression of the GluR2 subunit in AMPA receptors in motor neurones may make these receptors likely to permit calcium movement, thus rendering the neurone more susceptible to excitotoxic damage. It has been shown in a mouse model that normally non-toxic glutamate concentrations could be a major factor determining the selective vulnerability of motor neurones to excitotoxic injury via calcium permeable AMPA receptors. This may be linked to the higher expression of the EAAT2 transporter in astroglial cells in the vicinity of motor neurones that are affected in ALS compared with groups of motor neurones that are characteristically spared, including Onuf’s nucleus in the sacral spinal cord and the neurones that control eye movement. Furthermore, calcium binding proteins, which might buffer neurones against an increase in intracellular calcium allied to excitotoxicity, are expressed in neurones spared in ALS but absent in motor neurones that are typically affected. Oxidative damage to the EAAT2 glutamate transporter could feasibly lead to the accumulation of glutamate in the extracellular...
compartment and excitotoxic neuronal damage, thus linking oxidative damage with excitotoxicity. Cell culture systems also implicate ROS, including peroxynitrite, in impaired glutamate transporter activity, and loss of the EAAT2 transporter has been detected in the spinal cord of the mutant SOD1 transgenic mice in which raised ROS production has been documented. The fact that riluzole, a drug that inhibits glutamatergic transmission, impedes disease progression in humans strongly suggests a crucial role of excitotoxicity in ALS pathology. However, the failure of similar antiglutamatergic agents, including gabapentin, to be effective in human ALS suggests that riluzole may have a novel mode of action, or that other mechanisms of neurodegeneration are important in ALS. Furthermore, the absence of evidence specifically linking EAAT2 genetic polymorphism with ALS suggests that this protein may not be the key link between the two processes.

**Impaired mitochondrial function**

Mitochondria are the principal source of chemical energy within a cell. Mitochondrial degeneration, manifested by mitochondrial vacuolisation, has been reported in sporadic ALS motor neurones and is an early pathological feature in mutant SOD1 transgenic mice, preceding the onset of motor weakness. Mitochondrial function is known to decline with increasing age, which is the most robust risk factor for neurodegenerative diseases. Serial measurements of 8-hydroxy-2'-deoxyguanosine have provided direct evidence of an age dependent increase in oxidative damage to mitochondrial DNA. Abnormalities of the mitochondrial electron transfer chain, in addition to reduced or damaged mitochondrial DNA, have been detected in muscle and liver biopsies from patients with sporadic ALS. Reduced concentrations of manganese dependent SOD (another member of the SOD enzyme family located on mitochondrial membranes) have been detected in the muscle of patients with sporadic ALS. This enzyme is essential for ROS detoxification, and reduced concentrations would tend to exacerbate oxidative stress. Impaired mitochondrial function may lead to a cellular energy deficit. Indeed, neuronal metabolism is particularly vulnerable to acute disturbances of aerobically generated chemical energy, as exemplified by poor tolerance of ischaemia. It has been predicted that the large size of motor neurones (axonal processes in human motor neurones can approach 1 m in length) relative to other cell types and most other neurones, and consequently their high energy demands, may cause them to be particularly vulnerable to the adverse effects of mitochondrial impairment. Metabolically compromised neurones may be unable to maintain membrane potential, resulting in opening of voltage dependent NMDA subtype glutamate receptors and calcium influx. Therefore, a much lower concentration of extracellular glutamate may initiate the cascade of events leading to cell death. Treatment of mutant SOD1 transgenic mice with creatine, which buffers cellular energy values, has been shown to be partially neuroprotective; this is consistent with the hypothesis that neuronal energy depletion is pathologically relevant in ALS. Conversely, the failure to detect lactate peaks that might indicate anaerobic metabolism in ALS brain questions whether there is an energy deficit in ALS neurones because lactate peaks are readily detected by proton spectroscopy in Huntingdon’s chorea, a neurodegenerative disease in which cellular energy depletion is of pathological relevance.

**Aberrant protein aggregation**

Cytoplasmic inclusions in the proximal axon and cell body of degenerating motor neurones are a pathological hallmark of ALS and are also evident in mutant SOD1 transgenic mice. Several components have been identified including disorganised aggregates of neurofilament proteins. However, it is not understood how neurofilament aggregates develop and whether they are the cause or consequence of neuronal dysfunction. No gross developmental defects or clinical signs of motor neuron disease are apparent in knockout mice for any of the three neurofilament subunit genes, although in mice knocked out for NF-L or both NF-M and NF-L, losses of up to 50% of motor neurones have been detected. Expression of the NF-L subunit appears to be crucial for normal NF assembly. In NF-L knockout mice, NF-M and NF-H subunits were unable to assemble into organised 10 nm filaments and consequently aggregated in the perikarya. In situ hybridisation studies have revealed an approximately 60% reduction in NF-L mRNA in pathologically affected motor neurones of patients with ALS. Overexpression of any single NF subunit in transgenic mice also provoked the formation of perikaryal accumulations. This could be rescued by similarly increasing the expression of the other subunits, underlying the importance of subunit stoichiometry for NF assembly and transport.

SOD1 transgenic mice have been crossed with transgenic mice expressing various NF subunits and NF knockout mice as a means of investigating the possible neurotoxic role of NF in SOD1 associated disease. LacZ mice express an NF-H–β-galactosidase fusion protein. In this model, NFs are not exported to the axonal compartment and large perikaryal swellings develop in motor neurones. When crossed with G37R mutant SOD1 mice, expression of this dysfunctional NF did not influence the onset or progression of disease, suggesting that an intact NF network is not required for SOD1 mediated disease. In contrast, G37R mice crossed with transgenic mice overexpressing the human NF-H subunit led to a 65% increase in the mean lifespan of these mice. Crossing with NF-L null mice also extended the lifespan of G85R mice by approximately 15%. Therefore, the overexpression of NF-H or lack of NF-L, which tends to result in perikaryal NF accumulations, appears to be well tolerated by neurones and indeed is even neuroprotective, even though this is inconsistent with the observation that expression of a NF-H...
fusion protein did not extend the survival of SOD1 transgenic mice. It should be noted that neuroprotection was only apparent in mice with moderate expression of mutant SOD1 and not in line with a high transgene copy number. The apparent neuroprotective capacity of perikaryal NF accumulation is not clearly understood. NFs possess multiple calcium binding sites; thus, NF accumulations may protect against neuronal injury resulting from increased intracellular calcium. This concept is supported by the observation that forced overexpression of a calcium binding protein, calbindin D, conferred protection against SOD1 mutant mediated death of transfected PC12 cells. An alternative theory is that NFs act as a sink for ROS, thereby protecting other cellular components from damage; however, others have reported that numbers of NF associated nitrosylated tyrosine residues in ALS spinal cord did not differ significantly from controls. Mutations of the NF-H gene, resulting in deletion of the NF-H subunit tail, have been found in association with a very small number of sporadic ALS cases; these are believed to be a primary event, but are of unknown importance in the aetiology of the disease. Peripherin has been detected in almost 90% of motor neurone inclusions in ALS. It is a type III intermediate filament (57 kDa) that is normally expressed in autonomic nerves and peripheral sensory neurones, with only low amounts usually detectable in spinal motor neurones. Studies of peripherin–NF interactions in the SW13 cell line (which lacks endogenous intermediate filament proteins) have shown that peripherin may self assemble to establish an intermediate filament network or heterodimerise with each of the NF subunits. However, in the absence of the NF-L subunits, the interaction between peripherin and NF-H or NF-M results in a disorganised network and protein aggregation. Overexpression of peripherin in a transgenic mouse model caused late onset and selective motor neurone death. This deleterious effect was presumed to relate to the relative deficiency of NF-L protein, which is required for successful intermediate filament organisation; indeed, neuronal death was accelerated by the depletion of NF-L. Before motor neurone loss and clinical symptoms, small inclusions containing peripherin, NF-M, NF-H, and membranous elements including mitochondria were detectable in the neurone cell body and axon. These inclusions bear greater similarity to those detected in human ALS and mutant SOD1 transgenic mice than the large perikaryal inclusions observed previously in NF-H- and NF-L over-expressing mice. Peripherin inclusions are associated with subsequent axonal degeneration and massive death of motor neurones. The precise mechanism of cell death remains to be elucidated, but may relate to blockade of intracellular transport by multiple intermediate filament inclusions in the axon or the sequestration of cellular components, including mitochondria, that are essential for cell survival. Peripherin gene expression is known to be upregulated in response to cytokine expression, including interleukin 6 (IL-6) and leukaemia inhibitory factor (LIF) via the Janus associated kinase/signal transducers and activators of transcription signalling pathway. This occurs after neuronal injury and may be a general response of neurones to noxious stress. Sekizawa et al have demonstrated raised concentrations of IL-6 in CSF in a small group of patients with ALS, and increased LIF concentrations have been reported in relation to glutamate toxicity. Inflammatory cytokine release is associated with astrogliosis, which is a common phenomenon in ALS, and coincides with the onset of clinical disease and mitochondrial vacuolisation in mutant SOD1 transgenic mice. The concept of peripherin mediated neuronal death is compatible with the fact that approximately 90% of ALS is sporadic, in that no genetic mutations are required for the development of inclusion bodies. NF subunits and/or peripherin may be subject to post-translational modifications, including phosphorylation and nitrosylation, which favour the interaction of peripherin with the higher molecular weight NF subunits, resulting in the formation of noxious NF inclusions. For example, cyclin dependent kinase 5 (CDK5) phosphorylates NF, generating an epitope that has been detected in NF accumulations in patients with ALS. CDK5 is often present in accumulations and may be associated with lipofuscin, a protein associated with oxidative stress and ageing. The inherent stability of NF protein renders it susceptible to glycation by a non-enzymatic reaction with reducing sugars, which creates advanced glycosylation end products.

Conclusion
In conclusion, there is convincing evidence from studies of human ALS and animal models of motor neurone disease of the importance of several distinct molecular mechanisms in the pathogenesis of ALS. Not all such mechanisms have been considered here. Others that may be of importance are the contributions of the various DNA repair mechanisms and the generation of somatic DNA mutations, and the role of DNA methylation in the regulation of genes vital to the functioning of motor neurones. The fact that often only a subset of cases can be attributed to one particular molecular defect— for example, genetic mutation of the Cn/Zn SOD enzyme—suggests that the aetiology of ALS is likely to be multifactorial, a complex interplay between genetic factors, oxidative stress, glutaminergic excitotoxicity, damage to vital organelles, and aberrant protein aggregation. In each case, there are feasible explanations for the apparent selective vulnerability of motor neurones that characterises this disease. When faced with a problem like ALS, which appears to be the result of numerous components, it is tempting to fall back on the concept that these either individually or in concert contribute lethally to injure motor neurones. However, if one applies Williams of Ockham’s razor—when faced with several explanations of
increasing complexity, the simplest is usually the most probable—one is forced to return to the role of SOD1. As yet, this still is the only recognized cause of fALS. Despite much effort its role in sporadic ALS has not been proved, but it is still the best lead we have. It is hoped that a greater understanding of the mechanisms of neurodegeneration will lead to the development of more effective, even curative, treatments for ALS and other neurodegenerative disorders.

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