Superoxide Dismutase 1 and Amyotrophic Lateral Sclerosis

by

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Cover illustration: Lumbar spinal cord section from an ALS patient with sclerosis of the corticospinal tract. Klüver–Barerra staining. Kindly provided by Dr. Thomas Brännström

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Things should be made as simple as possible – but not any simpler

_Albert Einstein_
ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting motor neurons in the spinal cord, brain stem and motor cortex, leading to paralysis, respiratory failure and death. In about 5% of ALS cases, the disease is associated with mutations in the CuZn-superoxide dismutase (hSOD1) gene. As a rule, ALS caused by hSOD1 mutations is inherited dominantly and the mutant hSOD1s cause ALS by the gain of a noxious property.

The present study focused on two hSOD1 mutations with widely differing characters. In Scandinavia, ALS caused by the D90A mutation is inherited in a recessive pattern. Elsewhere, families with dominant inheritance have been found. The properties of D90A mutant hSOD1 are very similar to those of the wild-type protein. The G127insTGGG (G127X) mutation causes a 21 amino acid C-terminal truncation which probably results in an unstable protein.

The aim of this thesis was to generate transgenic mice expressing D90A and G127X mutant hSOD1s and to compare these mice with each other and with mice expressing other mutant hSOD1s, in search of a common noxious property. The findings were also compared with the results from studies of human CNS tissue.

The cause of the different inheritance patterns associated with D90A mutant hSOD1 was investigated by analyzing erythrocytes from heterozygous individuals from dominant and recessive pedigrees. There was no evidence that a putative protective factor in recessive pedigrees acts by down-regulating the synthesis of D90A mutant hSOD1.

In cerebrospinal fluid, there was no difference in hSOD1 content between homozygous D90A patients, ALS patients without hSOD1 mutations and controls. hSOD1 cleaved at the N-terminal end was found in both controls and D90A patients, but the proportion was significantly larger in the latter group. This indicates a difference in degradation routes between mutant and wild-type hSOD1.

Both D90A and G127X transgenic mice develop an ALS-like phenotype. Similar to humans, the levels of D90A protein were high. The levels of G127X hSOD1 were very low in the tissues but enriched in the CNS. Similarly, in an ALS patient heterozygous for G127X hSOD1, the levels of the mutant protein were overall very low, but highest in affected CNS areas. Despite the very different levels of mutant hSOD1, both D90A and G127X transgenic mice developed similar levels of detergent-resistant aggregates in the spinal cord when terminally ill. Surprisingly, mice overexpressing wild-type hSOD1 also developed detergent-resistant aggregates, although less and later. Most of the hSOD1 in the CNS of transgenic mice was inactive due to deficient copper charging or because of reduced affinity for the metal. The stabilizing intrasubunit disulfide bond of hSOD1 was partially or completely absent in the different hSOD1s. Both these alterations could increase the propensity of mutant hSOD1s to misfold and form aggregates.

The results presented here suggest that the motor neuron degeneration caused by mutant hSOD1s may be attributable to long-term exposure to misfolded, aggregation-prone, disulfide-reduced hSOD1s and that the capacity to degrade such hSOD1s is lower in susceptible CNS areas compared with other tissues. The data also suggest that wild-type hSOD1 has the potential to participate in the pathogenesis of sporadic ALS.

Keywords: aggregates, ALS, amyotrophic lateral sclerosis, cerebrospinal fluid, disulfide-reduced, inclusions, misfolded, protective factor, SOD1, transgenic
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ABBREVIATIONS

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<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
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<tr>
<td>ALS-PDC</td>
<td>ALS Parkinson dementia complex</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>CCS</td>
<td>copper chaperone for superoxide dismutase</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>cM</td>
<td>centimorgan</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>COX</td>
<td>cytochrome C oxidase</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>CT</td>
<td>computed tomography</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>EAAT2</td>
<td>excitatory amino acid transporter 2</td>
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<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<tr>
<td>EMG</td>
<td>electromyography</td>
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<td>FALS</td>
<td>familial ALS</td>
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<td>fMRI</td>
<td>functional magnetic resonance imaging</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
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<tr>
<td>HMW</td>
<td>high molecular weight</td>
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<td>hSOD1</td>
<td>human SOD1</td>
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<td>HSP</td>
<td>heat shock protein</td>
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<td>JALS</td>
<td>juvenile ALS</td>
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<tr>
<td>kb</td>
<td>kilo base pairs</td>
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<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LBHI</td>
<td>Lewy body-like hyaline inclusion</td>
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<td>LMN</td>
<td>lower motor neuron</td>
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<td>LOD</td>
<td>log of odds</td>
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<tr>
<td>Mb</td>
<td>mega base pairs</td>
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<tr>
<td>MND</td>
<td>motor neuron disease</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>mSOD1</td>
<td>murine SOD1</td>
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<td>mutSOD1</td>
<td>mutant SOD1</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>NF-H</td>
<td>heavy neurofilament</td>
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<tr>
<td>NF-L</td>
<td>light neurofilament</td>
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<tr>
<td>NF-M</td>
<td>medium neurofilament</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBP</td>
<td>progressive bulbar palsy</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>Abbreviation</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PET</td>
<td>positron emission tomography</td>
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<td>PLS</td>
<td>primary lateral sclerosis</td>
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<td>PMA</td>
<td>progressive muscular atrophy</td>
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<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
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<tr>
<td>SALS</td>
<td>sporadic ALS</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SMA</td>
<td>spinal muscular atrophy</td>
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<tr>
<td>SOD1</td>
<td>CuZn-superoxide dismutase</td>
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<td>SPECT</td>
<td>single photon emission computed tomography</td>
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<tr>
<td>SSCP</td>
<td>single strand conformational polymorphism</td>
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<tr>
<td>UMN</td>
<td>upper motor neuron</td>
</tr>
<tr>
<td>wt-hSOD1</td>
<td>wild-type hSOD1</td>
</tr>
<tr>
<td>ww</td>
<td>wet weight</td>
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Preface

The topic of this thesis is superoxide dismutase 1 and amyotrophic lateral sclerosis. Within this framework account, I have attempted to provide an overview of past and current research in the field, ranging from the more clinical aspects of the disease to the molecular studies of wild-type and mutant superoxide dismutase 1. I have focused on some areas of great interest, such as hereditary factors, the biophysical properties of wild-type and mutant superoxide dismutase 1, aggregation of mutant superoxide dismutase 1, mitochondrial pathology, apoptosis and glutamate excitotoxicity. Other important research areas have been covered in less detail. They include the areas of autoimmunity, axonal transport, inflammation, neurotrophic factors, viral infections and recent therapeutic developments. In the results and discussion section, the results from the papers included in the thesis are summarized and briefly discussed. The papers included in the thesis are enumerated with the following Roman numerals throughout the thesis.


III. P. Andreas Jonsson, Karin S. Graffmo, Thomas Brännström, Peter Nilsson, Peter M. Andersen, Stefan L. Marklund. Motor neuron disease in mice expressing the wild type-like D90A mutant superoxide dismutase-1. In manuscript.


V. P. Andreas Jonsson, Karin S. Graffmo, Peter M. Andersen, Thomas Brännström, Mikael Lindberg, Mikael Oliveberg, Stefan L. Marklund. Disulfide-reduced superoxide dismutase-1 in CNS of transgenic amyotrophic lateral sclerosis models. Submitted.

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REVIEW OF THE LITERATURE

Amyotrophic Lateral Sclerosis – The Disease

Clinical signs and symptoms of ALS
Amyotrophic lateral sclerosis or ALS is a progressive neurodegenerative disease mainly affecting lower motor neurons in the spinal cord and brain stem and the upper motor neurons of the precentral cortex. The disease was first described in the mid-19th century by Aran, Charcot and others. The symptoms of ALS are usually restricted to the motor system and should include symptoms from both upper and lower motor neurons. The degeneration of motor neurons causes weakness and muscular wasting ultimately leading to death, often due to respiratory failure. Symptoms and signs from the lower motor neurons include weakness, atrophy, cramps, fasciculations and the suppression of reflexes, while upper motor neuron symptoms are characterized by weakness, spasticity, stiffness and brisk reflexes.

El Escorial criteria: Diagnostic criteria for ALS were not established until 1990, when the World Federation of Neurology established the El Escorial criteria, to be used for the diagnosis of ALS in a research setting. These criteria were updated in 1998 at Airlie House, Virginia, USA, and mainly rest on clinical, neurophysiological and neuropathological examinations. For the diagnosis of ALS, the El Escorial/Airlie House criteria require the presence of:

A1 evidence of lower motor neuron (LMN) degeneration by clinical, electrophysiological or neuropathological examination,
A2 evidence of upper motor neuron (UMN) degeneration by clinical examination, and
A3 progressive spread of symptoms or signs within a region or to other regions, as determined by history or examination,

together with the absence of:

B1 electrophysiological or pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration, and
B2 neuroimaging evidence or other disease processes that might explain the observed clinical and electrophysiological signs.

According to these criteria, ALS is classified into various categories depending on the certainty of the diagnosis:

1. Clinically definite ALS
2. Clinically probable ALS
3. Clinically probable ALS – laboratory supported
4. Clinically possible ALS
In clinical practice, however, these strict criteria are less useful because a patient must have a fairly advanced disease to fulfill the criteria for clinically definite or probable ALS at diagnosis\(^6\) and about 10% of patients never fulfill these criteria\(^7\).

**Motor neuron degeneration in ALS:** Since the diagnosis of ALS requires both UMN and LMN symptoms, it excludes related disorders, such as progressive muscular atrophy/bulbar palsy (PMA/PBP) and primary lateral sclerosis (PLS), where only lower and upper motor neurons respectively are affected. Since PMA/PBP and PLS probably share many etiological mechanisms with ALS or form a disease continuum with ALS\(^5\)-\(^11\), the term motor neuron disease (MND) is often used to include these disease entities\(^12,13\).

ALS patients have often been ascribed preserved ocular muscular functions, as well as normal function of the voluntary sphincters of the bladder and bowel, suggesting that the CNS motor areas innervating these muscles, the oculomotor nuclei (cranial nerves III, IV, VI) and the nucleus of Onufrowicz, are spared in ALS\(^14\)-\(^16\). There are, however, some reports of complete or incomplete ophthalmoplegia in ALS patients\(^17\)-\(^19\), especially in cases where the disease duration has been prolonged due to mechanical ventilation\(^18,20\)-\(^22\). Upon testing, more subtle eye movement abnormalities can also be detected in ALS patients\(^19,23\),\(^24\). In ophthalmoplegic cases, histopathological evidence of neuronal loss and gliosis in the oculomotor nuclei has been seen\(^17,20\), but at present it is unclear whether the dysfunction is nuclear, supranuclear, or both\(^18,25\).

There are also some reports of ALS patients suffering from incontinence or other sphincter disturbances, but this appears to be a fairly infrequent problem\(^26,27\). Current evidence, however, suggests that the motor neurons innervating the sphincters could be sub-clinically affected in ALS. Histopathological studies have shown that inclusions characteristic of ALS (Bunina bodies, Lewy body-like hyaline inclusions and ubiquitin-positive inclusions – see below) frequently occur in the Onufrowicz nucleus of ALS patients but without neuronal loss\(^28,29\). Further evidence supporting the involvement of these motor neurons in ALS comes from electrophysiological studies. EMGs of the external anal sphincter in a small group of ALS patients without sphincter disturbances showed mild abnormalities in 9 of 16 patients, suggesting that these motor neurons were indeed affected\(^30\). However, in overall terms, it appears as if these motor neurons, for unknown reasons, are more resistant than other motor neurons.

**Involvement of CNS non-motor areas in ALS:** There is now also substantial evidence that the degenerative process in ALS is not limited to motor neurons. Although dementia has been described as being relatively rare in ALS\(^26,27,31\), it has now become evident that more subtle cognitive changes often occur in ALS patients. Results from neuropsychological testing have demonstrated that cognitive impairment in ALS could be as frequent as 30-60% of ALS patients\(^32\)-\(^34\). These cognitive deficits are possibly more common in ALS patients with bulbar symptoms\(^32\)-\(^34\). Most typically, the deficit is of the frontotemporal type with defects in verbal and non-verbal fluency, abstract reasoning, mental flexibility and judgment\(^32\)-\(^34\). Parkinsonism in ALS is rare, but there are some reports of ALS patients suffering from Parkinsonian symptoms such as bradykinesia, rigidity and gait abnormalities.
Tremor is only seen in a few of these patients and the efficacy of levodopa treatment is often poor\textsuperscript{31,35-37}.

Neuroimaging has provided additional support for the involvement of brain extra-motor areas in ALS. Although studies using conventional MRI and CT scans of the CNS have shown conflicting results and therefore are difficult to interpret\textsuperscript{38}, other neuroimaging techniques have provided evidence that suggests the involvement of regions beyond the motor areas of the brain. PET, SPECT and fMRI studies have shown reduced blood flow in extra-motor areas, particularly the frontal lobe, in the brains of ALS patients, with a greater reduction in cognitively impaired ALS patients\textsuperscript{39-42}. Similarly, Lloyd and co-workers used PET and saw a decrease in the amount of GABA\textsubscript{A} receptors in the prefrontal cortex and other areas of the brain in ALS patients without any signs of cognitive impairment\textsuperscript{43}. Also using PET, Turner demonstrated a global reduction of 5-HT\textsubscript{1A} receptors in the brain\textsuperscript{44} and activation of microglia in the prefrontal cortex, pons, motor cortex and thalamus of non-demented ALS patients\textsuperscript{45}. Finally, Takahasi and co-workers, also using PET, saw a negative correlation between disease duration and the uptake of 6-fluorodopa in ALS patients without clinical signs of extrapyramidal symptoms, suggesting a reduction in the number of neurons in the substantia nigra\textsuperscript{46}.

Degenerative processes are also seen in tissue sections of non-motor brain areas of ALS patients. These alterations appear to be more common or more pronounced in patients that are cognitively impaired. For example, there are several reports of ubiquitin-positive inclusions that are negative for tau and \(\alpha\)-synuclein in neurons in the frontal lobe neocortex and the dentate gyrus of the hippocampus\textsuperscript{47-52}. Other reports include cortical spongiosis and gliosis in non-motor brain areas of ALS patients\textsuperscript{47,48,50,51} and cases with degeneration of the substantia nigra without evidence of Lewy bodies have been described\textsuperscript{51,53}.

In the difficult task of cell counting in the brain, there are few reports from ALS patients. Maekawa and co-workers used an antibody for non-phosphorylated neurofilament heavy (NF-H) as a marker for pyramidal neurons and found neuronal loss not only restricted to the motor areas of the CNS but also in the frontal and cingulate cortex of ALS patients\textsuperscript{54}. Gredal et al., on the other hand, were not able to detect any neuronal loss in the motor cortex of ALS patients using a stereological 3-dimensional technique\textsuperscript{55}. Additional studies in this context appear to be needed. In ALS patients with prolonged disease duration due to the use of mechanical ventilation, widespread neuronal degeneration is often seen\textsuperscript{20,21}.

To summarize; the preponderance of evidence suggests that ALS should be regarded as a multisystem disease, affecting many different parts of the CNS but with a predilection for motor neurons in the spinal cord, brain stem and motor cortex. This implies that ALS represents one end of a spectrum of neurodegenerative disease entities that only differ by their particular susceptibility to cell toxic events. It therefore seems possible or even likely that disease mechanisms in other neurodegenerative diseases could also apply to ALS and that knowledge gained about the pathogenic mechanisms in ALS also could be applied to other neurodegenerative diseases. It also seems possible that therapies developed for ALS or other neurodegenerative disorders could be used for the treatment of a range of different neurodegenerative diseases.
Epidemiology

A large number of epidemiological studies of ALS have been published. The diagnostic criteria, study designs, methodologies and the populations studied vary considerably, making comparisons difficult. However, some conclusions can be drawn from these studies.56-58

Incidence: In most studies, the incidence of ALS has been reported to be between 1.5-2.5 per 100,000 people a year and the occurrence of ALS appears to be geographically uniform, at least in Europe and North America, where most studies have been conducted.58-63 Some clustering of a special form of ALS has previously been seen in the western Pacific island of Guam and in areas of the Kii peninsula of Japan (ALS Parkinson dementia complex – ALS-PDC), but now the incidence in these two regions appears to have declined.64-66 There is a tendency towards an increase in incidence in later years, especially among women. One explanation for this increase is that it is a consequence of an increase in life expectancy and/or a more complete assessment of cases, but it is also possible that this represent a true increase in incidence.56-58,66-68

ALS is somewhat more common in men than in women and studies indicate a ratio of around 1.4:1.7,27,31,58,59,61,63,69-73 The gender ratio decreases at higher age, however, and approaches 1:1 after the age of 65.31,57,67,70,71 The reason for this is not known, but it has been speculated that the difference in lower age groups may be due to hormonal differences.

Age at onset: The age at onset shows great variation, ranging from juvenile forms of ALS (JALS) to patients contracting the disease at a very advanced age, but the incidence appears to peak in the 6th to 8th decades of life58,61,63,74 and the mean age of onset in most studies is between 56 and 63 years of age.27,31,60,61,71-73,75,76 In analogy to a gender ratio of 1:1 in older age groups, it appears as if the mean age of onset is higher for women.59,60,70,71,76,77

Survival: Survival has been described in the form of both mean and median values in the literature. However, there appears to be little difference between these two measures and the mean/median survival from onset of symptoms to death is frequently reported to be between 2.5 and 3 years.7,27,31,60,61,71-73,76,78 In about 25% of cases, the presenting symptom is bulbar7,27,31,58,69-72,76,78 and these patients show a somewhat more rapid disease progression, as do older patients.26,27,31,56,57,69,70,72,73,77,78 With regard to survival, it is likely that these two factors, at least in part, should be regarded as confounding variables, since bulbar onset appears to be more common in the elderly.69,72

Neuropathology

General features: The key neuropathological feature of ALS is a loss of motor neurons which reflects the clinical symptoms of the patient. Macroscopically, this loss can be manifested as atrophy of the spinal cord, ventral roots, and, in a few patients, the precentral gyrus.79 Microscopically, loss of motor neurons is often most discernable in the lumbar and cervical ventral horns of the spinal cord but in patients with mainly bulbar symptoms motor neuron loss could be more or less confined to
the motor nuclei of the brainstem. Upper motor neurons loss is often more difficult to appreciate\textsuperscript{80}, most likely due to the normal low “density” of motor neurons in the motor cortex. Upper motor neuron degeneration is, however, reflected by myelin pallor in the cortico-spinal tract\textsuperscript{80} (hence amyotrophic lateral sclerosis, also see cover illustration). Remaining ventral horn motor neurons are often shrunken and show signs typical for apoptosis\textsuperscript{79}. Additional neuropathological features of ALS include reactive gliosis and the occurrence of different kinds of inclusions with varying degree of specificity for ALS.

**Inclusion pathology in ALS:** The following inclusions are known to occur in the spinal cords of ALS patients:

- **Bunina bodies:** These are small (1-4 µm) eosinophilic granular inclusions within the cytoplasm of ventral horn motor neurons. They consist of an amorphous material with tubules and vesicular structures and stain for cystatin C. There are indications that this inclusion is derived from lysosomes. Bunina bodies are perhaps the most ALS-specific inclusion, but not all ALS patients have this type of inclusion\textsuperscript{52,79-81}.

- **Skein-like inclusions:** The appearance of this inclusion is similar to skeins, hence the name. Skein-like inclusions are only detected when stained for ubiquitin (see below). The inclusions contain bundles of filaments with granules, 15-25 nm in diameter. Although characteristic for ALS they are not specific for the disease\textsuperscript{79,81,82}.

- **Spheroids:** These are eosinophilic and argyrophilic inclusions, primarily found in the proximal axons and perikaryon, that contain phosphorylated neurofilaments (see below). This type of inclusion can be as large as 20 µm, but smaller spheroids also exist and are sometimes referred to as globules. The specificity of these inclusions is low and spheroids/globules can be seen in normal subjects but the number appears to be increased in ALS\textsuperscript{79,80}.

- **Lewy body like hyaline inclusions (LBHI):** LBHIs exists in both neurons and astrocytes and are hyaline inclusions with a peripheral halo similar to the Lewy bodies seen in Parkinson disease. Initially it was believed that this type of inclusion was specific for certain types of familial ALS but it is now clear that LBHIs also can occur in also in sporadic ALS\textsuperscript{83-85}. The inclusions are 3-15 µm in diameter and are composed of 15-25 nm granule-coated fibrils and, in the periphery, of 10 nm neurofilaments\textsuperscript{79,81,83}. Besides neurofilaments, LBHIs also stain for other proteins (see below).

**Etiology and pathogenesis**

The cause of ALS is still largely unknown. When it comes to the pathogenesis of ALS, a number of disease mechanisms have been proposed and some of the most studied are discussed here.
Glutamate excitotoxicity: Glutamate is one of the most important excitatory amino acids in the CNS. At high concentrations, this amino acid is known to be toxic to neurons, probably by overexciting the cells. The toxic mechanism is likely to involve cellular calcium influx, the production of reactive nitrogen and oxygen species and the initiation of the apoptosis machinery, as has been recently reviewed\(^8\).

Because of its inherent toxic properties and experimental findings indicative of an abnormal glutamate metabolism in ALS patients, glutamate has attracted a great deal of interest from ALS researchers in the last couple of decades. There are several reports suggesting increased levels of glutamate in extracellular compartments, such as blood\(^87,88\) and cerebrospinal fluid (CSF)\(^89-91\), and reduced levels of glutamate in CNS tissues, representing the intracellular compartment, of ALS patients\(^92-94\). The results in this area of research have, however, been conflicting\(^88,89,95,96\), possibly as a result of the difficulties in measuring glutamate.

Albeit conflicting results, it has been suggested that ALS is associated with a shift in glutamate from the intracellular to the extracellular space, possibly resulting in the exposure of glutamate receptors on neurons to abnormally high concentrations of extracellular glutamate. In support of these findings, a deficiency in the capacity to transport glutamate from the extracellular to the intracellular space was demonstrated in ALS patients\(^97\) and, subsequently, a selective loss of the astrocytic glutamate transporter, EAAT2, in affected CNS areas of ALS patients was reported\(^98-100\). It was therefore suggested that the loss of EAAT2 could be responsible for the altered glutamate metabolism in ALS patients. The reasons for the loss of EAAT2 protein were (are) not known, since the levels of EAAT2 mRNA in the motor cortex of ALS patients was not significantly altered\(^101\) and screening for mutations in the EAAT2 gene gave no explanation\(^102-104\). In 1998, one report suggested that the low levels of EAAT2 protein in ALS patients were due to aberrantly spliced forms of EAAT2 mRNA\(^105\). Later studies have, however, reported a similar degree of alternatively spliced EAAT2 mRNA also in controls\(^106-109\).

Glutamate binds to three different types of receptors: NMDA, AMPA and kainate receptors. One of these receptors, the AMPA receptor, has been particularly implicated in ALS. For example, it was recently shown that the mRNA editing of the GluR2 subunit of the AMPA receptor was defective in the motor neurons of ALS patients\(^110\). Perhaps the most convincing evidence that glutamate participates in the process of motor neuron degeneration in ALS comes from the anti-glutamatergic drug riluzole. Clinical trials with this drug have shown a positive effect on survival and riluzole is so far the only drug approved for the treatment of ALS\(^111\). The precise mechanism of this drug is uncertain, but one of its actions is probably to inhibit presynaptic glutamate release\(^112,113\).

Intermediate filaments: In differentiated neurons, five different intermediate filament proteins are expressed – three neurofilament proteins, peripherin and α-internexin. The three different subunits of neurofilaments are termed heavy, medium and light (NF-H, NF-M and NF-L) in reference to their molecular weight\(^114\). The functions of the intermediate filaments are both to participate in axonal transport and to uphold axonal caliber. Intermediate filaments have been implicated in ALS pathogenesis by the occurrence of conglomerates (spheroids, see above) of 10 nm phosphorylated neurofilaments in the proximal axons, perikaryon and, somewhat more diffuse, in the somas of motor neurons. These conglomerates can also contain
peripherin. It has been suggested that the occurrence of these conglomerates is a consequence of poor axonal transport.

A number of transgenic mouse strains null for or overexpressing the different neurofilament proteins have been generated. Generally, these mouse strains do not develop an overt MND phenotype but in some strains an axonopathy and motor neuron dysfunction is seen, e.g. in mice that are null for NF-L or both NF-M and NF-H and in mice overexpressing peripherin or NF-H. In mice overexpressing L394P mutant murine NF-L a more overt MND phenotype is seen with motor neuron loss and neurogenic muscular atrophy.

Surprisingly, deletion of NF-L or overexpression of NF-H in the G85R and G37R, respectively, hSOD1 mouse models of ALS (see below) resulted in accumulations of neurofilaments in the perikarya and, importantly, significantly prolonged survival. The reasons for these surprising results are not known but the results indicate that the stoichiometry between the different intermediate filaments is important for motor neuron survival and that perikaryal accumulations of neurofilaments in some situations actually could be beneficial (e.g. by acting as a “phosphorylation sink”).

Environmental and lifestyle-related risk factors: Many epidemiological studies have been performed to try to identify environmental and lifestyle-related risk factors for ALS. Generally, these studies are hampered by the heterogeneity of ALS and the relative rarity of the disease. A number of such risk factors have, however, been suggested; they include exposure to heavy metals and trace elements (such as lead, mercury and selenium), exposure to chemicals, working with leather, welding, heavy labor, sports activities, physical trauma (including surgery), electrical trauma, farming, smoking, alcohol, excess body mass and participation in the Persian Gulf war. Despite a plethora of studies in this area, the results have been far from conclusive and today the only established risk factors are age, gender (see above) and hereditary factors (see below), which have recently been reviewed.

Familial ALS: In at least 5-10% of ALS cases, there is a family history of ALS in at least two blood relatives and in these instances the disease is regarded as familial (FALS). In most cases, FALS is indistinguishable from sporadic ALS (SALS), but it is generally recognized that FALS has a few years earlier onset, more equal gender distribution and perhaps a higher frequency of concurrent dementia, Parkinsonism and sensory disturbances. Histopathologically, the involvement of the posterior columns, dorsal spinocerebellar tracts and Clarke’s nucleus is probably also more frequent in FALS. In certain types of FALS, however, a more divergent phenotype is seen.

Today, 10 different gene loci (Table 1) have been linked to familial ALS (FALS). In four of these loci, the gene responsible for the disease has been identified. Two of these genes are involved in JALS, a condition that is relatively rare. In JALS, onset should take place before the age of 25 and the progression of the disease is generally much slower. The other two disease-causing genes have been found in families with classical ALS (ALS1) or a heterogeneous ALS/SMA disease (ALS8). The 10 different genetic loci are discussed below.
Table 1. Familial ALS loci

<table>
<thead>
<tr>
<th>Disease Type</th>
<th>gene</th>
<th>Locus</th>
<th>Inheritance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS1 Classical ALS</td>
<td>SOD1</td>
<td>21q22.11</td>
<td>Dominant/Recessive</td>
<td>133</td>
</tr>
<tr>
<td>ALS2 JALS, PLS, HSP</td>
<td>ALS2</td>
<td>2q33.1</td>
<td>Recessive</td>
<td>134, 135</td>
</tr>
<tr>
<td>ALS3 Classical ALS</td>
<td></td>
<td>18q21</td>
<td>Dominant</td>
<td>136</td>
</tr>
<tr>
<td>ALS4 JALS, slow progression</td>
<td>SETX</td>
<td>9q34.13</td>
<td>Dominant</td>
<td>137</td>
</tr>
<tr>
<td>ALS5 JALS</td>
<td></td>
<td>15q15.1-q21.1</td>
<td>Recessive</td>
<td>138</td>
</tr>
<tr>
<td>ALS6 Classical (and FTD?)</td>
<td>VAPB</td>
<td>16q12.1-q12.2</td>
<td>Dominant</td>
<td>139-141</td>
</tr>
<tr>
<td>ALS7 Classical</td>
<td></td>
<td>20ptel-p13</td>
<td>Dominant</td>
<td>140</td>
</tr>
<tr>
<td>ALS8 Heterogeneous ALS/SMA</td>
<td></td>
<td>20q13.33</td>
<td>Dominant</td>
<td>142</td>
</tr>
<tr>
<td>ALS-X Late onset</td>
<td></td>
<td>Xp11-q12</td>
<td>X-linked dominant</td>
<td>143</td>
</tr>
<tr>
<td>ALS/FTD Classical ALS and/or FTD</td>
<td></td>
<td>9q21-q22</td>
<td>Dominant</td>
<td>144</td>
</tr>
</tbody>
</table>

FDT = frontotemporal dementia, HSP = hereditary spastic paraplegia

**ALS1**: In 1991, the first genetic locus linked to ALS was reported by Siddique and co-workers. They found linkage to a 10 cM region of chromosome 21q22.1-q22.2 in families with autosomal dominant ALS. A few years later, in a pivotal paper by Rosen et al., it was shown that mutations in the gene encoding for superoxide dismutase 1 (hSOD1) was responsible for the disease in these families. This discovery gave rise to a surge in research into the pathogenesis of ALS and mutations in this protein are also the topic of this thesis. A detailed description of mutant SOD1 (mutSOD1) and ALS is given below.

**ALS2**: The second locus linked to ALS was reported in 1994, when linkage to an 8 cM large region of chromosome 2q33-q35 was shown in a consanguineous Tunisian family with juvenile-onset, recessive ALS. The ALS in this family was predominantly a slowly progressing UMN disease, but there were also signs of LMN involvement. In 2001, it was shown that a one base-pair deletion in the ALS2 gene encoding for alsin was responsible for the disease in this family. An additional two families with mutations in ALS2 were also reported, both of whom had the diagnosis juvenile-onset PLS (i.e. without signs of LMN involvement). Since then, another six mutations in the ALS2 gene have been discovered, all in families with infantile recessively inherited ascending spastic paraplegia. So far, all the detected mutations result in the truncation of the protein and it appears as if the mutant protein is highly unstable and rapidly degraded. These findings and the recessive inheritance pattern make it likely that mutant alsin causes motor neuron disease by a loss of function. The function of the alsin protein is not completely known, but alsin contains three guanine exchange factor (GEF) domains that are generally involved in the cycling of GTP/GDP for GTPases (presumably Rab5). The protein also contains several membrane occupation and recognition nexus (MORN) motifs, which suggests binding to membranes, and recent studies have suggested that alsin is involved in endosome dynamics. The reason for the particular vulnerability of motor neurons is not known and there is no clue in the
expression pattern of alsin, since the highest expression appears to be in the cerebellum, a part of the CNS that does not appear to be affected in ALS2\textsuperscript{135,150,151}. Alsin knockout mice have recently been reported. These mice show signs of cerebellar dysfunction, such as impaired motor coordination and motor learning deficits and an elevated anxiety response. There were, however, no neuropathological signs of motor neuron disease\textsuperscript{152}.

The importance of ALS2 mutations in ALS remains to be evaluated, but so far it seems as if mutant alsin is not a common cause of ALS, even in atypical cases with young onset, slow progression and predominantly UMN symptoms\textsuperscript{153,154}.

ALS3: In 2002, one family fulfilling the El Escorial criteria for adult onset ALS was described and linkage to an approximately 8 Mb large region of chromosome 18q21 with a LOD score of 4.5 was shown\textsuperscript{136}. The clinical picture was classical ALS but with a somewhat early onset (mean 45 years of age), but, as stated above, this is commonly seen in FALS. The mean disease duration for this family was 5 years and the inheritance pattern was autosomal dominant. The gene responsible for the disease has so far not been identified.

ALS4: This is an apparently rare autosomal dominant form of ALS with childhood or adolescent onset. There are signs of both upper and lower motor neuron lesions and there are also some indications of the involvement of sensory neurons. Bulbar and respiratory symptoms are not seen. The disease progression is very slow, with an apparently normal life expectancy, and there is a large variation in the severity of symptoms\textsuperscript{155,156}. ALS4 should thus be regarded as atypical ALS. There are three known ALS4 families with linkage to chromosome 9q34 and it was recently shown that missense mutations in the \textit{SETX} (senataxin) gene co-segregated with the disease\textsuperscript{137}. Interestingly, families afflicted by another neurodegenerative disease, autosomal recessive ataxia-oculomotor apraxia type 2 (AOA2), also carry mutations in this gene and at present it is unclear whether there is any relationship between these two disease entities\textsuperscript{157,158}. The function of the senataxin protein is not known, but it has been suggested that senataxin is involved in DNA/RNA maintenance\textsuperscript{137,157}. The mechanism by which mutant senataxin causes disease is not known and both loss and gain of function are conceivable\textsuperscript{137}.

ALS5: Patients with ALS5 suffer from JALS and, in 1998, linkage to a 6 cM large region of chromosome 15q15-q22 was reported in families from Germany and Tunisia\textsuperscript{138}. The inheritance pattern was recessive and patients suffered from both spinal and bulbar symptoms. Both UMN and LMN signs were present. The Tunisian patients had a slow disease progression, while the German family had a much more rapid progression. Signs of mental retardation were also seen in the two German siblings\textsuperscript{131,138}. No further reports of JALS linked to chromosome 15 have been published.

ALS6: In the August 2003 issue of the \textit{American Journal of Human Genetics}, three papers were published that showed linkage of autosomal, dominant, adult-onset ALS in four families to chromosome 16q12.1-12.2. The symptoms displayed were mainly classical ALS, but signs of frontotemporal lobe dementia were seen in some
patients\textsuperscript{139}. The chromosomal region of interest spans about 4.5 Mb\textsuperscript{139}, but so far the gene responsible for ALS\textsuperscript{6} has not been reported.

**ALS\textsuperscript{7}**: In their 2003 paper, Sapp and his co-workers also reported linkage to chromosome 20ptel in one family fulfilling the El Escorial criteria for adult-onset ALS\textsuperscript{140}. The maximum LOD score was 3.46 and the haplotype under scrutiny covers around 1 Mb\textsuperscript{140}. It should be noted that the linkage analysis was only based on two affected individuals in the same generation of a family. No further data on this family have been published.

**ALS\textsuperscript{8}**: In 2004, Nishimura and co-workers described a Caucasian Brazilian family with adult-onset ALS and dominant inheritance and linkage to chromosome 20q13 was demonstrated\textsuperscript{159}. Later that year, they were able to reveal that a missense mutation in the \textit{VAPB} (vesicle-associated membrane protein-associated protein B) gene segregated with disease in the family. In this report, an additional six families were also reported, all showing the same mutation in the \textit{VAPB} gene\textsuperscript{142}, and a founder effect in the mid-15\textsuperscript{th} century is suspected\textsuperscript{160}. The clinical phenotype was heterogeneous. Three families had a slowly progressing atypical ALS, three families had symptoms corresponding to adult-onset spinal muscular atrophy (SMA) and, in one family, patients suffered from both atypical and classical ALS. It is believed that the function of the VAPB protein is to act during ER-Golgi transport and secretion\textsuperscript{142}. It is not known why a mutation in this ubiquitously expressed protein is toxic to motor neurons and so far, no more data have been published on ALS and mutation(s) in \textit{VAPB}.

**ALS-X**: One family consisting of 11 affected individuals with linkage to the X chromosome has been described in one abstract. There was no male-to-male transfer. The maximum LOD score was 3.8, which was close to the maximum LOD score for this family\textsuperscript{143}. No additional data concerning this family have been described.

**ALS-FTD**: There are many reports on the nosological overlap between ALS and frontotemporal dementia (see above). The co-existence of ALS and frontotemporal dementia has also been reported in FALS. In 2000, Hosler et al. showed linkage between dominantly inherited ALS/FTD and a 17 cM large region of chromosome 9q21-q22. Five families in which individuals had ALS and/or frontotemporal lobe dementia were reported. The ALS disease seen in these families was reported to be classical ALS\textsuperscript{144}. No further reports of ALS-FTD linked to chromosome 9q21-q22 have been published.

There are two other genes involved in familial neurodegenerative diseases that are of potential importance for ALS. Dynactin is a multiprotein complex involved in axonal transport. Heterozygous mutations in the gene for the p150 subunit of the dynactin complex (\textit{DCTN1}) have been linked with a LMN disease with prominent bulbar symptoms similar to spino-bulbar muscular atrophy (SBMA or Kennedy’s disease – a hereditary disease caused by trinucleotide repeats in the androgen receptor gene)\textsuperscript{161}. Mutations in the \textit{DCTN1} gene have occasionally also been detected in ALS patients\textsuperscript{162}. Interestingly, perturbation of proteins involved in the dynactin complex in mice (e.g. \textit{Loa} or \textit{Cral} mice and transgenic mice overexpressing the p50 subunit of
the dynactin complex – dynamitin) results in a motor neuronopathy with features similar to ALS\textsuperscript{163,164}. It is unclear whether alterations in this gene should be regarded as direct causative genes with reduced penetrance or merely as a genetic risk factor.

The other gene is \textit{tau}, which encodes a microtubule-associated protein. Mutations in this gene cause dominantly inherited FTDP-17 (frontotemporal dementia with Parkinsonism linked to chromosome 17), which is, as the name implies, a disease characterized by frontotemporal dementia and Parkinsonism\textsuperscript{165}. Rarely, signs of motor neuron degeneration are also seen in these patients\textsuperscript{166,167}. Tau is also found in the neurofibrillary tangles of Alzheimer’s disease patients. In analogy to what is seen for dynactin, transgenic mice overexpressing mutant tau suffer from motor neuron loss and have a phenotype similar to ALS\textsuperscript{168}. It is, however, important to mention that tau inclusions and neurofibrillary tangles are not usually seen in classical ALS, although patients with the western pacific ALS-PDC display tau-positive inclusions\textsuperscript{82,169}. There is, however, little evidence that the \textit{tau} gene is involved in ALS-PDC\textsuperscript{170,171}, although one study has indicated that the \textit{tau} loci could be a susceptibility factor for ALS-PDC\textsuperscript{172}.

The genetic heterogeneity in all forms of FALS has thus proved to be much larger than previously anticipated and only a minority of FALS cases can so far be associated with any of these loci. It is therefore evident that there are several additional loci to be discovered\textsuperscript{140,173}. Even though some of the mutant genes involved in FALS are very rare causes of ALS, much could probably be learnt from these discoveries with regard to motor neuron biology and pathology. It is also possible or even likely that some of these proteins are involved in SALS, as is discussed below.

**Genetic risk factors:** In addition to the genes involved in familial forms of ALS, a number of polymorphic genes have been proposed as causative genes or susceptibility factors in ALS. For many of these genetic risk factors, studies have not been conclusive, due mainly to small sample sizes and ethnical heterogeneity in the population. Further investigations using larger sample sizes are therefore needed. The main findings of some of the most studied of these genes are summarized below.

\textit{APEX}: This is an enzyme involved in DNA repair as an endonuclease. Olkowski detected missense mutations in the coding region of this gene in six of eleven ALS patients. No mutations were seen in five controls\textsuperscript{174}. A much larger study consisting of 153 ALS patients and 58 controls was performed by Hayward and colleagues. In addition to rare polymorphisms in the gene in both controls and ALS patients, they detected a significantly higher frequency of a homozygous D148E polymorphism in SALS patients than in controls, suggesting that this polymorphism may be a risk factor for ALS\textsuperscript{175}. Tomkins and co-workers, on the other hand, were unable to find any significant differences when investigating 84 ALS patients and 154 controls. Some rare polymorphisms of unknown importance were seen in both ALS patients and controls, also in this study\textsuperscript{176}. In the brains of ALS patients, the amount and activity of APEX have been reported to be reduced\textsuperscript{177}.

\textit{APOE}: The \(\varepsilon4\) allele of \textit{APOE} (\textit{APOE4} – apolipoprotein E) is significantly over-represented in Alzheimer’s disease and has been associated with an early onset of the
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disease, which could also be the primary effect of this genotype, as has recently been reviewed\textsuperscript{178,179}. It has therefore been speculated whether this allele could also be a risk factor for ALS and several studies have addressed this aspect.

The first published study was that of Mui and colleagues, who screened 72 SALS and 98 FALS patients and 60 unaffected relatives for the \textit{APOE4} allele. In this study, they were unable to show any increased risk associated with the \textit{APOE4} allele\textsuperscript{180}. Similar negative findings have subsequently been confirmed by others\textsuperscript{181-188}. However, some studies have suggested that the \textit{APOE} genotype could be important for the age at onset. Li and co-workers demonstrated a later onset of ALS with an increasing number of \textit{APOE2} alleles\textsuperscript{181} and Moulard et al. showed that, in bulbar onset ALS, the \textit{APOE4} allele was associated with an earlier onset\textsuperscript{187}. Other studies have been unable to verify a correlation between \textit{APOE} genotype and age at onset of symptoms, although all stratifications were not made\textsuperscript{180,182-186}. Some studies have suggested that the \textit{APOE} genotype is important for disease progression, with the \textit{APOE4} allele associated with a more rapid disease progression\textsuperscript{183} or the \textit{APOE2} allele with a slower course\textsuperscript{187}, but survival was not controlled for other factors in these studies. Other studies, some of which have performed a multivariate analysis, have been unable to detect significant differences\textsuperscript{180,182,188}. Lacomblez et al., however, seeing no association between genotype and survival, showed that a low value of plasma apoE correlated positively with survival\textsuperscript{188}. It has also been suggested that the \textit{APOE4} allele is over-represented in ALS patients with bulbar instead of spinal onset\textsuperscript{182,187}, but again this has not been seen in other studies\textsuperscript{184-186}.

All these studies are relatively small, a frequent problem when studying ALS, and of different methodological qualities. This and the contradictory results make it difficult to draw safe conclusions. However, it seems reasonable to assume that, if the \textit{APOE} genotype has any importance in ALS, the overall effect of the genotype is small.

\textit{CNTF}: Ciliary neurotrophic factor (CNTF) is a protein whose main function appears to be to promote neuronal survival\textsuperscript{189}. In the spinal cords of ALS patients, reduced levels of this neurotrophic factor have been reported\textsuperscript{190}, although levels in the sciatic nerves of patients were unchanged\textsuperscript{191}. A one base pair insertion in the intronic sequence of this gene, resulting in a null mutant, has previously been described. The null mutant exists as a polymorphism in the population, with a few percent being homozygotic for null CNTF\textsuperscript{192}. Because of the role played by CNTF in neuronal survival and the motor neuron loss seen in CNTF knockout mice\textsuperscript{193} it has been speculated that the null mutant could be a susceptibility factor for ALS. In a small study consisting of 49 FALS patients, Orrell and co-workers were not able to show any such association\textsuperscript{194} and similar results were demonstrated by Giess and colleagues in an unspecified group of 98 ALS patients\textsuperscript{195}. However, in the latter study, all the ALS patients who were homozygotic null for CNTF had younger onset (42, 14 and 23 years of age) suggesting that the null mutant is a risk factor for early-onset disease\textsuperscript{195}. In the largest study so far, comprising 400 ALS patients (351 SALS and 49 FALS) and 217 controls, there were no differences, either in frequency or disease onset, between ALS patients and controls\textsuperscript{196}. It thus appears unlikely that the CNTF null mutation is of major importance in ALS pathogenesis.
**HFE**: Autosomal recessive hemochromatosis is caused by mutations in the *HFE* gene. Two mutations are particularly prevalent, H63D and C282Y with C282Y being associated with a higher risk for iron accumulation. Interestingly, mutations in this gene have also been associated with an earlier onset of Alzheimer’s disease. Wang and colleagues investigated the prevalence of *HFE* mutations in 121 ALS patients and 133 controls and found a significant association between the H63D mutation and ALS. In the larger study of Goodall et al., comprising 379 ALS patients and 400 controls, a similar significant association with H63D (odds ratio 1.85 for one mutant allele), but surprisingly not with C282Y, was seen. In a previous study by Yen and co-workers, who screened 51 ALS patients and 47 controls for these two polymorphisms, no such association was seen. The reasons for these discrepancies are not known but the latter study is of smaller size and is possibly also hampered by ethnical heterogeneity. The mechanism by which H63D HFE could increase the risk to develop ALS is not known, but it has been speculated that the mutation could lead to an altered iron transport across the blood-brain barrier since the H63D mutation is known to affect binding to the transferrin receptor. Interestingly, the *HFE* locus is located on chromosome 6p21.3 close to the major histocompatibility complex (MHC) region. One possibility is therefore that the H63D mutation is in linkage disequilibrium with a *HLA* haplotype that increases the risk to develop ALS. This would of course implicate the immune system in ALS pathogenesis.

**NFH**: The central domain of the five different intermediate filaments expressed in neurons is conserved, but in the N and C-terminal ends of the proteins (also known as head and tail respectively) there are less conserved domains. At the C-terminal end of NF-H and NF-M, there are multiple repeats of amino acids Lys-Ser-Pro or KSP repeats. In humans, the number of KSP repeats in NF-H is polymorphic with either 44 or 45 (previously stated 43 or 44) repeats. The phosphorylation of these repeats is important for neurofilament spacing and axon caliber and probably also for axonal transport, since hyperphosphorylation decreases transport velocity. Three studies have suggested that alterations in this domain of the *NFH* gene are associated with SALS. Figlewicz and co-workers reported heterozygous deletions in 5 of 356 ALS patients (1.4%), while none of the 306 controls carried a deleted copy. However, healthy relatives of ALS patients with mutant *NFH* also carried the mutant gene. Tomkious and colleagues screened 164 ALS patients and found one heterozygous insertion in one SALS patient causing an extra four KSP repeats. No alterations were seen in 209 age-matched controls. In the largest study so far, 530 ALS patients and 366 controls were screened for mutations using SSCP and RFLP. Four different tail deletions in three ALS patients and one FALS patients were seen. It could not be determined whether the mutation in the FALS patient segregated with disease. No linkage to the *NFH* locus at chromosome 22q12.2 has, however, been reported in FALS. Deletions in three unaffected relatives of these patients and in two controls were also detected. There was no association between the 44 or 45 repeats polymorphism and ALS. In two smaller studies, no association between mutations in the *NFH* gene and ALS were seen.

In the study by Vechio and colleagues, *NFL* and *NFM* were also investigated without significant associations. Garcia and co-workers recently sequenced the *NFL*, *NFM* and *NFH* genes, but excluded the KSP region of *NFH* in 100 SALS, 100 FALS patients and 100 controls. They found a few different mutations in all three genes in
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both controls and ALS patients. The data were somewhat difficult to interpret due to the small sample size, but it was suggested that mutations in neurofilaments could be possible risk factors\(^{207}\). A similar picture has been reported for another intermediate filament, peripherin\(^{208,209}\).

In all, the collective studies suggest that mutations in the KSP domain of NFH may be a rare susceptibility factor for sporadic ALS. Two negative studies have been published, but they appear to have been underpowered, considering that KSP mutations in NFH only appear in about 1% of ALS cases (and in 0.2% of controls). Findings from studies in transgenic mice and histopathology from ALS CNS tissue (see above) add further support for the role of neurofilaments in motor neuron degeneration. An interesting observation is that heterozygous mutations in the NFL gene are linked to Charcot-Marie-Tooth type 2E\(^{114,201,202}\).

**SMN1/SMN2/NAIP:** The survival motor neuron protein (SMN) is a protein that is most probably involved in the assembly of small nuclear ribonucleoproteins in a protein complex called Gems\(^{210}\). The SMN gene exists in two almost identical copies on chromosome 5q13. The telomeric copy of the gene has been named SMN1 and the centromeric SMN2. Homozygous deletions or conversions of SMN1 are responsible for more than 95% of spinal muscular atrophy (SMA) cases, but curiously it also appears as if the phenotype of SMA is correlated to the copy number of SMN2\(^{211}\). Because of the degeneration of lower motor neurons seen in SMA, it has been speculated if deletions or other major alterations in the SMN1 or SMN2 genes could be associated with ALS. There are today clear evidence that homozygous deletions of the SMN1 gene are not associated with ALS\(^{212-218}\). However, a study by Corcia and colleagues reported an association between ALS and one or three copies of the SMN1 gene\(^{213}\). No such association was, however, seen in the study by Veldink et al.\(^{214}\). On the other hand, this latter group reported that homozygous SMN2 deletions were four times more common in ALS than in controls and that these patients also had a significantly shorter survival\(^{214}\). Mouland and co-workers also reported an association with SMN2 but only in a subgroup of patients with a pure LMN disease\(^{217}\), which also was seen by Echaniz-Laguna et al.\(^{219}\). Other studies have not been able to confirm these findings\(^{212,213,216}\).

The gene for the neuronal apoptosis inhibitory protein (NAIP) is in the close vicinity of the SMN genes and has also been implicated in SMA pathogenesis, since this gene is deleted in a large proportion of SMA cases. However, there is no evidence of the involvement of this gene in ALS\(^{212,214-218}\).

The data so far have thus been contradictory and difficult to evaluate and larger studies are needed to draw safer conclusions about the role of these genes in ALS pathogenesis.

**SOD2:** Manganese superoxide dismutase or SOD2 is one of three human superoxide dismutases the main function of which is to catalyze the degradation of the superoxide anion (see below). SOD2 is a mitochondrial protein, but the gene for SOD2 is located on chromosome 6q25.3. After translation, the SOD2 protein is transported to the mitochondrial matrix, guided by a signal peptide where the protein is metallated with manganese\(^{220}\). The finding that mutations in the hSOD1 gene cause ALS and that SOD2 knockout mice develop a dramatic phenotype with, among other things, limb weakness\(^{221}\) made it plausible that mutant SOD2 could be associated
with ALS. Parboosingh and colleagues screened the 3’ end of the SOD2 gene using single strand conformation analysis (SSCA) and found no association with ALS. Tomblyn et al., on the other hand, sequencing the coding region, found homozygosity for an Ala-9Val substitution in the mitochondrial targeting sequence in 11 of 20 SALS patients and 3 of 10 controls. Although this study was very small, this suggested an association between the Ala-9Val polymorphism and ALS. This finding was further investigated by Van Landeghem et al., who, in a somewhat larger study involving 72 ALS patients and 136 controls, came to the opposite conclusion. Here, the Ala allele was associated with a greater risk of contracting ALS.

Tombkins et al., on the other hand, detected no significant association between ALS and this polymorphism or any other mutations in the gene, but there was a trend towards a lower frequency of Ala in ALS cases ($p=0.08$).

The significance of the Ala-9Val polymorphism is not fully understood. In vitro, the Val allele has been shown to be less efficiently imported into the mitochondrial matrix leading to lower SOD2 activity, but this has not yet been shown in vivo.

The frequency of the polymorphism shows great ethnic variability and this probably explains the divergent results in these studies and also stresses the importance of choosing the correct controls. All in all, the current evidence does not favor the involvement of this gene in ALS pathogenesis.

**VEGF**: Vascular endothelial growth factor (VEGF) is a growth factor for the vasculature that promotes neovascularization. The expression of this gene is partly regulated by hypoxia. A deletion of the hypoxia–response element in the promoter region of this gene in mice surprisingly caused a disease-phenotype similar to motor neuron disease. It was unclear whether the motor neuron death was caused by a decreased vascularization or by a reduction of a more direct neurotrophic effect of VEGF.

This finding suggested that polymorphisms in the promoter region of the human VEGF gene could be associated with ALS. In a large study by Lambrechts et al., it was reported that homozygosity for two specific haplotypes in the VEGF promoter were significantly more frequent in ALS patients than in controls. These haplotypes lead to decreased levels of plasma VEGF and indeed ALS patients also had lower levels of plasma VEGF, reaching only ~50% of controls. It was estimated that the “at risk” haplotypes increased the risk of contracting ALS by 80%. There were no other polymorphisms in other parts of the VEGF gene that were associated with ALS.

Two smaller studies have since investigated the “at risk” VEGF polymorphisms and their relationship to ALS. Terry and co-workers saw a trend similar to that reported by Lambrechts et al., but the association between the haplotypes and ALS was not significant. Brockington and colleagues, on the other hand, reported no association between the VEGF promoter haplotype and ALS.

Other studies have also investigated VEGF levels in ALS patients. Contrary to the report by Lambrechts et al., two smaller studies have reported unchanged or higher levels of VEGF in plasma and serum respectively of ALS patients. The considerably smaller sample sizes in these studies make the results difficult to evaluate, however.

Currently, the evidence favors VEGF promoter polymorphism as a risk factor for ALS. Although the increase in risk seen by Lambrechts et al. was not large, it was
suggested that the “at risk” haplotypes could be responsible for as many as 5-10% of
ALS cases\(^2^{30}\), which is similar to that of mutant SOD1.

There are several other polymorphic genes that have been suggested as risk factors or
susceptibility genes for ALS; they include \textit{CYP2D6}\(^2^{34}\), \textit{MAOB}\(^2^{35}\), \textit{LIF}\(^2^{36}\) and
\textit{PSEN1}\(^2^{37}\). Only single studies have investigated these genes and the significance of
these genes remains to be determined.

To summarize: besides the genes involved in FALS, the only truly established risk
factors for ALS are age and gender. A number of environmental and lifestyle-related
factors have been proposed to provoke the disease, as have a number of polymorphic
genes, but in most cases the data relating to these factors are still inconclusive.
However, environmental and genetic risk factors cannot be totally dismissed. Since
the vast majority of ALS cases are sporadic, it is more than likely that, in sporadic
ALS, a combination of environmental and genetic factors provoke the disease,
perhaps in conjunction with the wild-type forms of proteins involved in FALS in
analogy to what is seen in the case of Alzheimer’s disease, Parkinson’s and
Creutzfeldt-Jakob disease, for example, where proteins clearly involved in sporadic
cases are found mutated in familial cases\(^2^{38}\).

**Superoxide Dismutase 1 – The Protein**

\textit{Free radicals and superoxide dismutases}

Originally described as a copper-containing protein with unknown function,
“hemocuprein” was first isolated from bovine erythrocytes by Mann and Keilin in
1939\(^2^{39}\). The function of this protein was unknown until 1969, when McCord and
Fridovich\(^2^{40}\) reported an enzyme, also purified from bovine erythrocytes, which
catalyzed the dismutation of superoxide radicals (O\(_2\)\(^•\)) to hydrogen peroxide (H\(_2\)O\(_2\))
and oxygen (O\(_2\)) (Reaction 1). This enzyme was shown to be identical to the protein
purified back in 1939 and was now renamed superoxide dismutase (EC 1.15.1.1) to
better reflect the enzymatic function of the protein.

\begin{align*}
\text{(Reaction 1)} \quad \text{2O}_2\text{•}^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\end{align*}

The superoxide radical is, as the name implies, a free radical, meaning that the
molecule has an unpaired electron\(^2^{41}\). The unpaired electron makes the molecule
more prone to react with other molecules (e.g. proteins and lipids) by oxidizing,
reducing, or in other ways modify the molecules. There is therefore an obvious need
for the removal of free radicals. Superoxide dismutases are the enzymes responsible
for the removal of superoxide. The bulk of the superoxide radicals are formed in the
electron-transport chain of mitochondria but superoxide can also be formed by
enzymes such as nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases,
xanthine oxidase, nitric oxide synthase and peroxidases\(^2^{20}\). Despite its name,
superoxide is not as reactive as many other radicals although it is known to react with
for example ascorbate, glutathione and FeS cluster proteins. Instead, superoxide main
toxic potential lays in the formation of more reactive intermediates such as
peroxynitrite (ONOO\(^-\), Reaction 2, also see below) or the hydroxyl radical (OH\(\cdot\)) by the metal (Me) catalyzed Haber-Weiss reaction\(^{220,241}\) (Reactions 3 and 4 and the combined Reaction 5).

\[
\begin{align*}
\text{(Reaction 2)} & \quad \text{NO} + \text{O}_2\cdot^- \rightarrow \text{ONOO}^- \\
\text{(Reaction 3)} & \quad \text{O}_2\cdot^- + \text{Me}^{2+} \rightarrow \text{O}_2 + \text{Me}^+ \\
\text{(Reaction 4)} & \quad \text{H}_2\text{O}_2 + \text{Me}^+ \rightarrow \text{OH}\cdot + \text{OH}^- + \text{Me}^{2+} \\
\text{(Reaction 5)} & \quad \text{O}_2\cdot^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}\cdot + \text{OH}^- \\
\end{align*}
\]

Since the discovery by McCord and Fridovich of CuZn-superoxide dismutase (CuZn-SOD or SOD1), two additional human SODs have been reported. As has already been mentioned, manganese-containing superoxide dismutase (Mn-SOD or SOD2) is found in the mitochondrial matrix\(^{242,243}\). The third human superoxide dismutase is extracellular superoxide dismutase (EC-SOD or SOD3)\(^{244}\). Like SOD1, this SOD is, a copper- and zinc-containing metallo-enzyme. SOD3 has a high homology to the C-terminal part of SOD1\(^{245}\), but, unlike SOD1, the protein is mainly found in the extracellular environment of tissues with a particular affinity for heparan sulfate proteoglycans on cell surfaces\(^{246}\). Tissue SOD3 levels are also more variable. In humans, high levels of SOD3 are, for example, seen in the blood vessel walls, thyroid gland, pancreas and lung tissue, while lower levels are seen in the CNS\(^{247,248}\). As previously discussed, the Ala-9Val polymorphism in the targeting sequence of SOD2 has been suggested as a susceptibility factor for ALS. SOD3 has so far not been implicated in ALS.

Although hydrogen peroxide does not have an unpaired electron (and therefore is not a free radical) it still has the ability to oxidize substrates. However, similar to superoxide, the main toxic potential of hydrogen peroxide lies in the formation of the hydroxyl radical from hydrogen peroxide and reduced transition metal ions (Me\(^{2+}\); Reaction 4), particularly Fe\(^{3+}\), in a reaction known as the Fenton reaction. The hydroxyl radical is highly reactive with almost everything and there is therefore a need to remove hydrogen peroxide. Another advantage of the removal of hydrogen peroxide is that the inactivation of SOD by hydrogen peroxide is reduced. In humans, several enzymes are involved in the removal of hydrogen peroxide. In the peroxisomes, the heme containing enzyme catalase decomposes hydrogen peroxide to water and oxygen (Reaction 6). Another important family of enzymes is the peroxidases, which remove hydrogen peroxide by oxidizing another substrate (S in Reaction 7). Perhaps the most important member of this family is the selenium-containing enzyme glutathione peroxidase (GPX), which catalyzes the oxidation of the substrate glutathione (GSH, Reaction 8). Other proteins involved in the removal of hydrogen peroxide include the thioredoxins and peroxiredoxins\(^{220,241}\).

\[
\begin{align*}
\text{(Reaction 6)} & \quad 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \\
\text{(Reaction 7)} & \quad \text{SH}_2 + \text{H}_2\text{O}_2 \rightarrow \text{S} + 2\text{H}_2\text{O} \\
\text{(Reaction 8)} & \quad 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \\
\end{align*}
\]
As has been discussed previously, SOD2 knockout mice have a severe phenotype with early postnatal lethality, probably due to the very high production of superoxide in the mitochondria. A similar devastating phenotype is not seen for SOD1 null mice, although a range of phenotypic features has been reported. For example, an increased vulnerability to axonal injury, a mild axonopathy, a reduced female fertility, a propensity to develop cataracts, an age-dependent hearing loss and a slight decrease in life span, possibly due to the development of hepatocellular carcinoma.

**Structure**

The crystallographic structure of hSOD1 was first reported in 1992 by Parge and co-workers. In overall terms, the structure was very similar to that of bovine SOD1, which had been thoroughly studied previously. The structure of holo wt-hSOD1 has subsequently been refined at 1.8 Å resolution and there is also an abundance of structural data for other variants of SOD1, including mutSOD1s.

SOD1 is a hydrophilic ellipsoidal dimeric protein about 30x40x70 Å in size. Each subunit consists of 153 amino acids and ligands one copper and one zinc ion. The general structure of the subunit is that of a flattened Greek-key β-barrel. The β-barrel is formed by eight antiparallel β-strands joined by seven external loops (Fig. 1). Loops I, II and V are short β-hairpin connections between adjacent β-strands. Loop IV (residues 49-84) makes up the disulfide bond, the zinc-binding domain and part of the active site cavity. Loop VI (residues 102-114) together with the smaller loop III (residues 37-40) provides the Greek-key connection across the β-barrel, while loop VII (residues 121-144) is an electrostatic loop implicated in substrate attraction.

![Fig. 1 Schematic view of the SOD1 structure as adapted from Parge and co-workers. β-sheets are depicted as open arrows and loops as lines. The β-sheets are enumerated in sequential order using Arabic numerals and the loops are enumerated using Roman numerals.](image-url)
The copper ion is coordinated by the four histidine residues at positions 46, 48, 63 and 120 and also binds a water molecule. Zinc is also coordinated by the histidine residue at position 63 but also by histidine residues 71 and 80 and the aspartate at position 83. The metal ions are buried inside the protein and only the active site copper ion is solvent exposed. To reach the copper ion, the superoxide radical has to pass through a narrow cavity in the protein which, in the end, at the site of the copper ion, is only about 3 Å in diameter. Several negatively charged residues surrounding the cavity and positive charges in the cavity provide an electrostatic field gradient, driving the negatively charged superoxide radical to the copper ion. This arrangement is likely to be important for the high rate of catalysis seen for SOD1 (2x10^9 M^-1s^-1). The purpose of the narrow cleft is probably to avoid unwanted redox reactions between the copper ion and other larger molecules. Zinc does not participate in the redox reactions, but is important for the stability of the enzyme.

There are four cysteines in the hSOD1 protein at positions 6, 57, 111 and 146. The cysteines at positions 57 and 146 form an intrasubunit disulfide bond, a rare feature for a protein mainly residing in the reducing intracellular environment. The bond is of structural importance and provides (un)folding constraints for the protein (see below).

Properties

Expression of SOD1: The hSOD1 gene is located on chromosome 21q22.11 (NCBI) and spans 11 kb of DNA. It consists of 5 exons separated by four introns (Fig. 2) and produces two transcripts 0.7 and 0.9 kb in size. Although the gene contains several regulatory elements, the transcription of the gene in vivo does not appear to be “actively” regulated (unlike SOD2 and SOD3) and SOD1 have even been regarded as a housekeeping gene. In spite of this, activity levels differ widely in different organs. In humans, the highest levels of hSOD1 activity are seen in the liver and kidney, while the brain only contains intermediate levels. The levels of hSOD1 within the human CNS have been measured with in situ hybridization and immunohistochemistry and hSOD1 expression appears primarily in neurons but could also be seen in glia. There is, however, no clear correlation between the amount of hSOD1 in different neuronal populations with the susceptibility to mutant hSOD1s.

SOD1 is mainly a cytosolic protein, but there are also indications, mainly from animal studies, that SOD1 can localize to other cellular compartments such as the mitochondrial intermembrane space, peroxisomes, lysosomes and in the nucleus. Results in this area have, however, been conflicting.

Chemical properties: The hSOD1 dimer has a molecular weight of approximately 32 kDa with a pI of 4.7-4.9. hSOD1 is therefore negatively charged at physiological pH, which could be important for substrate attraction as described above. The activity of the enzyme is, however, almost pH independent in the range of 5.3-9.5.

SOD1 is unusually resistant to proteolysis, thermal inactivation and inactivation by chemical denaturants such as 4% SDS (sodium dodecyl sulfate) and 10 M urea. Nor does long-term storage at 5°C appear to reduce activity. Known
inhibitors of the protein are hydrogen peroxide, cyanide, azide and dietyldithiocarbamate. Interestingly, the high stability of SOD1 is largely dependent on the metal occupancy and the disulfide status of the protein. In fact, disulfide reduction and zinc depletion of copper-deficient wild-type hSOD1 reduced the relative melting temperature from 74.6°C to 42.9°C (a reduction of 31.7°C). These modifications are cooperative, since disulfide oxidation or zinc loading alone only increased the relative melting temperature by 6.9°C and 15.5°C respectively. This means that the melting temperature of disulfide-reduced apo hSOD1 is only slightly higher than the physiological temperature in humans (~37°C). The disulfide status and metal occupancy of hSOD1 also appears to be of importance for the dimerization of the protein, since disulfide-reduced apo hSOD1 appears at least in part as a monomer. Metal depletion or disulfide reduction alone are not sufficient to monomerize the protein in vitro.

**Enzymatic function:** As described above, the enzymatic function of SOD1 is to dismutate the superoxide anion to hydrogen peroxide and water (Reaction 1). This is done in two steps. First, copper is reduced to Cu⁺ by the superoxide radical (Reaction 9). During this step, copper, when reduced, disassociates from the imidazolate ring of H63 and the water molecule. At the same time, the histidine ligand is protonated. Later, in the second step of the dismutation reaction, the copper ion is oxidized by an additional superoxide radical (Reaction 10) and re-associates with H63 and the water molecule.

\[
\text{(Reaction 9)} \quad \text{Cu}^{2+} + \text{O}_2\cdot^- \rightarrow \text{Cu}^+ + \text{O}_2
\]

\[
\text{(Reaction 10)} \quad \text{Cu}^+ + \text{O}_2\cdot^- + 2\text{H}^+ \rightarrow \text{Cu}^{2+} + \text{H}_2\text{O}_2
\]

The protons in Reaction 10 are probably donated from a water molecule and from the H63 residue. At low O₂•⁻ concentrations, the rate-limiting step of the reaction is probably the diffusion rate of the superoxide radical and at higher concentrations the transfer of the protons.

**Copper chaperone for superoxide dismutase**

Due to the high toxic potential of free copper, this cation is tightly regulated in cells. It has been estimated that the amount of intracellular free copper is less than one copper ion per cell. Since the levels of free copper ions in the cells are so low, there is an obvious need for copper transporters. In humans, the copper transporter responsible for the copper loading of SOD1 is termed copper chaperone for superoxide dismutase (CCS). Much of the research on CCS has been done on yeast CCS, but most of these data also appear to be valid for the human protein. Human CCS consists of 274 amino acids and sequence analysis has suggested that this protein consists of three distinct domains. This view has subsequently been confirmed by X-ray crystallography of domains I and II of yeast CCS and of domain II of human CCS.

The N-terminal domain (domain I) consists of residues 1 to 85 and shows high homology to the copper chaperone ATOX1, which is a protein involved in the
transport of copper to copper ATPases in the trans-Golgi network (ATP7A and ATP7B, or the Menkes and Wilson disease proteins). This domain contains an MXCXXC motif that is known to bind copper. The second domain of CCS (residues 86-234) is highly homologous to SOD1. Interestingly, a single mutation in this region of CCS (D201H) is sufficient to give CCS superoxide dismutase activity. The third and shortest domain (residues 235-274) is located at the C-terminal end of the protein. This domain is unique to CCS and, like domain I, this domain also contains a copper-binding CXC motif.

A model for the copper loading of SOD1 has been proposed whereby domain I of CCS acquires copper, probably from the CTR1 copper transporter in the plasma membrane. Domain II of CCS then transiently docks with zinc-charged SOD1 monomer and creates a disulfide-linked heterodimer that subsequently results in the transfer of copper from domain III to SOD1. Currently, the relationship and the handling of copper between the two copper-binding domains are unclear. The heterodimeric formation between human SOD1 and human CCS has been confirmed with X-ray crystallography and is not surprising, considering the high homology of SOD1 and CCS domain II. Human SOD1 is also relatively promiscuous and can dimerize with SOD1s from quite distantly related organisms. Interestingly, studies in yeast have suggested that CCS is also important for the formation of the intrasubunit disulfide bond of SOD1. The exact mechanism for this is not known, but it probably involves the exchange of the intermolecular disulfide bond between SOD1 and CCS to an intramolecular disulfide bond in SOD1.

The expression of human and mouse CCS mirrors that of SOD1 and the protein, like SOD1, appears to be mainly cytosolic. Levels of SOD1 are, however, 12 to 30 times higher than CCS. Recent findings in yeast suggest that about 1-5% of CCS is located, together with SOD1 (see above), in the mitochondrial intermembrane space. Intriguingly, the uptake of SOD1 into mitochondria appears to depend on CCS, since CCS null yeast has lower levels of SOD1 in the mitochondrial intermembrane space. The form of SOD1 that preferentially enters the mitochondria is (monomeric) disulfide-reduced apo SOD1 which, within the intermembrane space, is disulfide oxidized by CCS which helps retain SOD1 within the mitochondria. The copper loading of SOD1 by CCS is not necessary for retention, but SOD1 most probably has to be zinc loaded to interact with CCS. The mechanism by which disulfide-reduced SOD1 and CCS enter the mitochondria or the reason why the disulfide oxidized enzyme is retained is not known. Presumably, the function of the mitochondrial intermembrane space SOD1 is to detoxify superoxide radicals generated within the mitochondria, possibly as a second line of defense, where SOD2 is the first line.

CCS knockout mice are viable and show no overt phenotype. Like SOD1 knockout mice, these mice suffer from reduced female fertility and are extra sensitive to the free radical-generating substance paraquat. As expected, the SOD1 activity levels are dramatically decreased to about 15-30% of those of control mice, which corresponds to the decrease in copper loading of SOD1 that is seen. It has been suggested that the residual SOD1 activity seen in CCS null mice depends on reduced glutathione for the insertion of copper into SOD1.
Superoxide Dismutase 1 and ALS

Features of ALS caused by mutant hSOD1s

hSOD1 mutations: At present, 119 different mutations in the hSOD1 gene have been associated with ALS (P.M. Andersen personal communication). Most of the mutations are missense mutations, but some mutations cause alterations in the reading frame leading to C-terminal truncations and in some cases the insertion of amino acids. Mutations have been found in all five exons of the hSOD1 gene and are not restricted to a specific part of the folded protein. Relatively few mutations have, however, been found in exon 3 and the N-terminal part of exon 2 is so far completely devoid of mutations. There are some examples of intronic mutations that cause aberrant splicing of the hSOD1 transcript. Silent mutations with no amino acid alteration have been reported in some ALS patients, but the importance of these mutations is not known.

Pattern of inheritance: ALS caused by hSOD1 mutations is inherited dominantly, with the exception of ALS caused by the D90A mutation that is inherited both as a recessive and a dominant trait (see below). The penetrance of ALS caused by mutSOD1 is high. A large US study reported a penetrance of 92% at the age of 70. Some mutations, e.g. the I113T mutation, have, however, been associated with reduced penetrance in FALS. Interestingly, the I113T mutation is also surprisingly frequently seen in “SALS” but it appears as if some of these “SALS” cases actually represent FALS with reduced penetrance. A few I113T families with high penetrance have, however, been reported.

Epidemiology: In FALS, about 20% of the families have a mutation in the hSOD1 gene. The incidence of mutSOD1-FALS could thus be calculated at about 2% (20% × 10%) of ALS cases, which is often the number given in the literature. In reality, however, the incidence is probably much higher, since hSOD1 mutations are seen in about 3-7% of apparently sporadic ALS cases. In a recent large US study, comprising 2,045 unspecified ALS patients, the frequency of hSOD1 mutations in ALS was 7.2%, which supports the notion that about 5% of SALS cases are associated with hSOD1 mutations. It is likely that some of these SALS cases represent FALS with reduced penetrance, as discussed above, but there is also a possibility of de novo mutations. The most common mutation worldwide is the D90A mutation that exists as a polymorphism in the northern part of Scandinavia (see below). This mutation is probably also responsible for more ALS cases than any other mutation, given the reduced penetrance of this mutation (P.M. Andersen personal communication). In the US, the A4V mutation is the most common mutation and is found in about 50% of mutSOD1-FALS cases. Elsewhere in the world, there are only a few reports of this mutation. The high prevalence of this mutation in the North American population is probably the result of a founder effect during the early colonization of North America, since a FALS pedigree described as early as 1880 by William Osler, later turned out to carry this mutation. I113T appears to be the third most common mutation.

Phenotype of mutant hSOD1-FALS: In overall terms, ALS caused by mutSOD1s is indistinguishable from sporadic classical ALS but similar to adult-onset FALS.
linked to other genes (Table 1); the mean onset is somewhat earlier than for SALS and the gender ratio is closer to 1:1. The clinical phenotype is dominated by lower motor neuron symptoms and in some patients extramotor involvement is evident. Neuropathologically, mutSOD1-FALS patients are similar to SALS patients, but dorsal column involvement and LBHI are possibly more common and the corticospinal tract is less affected in analogy to the predominant LMN symptoms. In the spinal cord, inclusions containing SOD1 have been described, but similar inclusions have also been described in a few SALS cases.

In general, there is a poor correlation between genotype and phenotype for most mutant hSOD1s. Clinical characteristics, age at onset and disease duration differ widely between and among the different mutant hSOD1s, even within the same family. Environmental and/or other genetic factors most probably influence the phenotype. There are, however, some exceptions. Patients carrying the A4V mutation generally present with a rapidly progressing mainly lower motor neuron disease with a mean survival of only about one year and recessively inherited D90A ALS is associated with a slowly progressing stereotypic somewhat atypical phenotype (see below). Recent studies on recombinant proteins have also reported an association between the stability of the protein and disease duration (see below). Genotype-phenotype studies are, however, generally hampered by the small number of patients for most mutations.

Properties of mutant hSOD1s
The biochemical and biophysical properties of mutSOD1 have been investigated in a number of studies and have been shown to vary widely. In general, most mutSOD1s appear perturbed compared with wt-hSOD1. For example:

- The SOD activities of mutSOD1 in ALS patients range from 0% to close to 100% (see for references).

- Several mutSOD1s showed decreased resistance to proteolytic treatment with proteinase K, but G37R, G93C and I113T appeared to be as resistant as the wild-type protein.

- In cells transfected with mutant and wild-type hSOD1, the half-life was considerably shorter for most mutant hSOD1s, but some mutant hSOD1s, e.g. H46R and H48Q, appeared to be almost as stable as wt-hSOD1.

- There are indications that the zinc-binding affinity and metal ion specificities are weakened in mutSOD1 and some mutSOD1s also show a significant reduction in both copper and zinc content compared with wt-hSOD1 (sometimes referred to as metal-binding region mutSOD1s). Other mutSOD1s, however, have a metal-binding capacity similar to wt-hSOD1 (wild-type like mutSOD1s).

- “Metal-binding region” mutSOD1s have been shown to be more sensitive to disulfide reduction, which may lead to decreased resistance to
proteolytic digestion. Some mutSOD1s are, however, almost as resilient to disulfide reduction as wt-hSOD1 (e.g. D90A and G93A).  

- The thermal stability of as isolated mutant and wild-type hSOD1s has been evaluated with differential scanning calorimetry and found to reflect the metal-binding capacity of the different mutSOD1s (see above). Most mutant hSOD1s, however, display a decrease in stability compared with wt-hSOD1. In cases of disulfide-reduced, metal-deficient hSOD1, the mutant protein even unfolds at temperatures well below the physiological temperature in humans (~37°C). However, the melting point for as isolated D90A and G93A was not that unlike wt-hSOD1 and a recent study by Rodriguez and colleagues shows that some disulfide-reduced apo mutSOD1s (e.g. H48Q and D101N) have melting temperatures very similar to wt-hSOD1.

- The unfolding behavior of disulfide-reduced wild-type and mutant hSOD1s when exposed to guanidinium chloride or urea was evaluated by Lindberg et al. using circular dichroism. In this case, all mutSOD1s displayed a reduction in stability compared with wt-hSOD1, more when deficient in metal. The difference between the wild-type protein and D90A was, however, not that great. Similar results have also been reported by others.

- Lindberg et al. also measured the folding rates of monomeric and dimeric mutant hSOD1s using stopped flow technique, with urea as the denaturant, and compared the folding rates with wt-hSOD1. They were able to divide the mutants into three groups – one group that affected the stability of the monomer (class 1), the second group included one mutant (L144F) that only affected the stability of the dimer interface (class 2) and the third and dominant group included the mutants that affected both the stabilities of the monomer and the dimer interface (class 1+2). Interestingly, an association between the stability of the mutant protein and disease duration was suggested.

- Compared with wt-hSOD1, mutSOD1s have a greater affinity for phenyl-sepharose and bind 8-anilino-1-naphthalene-sulfonic acid (ANS) better suggesting an increase in hydrophobic surfaces.

**Mechanism of disease**

It is generally agreed that mutSOD1s cause ALS by the gain of a toxic property rather than a loss of enzymatic function. The main evidence for this is that:

1. The disease is inherited dominantly.
2. The D90A mutation retains full SOD activity in human erythrocytes and CNS.
3. Some mutSOD1 transgenic mice develop a phenotype similar to MND, despite higher than normal SOD1 activity.

4. SOD1 knockout mice do not develop a MND phenotype.

The nature of this toxic property of mutSOD1 is not known, but a number of disease mechanisms have been proposed and some of them are discussed below.

**SOD1 transgenic mice:** To facilitate research in this area, a number of model systems have been used to study the mutant protein and its consequences (as discussed in the methodology section). Transgenic mice and rats expressing different mutSOD1s have been very valuable for our understanding of the disease and the noxious property of mutSOD1s. A number of such strains have been generated and some of them are summarized in Table 2.

### Table 2. Mutant SOD1 transgenic rodents

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Promoter</th>
<th>Specie</th>
<th>MND phenotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>A4V hSOD1 gene</td>
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<td>Mouse</td>
<td>No</td>
<td>352</td>
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<td>G127insTGGG hSOD1 gene⁹</td>
<td>hSOD1</td>
<td>Mouse</td>
<td>Yes</td>
<td>This thesis</td>
</tr>
</tbody>
</table>

¹ In the CNS, expression is mainly in neurons and glia
² Expression in neurons only
³ Also known as the “Quad” mutant
⁴ Expression in neurons only. Green fluorescent protein containing construct
⁵ Expression in astrocytes only
⁶ Both high and low expressing lines are frequently used
⁷ Introns 3 and 4 were deleted in the construct. Stop at 126
⁸ Also with a FLAG containing construct. Stop at 131
⁹ Stop at 133
The neuropathology of these mouse strains mainly recapitulates the findings in ALS patients, including motor neuron loss, reactive gliosis, LBHIs, spheroids and ubiquitin-positive inclusions (including skein-like inclusions). Some deviating features are, however, seen. Perhaps the most conspicuous of these is the presence of vacuoles seen in the CNS of G93A, G37R and even wt-hSOD1\textsuperscript{368} transgenic mice and rats. These vacuoles are thought to emanate from swollen mitochondria\textsuperscript{353}. Interestingly, transgenic mice and rats expressing mutSOD1s that are deficient in copper binding (e.g. H46R, H46R/H48W and G85R) do not develop these vacuoles. Another deviating feature is the lack of Bunina bodies in the transgenic mice.

In mutSOD1 transgenic mice, there is an gene-dosage effect on the phenotype, since mice that are homozygous for the transgenic insertion show a much more rapid disease progression\textsuperscript{354,366,367}, while mice with a loss of copy numbers have a protracted disease course\textsuperscript{367}. Interestingly, double G93A/wt-hSOD1 transgenic mice have been reported to have an accelerated disease progression compared to G93A hSOD1 alone, suggesting that wt-hSOD1 itself could be harmful\textsuperscript{368,369} (see also Results and Discussion). This was not, however, seen in G85R/wt-hSOD1 transgenic mice\textsuperscript{370}. It has been suggested, although this was not convincingly demonstrated, that this difference was due to the stabilization of the G93A, but not the G85R protein, by heterodimerization with wt-hSOD1\textsuperscript{369}. A gene-dosage effect may also be relevant in humans. In the literature, there is one report of an ALS patient homozygous for mutant SOD1 (besides the special case of recessively inherited D90A discussed below). This patient, who was homozygous for the N86S mutation, suffered from an aggressive form of ALS and died at the age of just 13 years with a very short disease duration of only 14 weeks\textsuperscript{371}. Most interestingly, two recent studies have reported that \textit{hSOD1} gene silencing with RNA interference (RNAi) in G93A transgenic mice dramatically improves onset, progression rate and survival\textsuperscript{372,373}. A similar strategy could be feasible in humans and could potentially be a new therapeutic alternative for ALS patients.

The cell type(s) in which mutSOD1s are toxic is(are) not known. The specific expression of mutSOD1 in astrocytes\textsuperscript{363} or neurons\textsuperscript{355,361} in transgenic mice has failed to provoke disease. Studies of mutSOD1 chimeric mice\textsuperscript{374} and glial and neuronal co-culture experiments\textsuperscript{375} have suggested that mutSOD1 have to be present in multiple cell types to cause motor neuron degeneration. Concerns have, however, been raised about the expression levels of mutSOD1 in strains with cell-specific expression of mutSOD1\textsuperscript{365}. An absolute amount of mutant protein was only given in the report from Pramatarova et al. and, compared with our own measurement of hSOD1 in the spinal cord of D90A transgenic mice, the levels of \textit{NFL} regulated H46R\textsuperscript{355} correspond to about one quarter of the levels of D90A mutant SOD1 in homozygous D90A mice (Papers III, V). The levels of \textit{THY1} regulated G85R have now also been re-calculated to be less than 5% of endogenous mouse SOD1 (mSOD1)\textsuperscript{376}. These levels of the mutant protein could possibly be too small to cause disease. A recent study by Wang and colleagues showed that the expression of mutSOD1 in muscle cells, astrocytes and neurons but not microglia is sufficient to cause disease\textsuperscript{354}. This finding most likely excludes microglia as a primary participant in the motor neuron death mechanisms.
Apoptosis: Today, there is a considerable amount of evidence supporting a role for apoptosis (or programmed cell death) in ALS pathogenesis. Some of the main evidence is briefly summarized below.

In neuronal cells transfected with mutSOD1s, there are signs of DNA fragmentation characteristic of (although not exclusively seen in) apoptosis, as well as signs of caspase activation in distressed mutSOD1-expressing cells. Furthermore, treatment with caspase inhibitors or co-expression with the anti-apoptotic protein Bcl2 attenuates cell death in these cells.

In tissues from mutSOD1 transgenic mice (and humans), it is difficult to detect apoptotic cells, since the process itself is rapid and little trace can be discerned after the process is over. The daily rate of motor neuron loss is probably also quite low. The morphology of cells undergoing apoptosis in tissues is also somewhat unspecific. This may be the reason why some studies of apoptosis in mutSOD1 transgenic mice have been negative. It might therefore be a better choice to rely more on biochemical investigations in order to study apoptosis in vivo. The activation of caspases in neurons (and possibly also glia) in the CNS of mutSOD1 transgenic mice has been reported by several groups. Interestingly, in one study, the activation of caspase-1 was seen at just one month of age in G85R mice, with the activation of caspase-3 occurring much later. Vukosavic et al. reported similar results of the temporal activation of caspases in G93A mice. This sequence of activation is also in agreement with the known activation cascade of caspases. Dominant negative inhibition of caspase-1 in neurons significantly prolonged survival in G93A mice, as did intracerebroventricular treatment with the broad caspase inhibitor zVAD-fmk. The caspase-1 downstream mediator interleukin-1β (IL-1β) has also been reported to be upregulated in the spinal cords of mutSOD1 (G37R) transgenic mice, although on an IL-1β null background there was no difference in survival. G93A transgenic mice on a caspase 11 knockout background resulted in a decrease in caspase 1- and 3-like activities, but did not lead to enhanced survival. For this reason, caspase 11 does not appear to be needed to generate the MND phenotype of G93A transgenic mice. The lack of effect could represent redundancy in which other caspases may compensate for the lack of caspase 11.

Cytochrome C is important in mitochondrial-associated caspase activation and it has been shown that this protein translocates from the mitochondria to the cytosol in the spinal cords of G93A mice. One caveat is naturally that these mice have distended vacuolated mitochondria which could possibly interfere with the analysis. However, wt-hSOD1 transgenic mice, which also have vacuolated mitochondria, did not show any signs of cytochrome C translocation.

The levels of the anti-apoptotic proteins Bcl2 and Bcl-xL have been shown to be decreased in spinal cord neurons of mutSOD1 transgenic mice, while pro-apoptotic Bak and Bax are increased. A recent study also indicated that both wild-type and mutant hSOD1s were able to bind to Bcl-2. However, in mitochondrial preparations from mutSOD1 transgenic mice, only mutSOD1s co-precipitated with Bcl-2 and it was suggested that aggregated mutSOD1 could entrap Bcl-2 and thereby promote apoptosis. Several reports have shown that the overexpression of Bcl2 in mutSOD1 transgenic mice attenuates caspase activation and the amount of unbound Bax, resulting in a significant prolongation in survival.
In humans, there are few reports of apoptosis in mutSOD1-FALS. Pasinelli and colleagues investigated an A4V patient and showed that hSOD1 in this patient bound to Bcl-2. In sporadic ALS cases without hSOD1 mutations, the activation of caspases has been seen in the spinal cord and more specifically in motor neurons. The levels of proteins in the Bcl-2 family in ALS patients are more difficult to evaluate, but there are signs of upregulation of Bak and Bax and downregulation of Bcl-2 in the spinal cords of ALS patients. Finally, there is one report suggesting translocation of cytochrome C from the mitochondria to the cytosol in motor neurons of ALS patients.

**Mitochondrial pathology:** In several of the mutSOD1 transgenic mouse strains, there is evidence of vacuolated mitochondria. The temporal occurrence of these vacuoles appears to precede the occurrence of symptoms in mutSOD1 transgenic strains, which suggests the involvement of these organelles in mutSOD1-FALS pathogenesis. The vacuolation of mitochondria has been ascribed to the entry of hSOD1 into the intermembrane space of mitochondria. Vacuolization could therefore be dependent on CCS. However, overexpression of mutSOD1 on a CCS null background did not show any “gross differences” in histopathology.

There are several ways in which injured mitochondria may be toxic to motor neurons. One possibility is that cytochrome C in the intermembrane space leaks into the cytosol and thereby initiates apoptosis. Another possibility is that the normal mitochondrial function becomes impaired. In mitochondrial fractions from the CNS of G93A mice, a decrease in the maximum respiratory rate and a lowering of mitochondrial complex activities have been reported. A similar decrease in cytochrome C oxidase (COX or complex IV) activity was also seen in the motor neurons of ALS patients by staining tissue sections for COX activity. In CNS homogenates from ALS patients, the results have been conflicting. Two studies have shown a decrease in COX activity in the spinal cord of ALS patients but not when corrected for the amount of mitochondria by measuring citrate synthase. In the brains of ALS patients, studies of COX activity have been negative, while complex I-III activities have shown variable results. It is, however, possible that the defect in energy metabolism is only a secondary effect of already injured neurons. The fact that the effect on respiratory enzymes and rate was especially prominent in symptomatic mice is compatible with this theory. Other proposed mitochondrial disease mechanisms include the association between mitochondria and mutSOD1-containing aggregates in mutSOD1 transgenic mice, with a subsequent decrease in Bcl-2.

The findings described above suggested that treatment with mitochondrial protective agents would be beneficial. Indeed, supplementation with creatine, which could function as an energy buffer in cells and/or inhibit mitochondrial transition pore openings, prolonged survival in G93A mice and signs of free radical damage were attenuated. Similarly, treatment with the anti-inflammatory and anti-apoptotic drug minocycline, which has also been shown to inhibit mitochondrial cytochrome C release, significantly enhances survival in G37R and G93A transgenic mice. A drug cocktail using both these substances has shown an even greater effect on survival.
In human clinical trials, creatine has failed to prove effective\(^{419,420}\) and a minocycline phase III study is currently ongoing\(^{421}\). The failure of creatine suggests that the mitochondrial abnormalities seen in some mutSOD1 transgenic strains are not relevant to ALS pathogenesis. There are also other reasons why the vacuolated mitochondrial may prove to be irrelevant.

- Not all mutSOD1 transgenic strains develop mitochondrial vacuoles (see above).
- Vacuoles are almost never seen in spinal cord sections from ALS patients\(^{422}\).
- Mice transgenic for wt-hSOD1 also develop vacuoles but do not develop overt MND\(^{402}\).

**Oxidative stress:** It has been suggested that aberrant copper chemistry at the active site of mutSOD1s could be responsible for motor neuron death. Two substrates have been proposed for the copper ion, nitric oxide and hydrogen peroxide.

Nitric oxide can react with the superoxide radical and form the highly reactive peroxynitrite (Reaction 2) Peroxynitrite could subsequently, by widening of the active site cavity of mutSOD1, react with Cu\(^{2+}\) to form a nitronium intermediate (Reaction 11) that could nitrate tyrosine residues of proteins (Reaction 12), which could be harmful to neurons by blocking phosphorylation sites, for example\(^{423,424}\).

\[(\text{Reaction 11}) \quad \text{Cu}^{2+} + \text{ONOO}^– \rightarrow \text{CuO}···\text{NO}_2^+ \]

\[(\text{Reaction 12}) \quad \text{CuO}···\text{NO}_2^+ + \text{Tyr-H} \rightarrow \text{Cu}^{2+} + \text{HO}^– + \text{Tyr-NO}_2 \]

Another route in which nitric oxide reacts with a superoxide intermediate of SOD1 to create peroxynitrite has also been suggested (Reactions 13 and 14)\(^{425}\).

\[(\text{Reaction 13}) \quad \text{Cu}^+ + \text{O}_2 \rightarrow \text{Cu}^{2+}···\text{O}_2\cdot^– \]

\[(\text{Reaction 14}) \quad \text{Cu}^{2+}···\text{O}_2\cdot^– + \text{NO} \rightarrow \text{Cu}^{2+} + \text{ONOO}^– \]

Increased levels of free nitrotyrosine have been observed in the spinal cords of both mutSOD1 transgenic mice\(^{424,426}\) and ALS patients with and without hSOD1 mutations\(^{427}\). In spinal cord sections of both transgenic mice and ALS patients, there is also evidence of increased levels of nitrotyrosine\(^{424,427-429}\). With regard to the levels of protein-bound nitrotyrosine in the spinal cords of mutSOD1 mice and ALS patients, findings have been contradictory\(^{426,430}\).

Importantly, the survival of G93A mice that are null for the inducible\(^{431}\) or neuronal\(^{432}\) nitric oxide synthase is similar to that of ordinary G93A mice, suggesting that the levels of nitric oxide are not important in ALS pathogenesis. The levels of nitric oxide in these mice were, however, not reported.

The other proposed substrate is hydrogen peroxide. It is known that SOD1 can function as a peroxidase and thereby generate highly reactive and potentially toxic
hydroxyl radicals, similar to the Fenton reaction (Reaction 4). Wideau-Pazos and co-workers measured the hydroxyl radical production of recombinant A4V and G93A mutSOD1s using a DMPO spin trap assay and found an increase in hydroxyl radical formation compared with wt-hSOD1. Similar to the peroxynitrite hypothesis, they speculated that mutation could confer a widening of the active site cavity that would give the substrate greater access to the active site\(^{433}\). Evidence supporting an increase in hydroxyl radical formation from mutSOD1s has also been seen in the striatum of G93A transgenic mice using microdialysis\(^{434}\). Other groups have reported similar results\(^{435,436}\), but these results have also been questioned, since not all groups have noted such differences between the wild-type and the mutant enzyme\(^{281,426,437,438}\). The DMPO spin trap assay has also been criticized, since it may not be specific for free hydroxyl radicals\(^{438,439}\).

Today, the active site copper hypothesis is less favored. Some of the evidence speaking against the hypothesis has already been mentioned, but there is also additional evidence. For example:

- Some mutSOD1 have very low copper affinity\(^{343,345}\).
- Knocking out the CCS gene in mutSOD1 transgenic mice does not attenuate disease\(^{404}\).
- Mice transgenic for the artificial “Quad” mutant that is lacking all four copper-binding histidines still develop MND disease\(^{359}\).

**Mutant hSOD1-containing aggregates and inclusions:** One conspicuous finding in the spinal cords of older mutSOD1 transgenic mice is the occurrence of neuronal\(^{366,440}\) and astrocytic\(^{360,370,441}\) SOD1-containing inclusions. These inclusions can be found in the soma as well as in the neuropil and are sometimes associated with LBHIs\(^{366,440,442}\). Not all inclusions, however, stain for SOD1\(^{441}\). Interestingly, similar inclusions (predominantly LBHIs) are also seen in the spinal cords of ALS patients with\(^{442,443}\) and without SOD1 mutations\(^{44,85,422}\). These inclusion also stain positive for other proteins; they include ubiquitin\(^{360,422,440,441,443,444}\), neurofilaments\(^{422,440,443}\), CCS\(^{441,442}\), heat shock proteins\(^{441,444,445}\), proteasome 20S\(^{441}\), NEDL1\(^{446}\), dorfin\(^{447}\) and the glial fibrillary acidic protein (GFAP)\(^{360,441}\). Some spinal cord inclusions also stain with thioflavin, which is a specific stain for fibrillar aggregates that are rich in β-sheets\(^{354,358,359}\). Such staining, although very weak, was also seen for in vitro aggregated protein filaments of mutSOD1s\(^{346,348,350}\).

In spinal cord homogenates of symptomatic mutSOD1 transgenic mice, SOD1-containing detergent-resistant aggregates can be detected by filter trap assays\(^{354,358,359,444,448}\) or by different centrifugal techniques\(^{354,359,365,444,449,450}\). In the detergent-resistant aggregates, high molecular weight (HMW) species of SOD1 are seen, suggesting the enrichment of these species in aggregates\(^{354,359,451}\). Similar detergent-resistant aggregates have also been seen in an A4V patient\(^{359}\). It is probable that these aggregates, at least in part, represent the SOD1-containing inclusions discussed above.
Three major proteolytic systems participate in the turnover of proteins. The ubiquitin/proteasome pathway functions both as a degradation route for short-lived (regulatory) proteins and as a quality control system in which mis/unfolded proteins are degraded by the proteasome proteolytic complex after being covalently tagged with polyubiquitin. The process of ubiquitination is not known in detail but depends on three types of enzymes. An E1 ubiquitin-activating enzyme activates ubiquitin in an ATP dependent manner. Subsequently, the activated ubiquitin is transferred to the substrate by binding to an E2 ubiquitin-conjugating enzyme. The ubiquitin containing E2 enzyme then interacts with an E3 ubiquitin-protein ligase that is responsible for substrate recognition and ligation of ubiquitin to the substrate. The polyubiquitinated substrate is then recognized by the 19S proteasome of the 26S proteasomal complex, deubiquitinated, unfolded and degraded by the 20S proteasome. An increasing number of proteins are, however, recognized to be degraded by the 26S proteasome without prior ubiquitination and are, in some cases, degraded directly by the 20S proteasome without processing by the 19S proteasome. 

Calpains are a family of calcium-dependent cysteine proteases that participate in the degradation of proteins in the membrane or in the cytosol. The regulatory mechanisms of calpains are poorly understood. The third system is the autophagic/lysosomal system which is thought to play a major role in the turnover of long-lived proteins. Autophagy occurs when a membrane surrounds a part of the cytoplasm, creating an autophagosome, and then fuses with lysosomes where proteins are degraded by lysosomal proteases. Although mainly a non-selective process, autophagy can in some instances be more specific, for example by eliminating injured organelles. The signals regulating the process of selective autophagy are, however, unknown. The rate of autophagy could be regulated both positively and negatively.

Little is known about the way wild-type SOD1 is degraded. In vitro, it has been demonstrated that apo hSOD1 is degraded by the proteasome in an ubiquitin-independent manner. Additional data comes from cell culture studies using proteasome inhibitors where it has been demonstrated that the wild-type protein is at least partly digested by the proteasomal system but also that the system is responsible for the digestion of a much larger proportion of the mutant enzyme. In these cell-culture experiments, proteasome inhibition causes a change in the distribution of the mutant protein into inclusions or aggregates and decreases cell viability. The overexpression of mutSOD1 in cells has also been associated with a decrease in proteasome activity, but the results in this area are conflicting and a similar decrease in proteasome activity was not seen in the spinal cords of G93A mice, even though the levels of some proteasomal subunits were altered. Interestingly, the overexpression of dorfin, one of two known mutSOD1 E3-ubiquitin ligases (the other one is NEDLI) and also appearing in LBHIs of ALS patients and mice, in mutSOD1-expressing cells was shown to enhance survival and decrease the amount of inclusions.

There is also evidence to support a role for the phagocytic/lysosomal system in the degradation of SOD1. For example, immuno-electron microscopy has shown that SOD1 can be localized to lysosomes, as well as autophagic vesicles, and SOD1 was enriched in lysosomal fractions from density gradient centrifugation.
although the amount of SOD1 that was associated with lysosomes in some studies probably is an overestimation.

Heat shock proteins (HSP) are a group of highly conserved proteins that are induced in response to environmental stress by infection, ultraviolet radiation, chemicals, oxidative stress and of course heat, among other things. The HSP proteins are grouped in families according to their size (HSP 100, HSP90, HSP 70 etc.) and the smallest are referred to as the small HSPs (e.g. HSP27, HSP10 and αB-crystalline). Ubiquitin has also been included in this group of proteins. The classical role of HSPs is to function as molecular chaperones and to participate in the folding of proteins but also to help in the handling of misfolded proteins. Another function is to mediate in the degradation of proteins. Studies of cells expressing mutSOD1s have indicated that HSPs could be upregulated in response to the expression of mutSOD1. For example, Bruening and co-workers showed that mutSOD1-expressing NIH 3T3 cells had higher levels of HSP27, HSP70 and αB-crystalline, which corresponded well to the increase in chaperoning activity seen in these cells. Studies of mutSOD1 transgenic mice have shown a more complex picture. In the same paper, Bruening et al. reported a decrease in chaperoning activity in the lumbar spinal cords of presymptomatic G93A mice and it was therefore suggested that the abundant mutSOD1 protein depleted chaperones in ALS-affected areas. Similar results were also reported by Tummala et al. investigating spinal cord homogenates from G93A but also G85R mice. When examining the levels of the different HSPs, a temporal pattern of expression has been reported. In the spinal cords of presymptomatic mutSOD1 transgenic mice, the levels of HSP25/27 and αB-crystalline are equal to or lower than those of control mice, but the levels increase in symptomatic mice, possibly as a result of gliosis. The levels of HSP40, HSP60, HSP70 and HSP90 have been reported to be mainly unchanged. The pattern of expression of the small HSPs suggested that the high levels of expression of these proteins in glia protected these cells in mutSOD1 transgenic mice, while motor neurons had lower levels. In spinal cord sections of ALS patients, the relative HSP27 intensity was similar to that of controls.

It is difficult to understand why the spinal cords of mutSOD1 transgenic mice display a reduction in chaperone activity, but at the same time HSP levels are increased. Tummala et al. investigated this enigma and suggested that the lack of concordance could be due to the inactivation of HSPs by mutSOD1 and reported that, by adding recombinant mutSOD1s to spinal cord lysates, chaperone activity is inhibited dose dependently. In their assay, however, the possibility was not excluded that the mutSOD1s were preferentially chaperoned by HSPs rather than the reporter protein, catalase.

There is also more direct evidence of an interaction between mutSOD1 and HSPs. Co-immunoprecipitation experiments in cells transfected with different hSOD1s have shown that HSP25, HSP40, HSP70 and αB-crystalline interact with mutSOD1 but not wt-hSOD1. HSPs have also been found in mutSOD1-containing aggregates and inclusions, but Maatkamp et al. failed to see a more direct association between HSP25 and mutSOD1.

Interestingly, in an in vitro assay of mutSOD1 aggregation, it was found that αB-crystalline attenuated the formation of aggregates. Similarly, Bruening and
colleagues transfected a primary motor neuron culture with G93A and HSP70 and saw a decrease in inclusions and toxicity compared with cells expressing G93A alone\(^{466}\). Subsequent cell-culture studies have provided similar results\(^{382,470}\). Although overexpression of HSP70 alone in G37R, G85R and G93A mice did not alter disease\(^{445}\), treating G93A mice with arimoclomol, an inducer of HSPs, results in a remarkable improvement and an increase in mean survival from 125 days to 153 days\(^{471}\).

Recently, mutations in the two small HSPs, HSP27 and HSP22 was linked to two dominantly inherited neuromuscular diseases, Charcot-Marie-Tooth 2F\(^{472}\) and distal hereditary motor neuropathy type II\(^{473}\) respectively.

It is well known that almost all proteins, including proteins completely consisting of \(\alpha\)-helical structures, can form fibrils under certain circumstances\(^{474}\). The way this process occurs in the case of mutSOD1 is not known in detail. For the protein to form aggregates unfolding (or misfolding) of the protein is a necessity. As has been discussed above, SOD1 is considerably more prone to unfold in its disulfide-reduced apo form and, when mutated, the protein is even less stable. Different in vitro aggregation assays have also shown that the form of the protein most prone to aggregate is the monomeric mutant apo protein\(^{348,350,359,475}\). Strikingly, Stathopoulos et al. were able to demonstrate an inverse correlation between the thermal stability of mutated and wild-type apoSOD1s and the propensity to aggregate\(^{346}\). The importance of disulfide reduction in aggregation has not been reported.

One example of the way SOD1 aggregation may occur was given by Elam et al., who examined the crystal structure of apo S134N and H46R. They showed that these mutSOD1s crystallized into three different crystal forms through interactions between loops and the \(\beta\)-barrel. The filamentous structure was formed by conformational changes in loop IV and VII (Fig. 1) that caused the deprotection of the edges of \(\beta\)-strands 5 and 6, providing new contact surfaces leading to oligomerization\(^{476}\). Electron and atomic force microscopy of in vitro aggregated mutSOD1 have revealed both amorphous aggregates\(^{346,350}\) and more fibrillar structures\(^{346,348,350,477}\). Interestingly, similar fibrils, 15-25 nm in diameter and immunoreactive for SOD1, have also been seen in the hyaline inclusions of ALS patients\(^{478}\) and mutSOD1 transgenic mice\(^{478,479}\). Intriguingly, it has also been reported that metal-depleted wt-hSOD1 also aggregates, but that wt-hSOD1 was comparatively less prone to form fibrillar aggregates than mutSOD1\(^{348,350,475,477}\).

**D90A mutant hSOD1**

**D90A mutant hSOD1 in recessive ALS pedigrees:** Of all hSOD1 mutations identified so far, only D90A cause ALS with a recessive pattern of inheritance\(^{480}\). In the northern part of Scandinavia, this mutation exists as a polymorphism with an allele frequency of about 2% and, in Finland alone (population 5,236,611, Dec 31, 2004, Tilastokeskus), there should be approximately 99,000 carriers of this mutation\(^{481}\). Patients homozygous for the D90A allele have also been found in other parts of the world including Germany\(^{482}\), France\(^{483}\), Italy\(^{328,484}\), Russia\(^{485}\), Canada\(^{486}\) and the USA\(^{311}\). The penetrance of the disease in recessive D90A pedigrees has been calculated to 95% at the age of 70 years\(^{487}\). The mean age at onset is 44 years, ranging from 20-94 (!) years. The homozygous D90A patients suffer from a
characteristic stereotypic phenotype. Onset is characterized by stiffness and cramps in the legs and a feeling of general fatigue. Lumbar back pain is common. Paresis always starts in the lower limbs and then slowly progresses. Later, symptoms from the upper limbs and the brain stem nuclei are seen. UMN symptoms are abundant. Some patients also experience paresthesias and micturition problems. Mean survival has been calculated to 13 years, but some patients have a disease duration of more than 20 years.

**ALS in individuals heterozygous for D90A mutant hSOD1:** Most fascinating and probably unique to genetics, this mutation has also been associated with ALS in dominant FALS pedigrees and the mutation has also been found in SALS in geographical locations where the mutation is otherwise rare, suggesting that the mutation in these cases are disease-causative although with low penetrance. The dominant D90A ALS cases display a more variable and aggressive clinical picture, similar to that of SALS.

It has been proposed that the phenotype in recessive D90A pedigrees is modified by a cis-acting protective factor close to the D90A hSOD1 gene that could act by downregulating D90A expression in recessive cases. Despite considerable efforts, this putative protective factor has not yet been identified. Intriguingly, it appears as if both dominant and recessive cases are all descended from a single founder some 895 generations ago (~20,000 years) and that the recessive haplotype arose about 63 generations ago (~1,500 years).

**Properties of the D90A protein:** The SOD1 activity in hemolysates from homozygous D90A individuals is very similar (~92%) to the SOD1 activity of controls and for the purified enzymes there were no significant differences in copper and zinc binding or specific activity between wild-type and D90A hSOD1. On denaturing gel electrophoresis, the mobility of the D90A protein is somewhat higher than that of the wild-type protein.

The properties of the protein in vitro have also been shown to be very similar to those of wt-hSOD1. For example:

- The metallation, specific activity and the copper coordination are similar to wt-hSOD1.
- Metal-charged D90A exhibits a high degree of thermal stability, close to that of the wild-type protein.
- The D90A protein is also almost as stable as the wild-type protein when exposed to denaturants such as urea or guanidinium chloride.
- The disulfide bond of D90A mutant hSOD1 is nearly as stable as the bond in the wild-type protein when exposed to reductants.

**G127X mutant hSOD1**

Only one family has been reported with the Gly127insTGGG mutation (abbreviated G127X). This mutation inserts a repeat of four nucleotides (TGGG), which leads to
five novel amino acids and a premature stop codon resulting in a 21 amino acid truncation (128-Gly, 129-Gln, 130-Arg, 131-Trp, 132-Lys, 133-STOP). In the first report, the insertion was wrongly stated to be an insertion of ACCC, which is actually the anti-sense of TGGG, and to put a stop codon at position 132 when the correct number is 133\(^{492}\).

This Danish family has subsequently been described in more detail. Both UMN and LMN symptoms, as well as bulbar and spinal symptoms, are known to occur in G127X ALS patients. Reduced sense of vibration has also been reported, although cutaneous sensation was normal. The range of age at onset is 40-60 years of age and the disease duration has been \(~3\) years. Erythrocyte SOD1 activity was about 40\% of that of controls\(^{324}\).
AIMS

The main aim of this thesis was to investigate the mechanisms by which mutSOD1s cause ALS. The strategy was to investigate mutSOD1s with widely differing properties and compare these mutSOD1s with each other and with wild-type hSOD1 in the search for a common noxious property of mutSOD1s.

The more specific aims were:

- To investigate whether the putative protective factor in recessively inherited D90A FALS acts by downregulating synthesis, influencing degradation or in other ways altering D90A mutSOD1 (Paper I).

- To measure the levels of the three different SODs in cerebrospinal fluid from sporadic, familial and mutSOD1-FALS patients and controls (Paper II).

- To generate D90A and G127X mutSOD1 transgenic mice (Papers III, IV).

- To compare the in vivo properties of wild-type hSOD1 and wild type-like D90A mutSOD1 to find a clue to the toxicity of this mutSOD1 (Paper III).

- To validate the G127X mice model by comparing the findings in the model with findings in an ALS patient carrying the same mutation (Paper IV).

- To study and compare the biochemical properties of mutant and wild-type hSOD1s in transgenic mouse strains (Papers III-IV).
METHODOLOGY

A summary of the main materials and methods used in this thesis and some considerations relating to the methodology are given below.

Human samples

Sample collection
Blood was drawn into EDTA or acid citrate dextrose (ACD) containing vacuum tubes. Samples were then centrifuged and packed erythrocytes, buffy coat and plasma were collected separately and stored at -80°C until further analyzed (Papers I, II, IV).

CSF was collected using methods used in ordinary clinical practice. Samples were immediately frozen and stored at -80°C until further analyzed. Cases with headache or other neurological symptoms without underlying neurological disease served as controls (Paper II).

Skin punch biopsies were taken from a patient carrying the G127X SOD1 mutation to establish a fibroblast culture. The fibroblasts were grown in Ham’s F10 containing 10% fetal calf serum (Paper IV).

An autopsy on the same G127X patient (Paper IV) was performed within 20 hours after death. The whole brain and spinal cord were removed and frozen at -80°C. Further dissection of the tissue for analysis was performed on an aluminum plate at -20°C. For histological studies, pieces were immersion-fixed in 4% paraformaldehyde, 100 mM sodium phosphate, pH 7.4. As controls, five patients with no known neurological disease were used. The ages and postmortem times of these patients were similar.

Most of the individuals and/or their families in Papers I, II, IV have been described previously.

SOD1 genotyping
All the patients in Papers I, II, IV were screened for hSOD1 mutations. DNA was purified from buffy coats using the Nucleon BAAC2 kit (Amersham). The hSOD1 gene was analyzed with PCR and SSCP according to Andersen et al. Primers and PCR conditions were according to Table 3. The D90A allele was also identified by restriction enzyme cleavage of exon 4 PCR products using Fnu4HI, since this mutation introduces a new site for this restriction enzyme.

Ethical considerations
All the human samples were collected with informed consent and the studies adhered to the tenets of the World Medical Association Declaration of Helsinki. All the studies were approved by the medical research ethics committee at Umeå University and in Paper IV also by the ethics committee at the University of Copenhagen, Denmark.
Table 3. Primers and PCR conditions used for genotyping

<table>
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<tr>
<th>Exon</th>
<th>Direction</th>
<th>Oligonucleotide sequence</th>
<th>Product length</th>
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<tr>
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<td>Forward</td>
<td>5'-TTC CGT TGC AGT CCT CGG AA-3'</td>
<td>158 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGG CCT CGC AAC ACA AGC CT-3'</td>
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<td>Forward</td>
<td>5'-CAT AAT TTA GCT TTT TTT TCT TCT TC-3'</td>
<td>138 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CCA TTG ATT ACA AGA GTT AAG C-3'</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Forward</td>
<td>5'-CAT CAG CCC TAA TCC ATC TGA-3'</td>
<td>236 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGG GAC TAA CAA TCA AAG TGA-3'</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Forward</td>
<td>5'-GTT ATC TTC TTA AAA TTT TTT ACA G-3'</td>
<td>133 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CTC AGA CTA CAT CCA AGG GA-3'</td>
<td></td>
</tr>
</tbody>
</table>

PCR conditions were:
Exon 1: 95°C for 15' then 35 cycles of 94°C for 1', 65°C for 1', 72°C for 1' ending with 72°C for 10'
Exons 2-5: 95°C for 15' then 35 cycles of 94°C for 1', 55°C for 1', 72°C for 1' ending with 72°C for 10'

SOD1 transgenic mice

The mouse as a model system

In Sweden, about 200 people each year succumb to ALS. A fraction of these patients are autopsied, thereby making CNS tissue from ALS patients, especially those carrying SOD1 mutations, scarce. To be able to study the motor neuron pathology of ALS, we therefore have to rely on different model systems. These results then have to be compared with what we see in ALS patients. In our studies, we have used transgenic mice overexpressing different kinds of hSOD1s as a model system. The mouse has several advantages, for example:

- The physiology of the mouse is similar to that of humans.
- Mice have a rapid rate of reproduction.
- Mice are relatively easy to handle.
- The complete genome of the mouse has been sequenced.

An additional advantage of using these model systems is that tissues and cells can be sampled early in the disease process, which for natural reasons is impossible in humans. The model systems also give us the opportunity to manipulate cells and animals both genetically and chemically.

There are also other model systems which have been used to study mutSOD1 and ALS. They include in vitro models, such as cell-culture experiments and tissue-slice experiments, and in vivo models using other mutSOD1 transgenic animals, such as Caenorhabditis elegans, Drosophila melanogaster and Rattus norvegicus.
All these models have their advantages and disadvantages. Differentiated neurons do not divide and short-lived primary neuronal cultures or more artificial tumor cell lines therefore have to be used for cell culture experiments. These cells typically have a relatively low expression of mutSOD1, but toxicity is still seen in a matter of days, suggesting that the pathogenic process in the cell cultures could be different. Moreover, the system usually only includes one cell type which is different from the situation in the spinal cord. One advantage of this system is that it is easy to manipulate and that the biochemical properties of proteins are easy to study. Tissue slices more closely resemble the environment in the spinal cord since the neurons here are supported by glial cells, but the conditions are still fairly artificial. This system is also comparatively easy to manipulate and analyze. The transgenic expression of mutSOD1 in *C. elegans* and *D. Melanogaster* has been difficult to evaluate since no overt neurological phenotype has been seen, possibly due to the short life span of these animals. Transgenic rats expressing mutSOD1 have also been generated, with a phenotype very similar to that of transgenic mice. The only difference between the mouse and the rat models appears to be the size of the animals. The size of rats could in some instances be an advantage. For example, some therapeutic trials may be more easily performed on a larger animal and the sampling of tissue and fluids from rats could also be easier and richer. The larger size of rats could, however, make them more difficult to handle in some situations. One recent development is the use of chick embryos to study the effect of mutSOD1 in the spinal cord.

**Generation of transgenic mice**

The human SOD1 gene (Fig. 2) was chosen for the generation of transgenic mice to mimic the transcriptional regulation seen in humans. Subsequent studies by others have shown that cDNA constructs provoke disease as well\(^{354}\), but that the choice of promoter could be important since constructs that only express mutant SOD1 in certain cell types have failed to cause an ALS-like phenotype\(^{355,361,363}\) (see also above). The level of expression is also important\(^{354,366,367}\).

![Fig. 2](image-url)  
**Fig. 2** The human SOD1 gene. Coding regions are depicted in white with exon numbers and non-coding regions in grey. All cleavage sites for the restriction enzymes used for cloning and mutagenesis are shown.

An 11.6 kb fragment containing the whole human SOD1 gene was cloned into the plasmid vector pGEM3 (Promega). Subcloning was performed using a pSP73-P...
vector (Promega). Mutagenesis and construct for the D90A transgenic mice was performed according to Fig. 3 and according to Fig. 4 for the G127X mice.

PCR primers for D90A were: 5’-CAC TAG CAA AAT CAA TCA TCA-3’ and 5’-TCT TAG AAT TCG CGA CTA ACA ATC-3’ and for G127X: 5’-AAA GTA AGA GTG ACT GCG GAA CTA-3’ and 5’-CTG GCA AAA TAC AGG TCA TTG A-3’.

The entire 11.6 kb mutated SOD1 genomic fragment was then excised with EcoRI and BamHI, electroeluted and used for microinjections in ova from C57Bl6/CBA mice. Transgenic mice were identified by Southern blots and then mated with C57Bl/6JBon (B6) mice.

**Other mice studied**

In addition to the D90A and G127X transgenic mice, five other transgenic strains were studied. From Jackson Laboratories, a high (G93AGur) and a low (G93AGur dl) expressing line of G93A transgenic mice were acquired. The low-expressing G93A line is derived from the G93AGur strain but has a reduced copy number. Copy number loss is known infrequently to occur in this strain. Wild-type hSOD1 transgenic mice, strain N1029, were also acquired from Jackson Laboratories.
G85R transgenic mice were generously donated by Dr. D.W. Cleveland. The construct of these strains was almost identical to that of D90A and G127X mice. SOD1 null mice were generously donated by Dr. A.G. Reaume. Non-transgenic littermates were used as controls.

An exon 5 PCR fragment from a G127X patient was ligated into a PstI-BamHI subclone of the human SOD1 gene using Ncol-BclI.

The mutated PstI-BamHI fragment was then ligated with a PvuII-PstI subclone of the human SOD1.

Finally the PvuII-BamHI fragment was ligated into the pGEM vector containing the whole SOD1 gene using PvuII and BamHI.

Fig. 4 Mutagenesis and construct for G127X transgenic mice. Some cleavage sites of the restriction enzymes have been omitted.

**Handling of mice**

**Keeping mice**: Transgenic mice were kept at the animal facilities at Umeå University and the Swedish Defense Research Agency (FOI). Mice were maintained under a 12-hour light/dark cycle at 22°C and fed standard laboratory and water *ad libitum*. For experiments using a copper-enriched diet, chow enriched with 400 ppm copper (Lactamin) was used, starting at weaning. Controls were fed the same chow without enrichment.

**Genotyping**: G93A, G85R, wild-type hSOD1, SOD1 null and D90A transgenic mice were identified by ELISA or SOD activity measurement. Heterozygous G127X mice were identified with PCR of tail DNA, since the G127X protein is non-reactive in the ELISA and does not have any SOD activity.
The zygosity of D90A mice was determined by the amount of SOD1 in erythrocytes, as determined by the ELISA corrected for the amount of hemoglobin. D90A mice that could not safely be determined to be hetero- or homozygous were not used. The age at bleeding could be important when differentiating between hetero- and homozygous D90A mice, since older mice have lower levels of mutant SOD1 in erythrocytes than younger ones (see Paper III). Care was therefore taken to bleed the mice directly after weaning, at about three weeks of age.

**Breeding:** It is well known that the mouse background could be important for the phenotype of transgenic mice and this also appears be the case for mutSOD1 transgenic mice. In the studies presented here, mice were backcrossed into C57Bl/6JBom (B6) mice for at least 4 generations. G93A, G85R, wild-type hSOD1 and heterozygous G127X and D90A transgenic mice were continuously mated with B6 mice (Bomholtgård). G127X homozygous mice were maintained by homozygous mating and homozygous D90A mice were bred by mating two D90A heterozygous mice. The breeding of SOD1 null mice has previously been described.

**Sample collection**

Mice intended for biochemical analysis were sacrificed by an overdose of sodium pentobarbital injected intraperitoneally. Following dissection, pieces of tissue were frozen in liquid N2 and kept at -80°C.

For histopathological investigations, mice were anesthetized by an intraperitoneal injection of midazolam, fentanyl and fluanisone and subjected to perfusion fixation through the heart with 4% paraformaldehyde, 75 mM sodium phosphate (pH 7.2), and then stored in paraformaldehyde containing phosphate buffer until further dissected.

Animals were studied at 2, 50, 100, 200, 400, 600 and 700 days (if applicable) of age (± 5%) and when terminal.

**Ethical considerations**

Mice were handled according to Swedish laws and regulations and the studies were approved by the animal ethics committee in Umeå. Animal care and experiments were carried out in accordance with European Communities Council Directive (86/609/EEC). Care was always taken to avoid mice suffering unnecessarily. For humane reasons, mice were regarded as terminal when they were so ill that they were no longer able to reach the food in their cages.

**Homogenization of samples**

Generally, tissues were homogenized in 25 volumes of PBS (10 mM K phosphate, pH 7.0, in 150 mM NaCl with EDTA-free Complete (Roche Diagnostics) antiproteolytic cocktail added, using an Ultraturrax (IKA) for two minutes. This was followed by the sonication of the homogenate using a Sonifier Cell Disruptor (Branson) for 1 minute.
For the analysis of detergent-resistant aggregates (Papers III, IV, V), the PBS was supplemented with 0.1% NP40 (Roche Diagnostics) and, in studies of the status of the intramolecular disulfide bond (Paper V), 20 mM iodoacetamide was added to the buffer. For the quantification of tissue SOD1 and CCS content and activities, a buffer containing 50 mM K phosphate, pH 7.4, 3 mM DTPA, 0.3 M KBr and Complete with EDTA was used instead. The high concentration of KBr in this buffer increases the extraction of EC-SOD (SOD3) two to threefold and this buffer is therefore usually used for activity measurements in the laboratory.

Human packed erythrocytes were lysed by the addition of 20 volumes of 5 mM Na-HEPES buffer, 50 µM EDTA, pH 7.5 and, for mice, EDTA anticoagulated blood was lysed with 10 volumes of buffer. No precipitation of hemoglobin was performed unless otherwise stated.

**Generation of antibodies**

The SOD1 and CCS primary antibodies used in this thesis were polyclonal rabbit antibodies raised against keyhole limpet hemocyanin (KLH) conjugated peptides. All peptides have a cysteine on the N or C-terminal end of the peptide to be able to link the peptide to KLH or to a sulfolink coupling gel which was used for the purification of the antibodies. A list of the antibodies, the peptide sequence and their reactivity can be seen in Table 4. The purification process was performed in two steps. First, the rabbit antisera were purified on a protein A sepharose (Amersham), which was followed by purification on the sulfolink coupling gel (Pierce) with the corresponding peptides coupled.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Peptide sequence</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-hSOD1</td>
<td>aa 4-20 CAVCVLKGDPVQGIINF</td>
<td>Human/mouse SOD1</td>
</tr>
<tr>
<td></td>
<td>aa 24-39 ESNGPVKVWSIKGLTC</td>
<td>Human SOD1</td>
</tr>
<tr>
<td></td>
<td>aa 43-57 HGFHVHEFGDNTAGC</td>
<td>Human/mouse SOD1</td>
</tr>
<tr>
<td></td>
<td>aa 57-72 CTSAGPHFNPLSRKHG</td>
<td>Human/mouse SOD1</td>
</tr>
<tr>
<td></td>
<td>aa 80-96 CHVGDGLNVTADDKGVA</td>
<td>Human/mouse SOD1</td>
</tr>
<tr>
<td></td>
<td>aa 100-115 CEDSVISLSGDHCIIGR</td>
<td>Human/mouse SOD1</td>
</tr>
<tr>
<td></td>
<td>aa 131-153 CNEESTKTGNAGSRLACGVIGIAQ</td>
<td>Human/mouse SOD1</td>
</tr>
<tr>
<td>wt-mSOD1</td>
<td>aa 24-36 ASGEPVVLSEQITC</td>
<td>Mouse SOD1</td>
</tr>
<tr>
<td>G127X hSOD1</td>
<td>aa 111-132 &quot;Agaz&quot; CIIGRTLVVHEKADDLGGQRWK</td>
<td>G127X/wt-SOD1</td>
</tr>
<tr>
<td></td>
<td>aa 123-132 &quot;Inger&quot; CADDLGGQRWK</td>
<td>Human G127X</td>
</tr>
<tr>
<td>Human CCS</td>
<td>aa 252-270 CWEERGRIAGKGRKESAQP</td>
<td>Human/mouse CCS</td>
</tr>
</tbody>
</table>
The five novel amino acids seen in the G127X protein made it possible to generate G127X specific antibodies to be used for immunoblots and immunohistochemistry. Since the antigenicity of only five amino acids is very low, some wild-type sequence was also included in the peptides used for immunization (Fig. 5). The wild-type reactivity of the antibodies is then blocked with wt-hSOD1 that has been denatured by exposure to 6 M guanidinium chloride and 3 mM DTPA followed by dialysis. Two G127X specific antibodies were generated, the longer (Agaz antibody) shows some reactivity to wt-hSOD1, while the shorter (Inger antibody) has no such reactivity.

Fig. 5 C-terminal end of the G127X protein. Novel amino acids have a light grey background. The four nucleotides insertion is underlined.

### Analysis of SOD isoenzymes

**SOD activity**

SOD activity was determined using three different assays. The direct spectrophotometric method using KO₂ (Papers I-V), the pyrogallol method (Paper IV) and the nitroblue tetrazolium gel assay (Papers I, IV).

**Direct spectrophotometric method:** Most assays of the superoxide anion are indirect assays because of the very rapid spontaneous disproportionation of the anion radical in aqueous solutions at neutral pH. Under alkaline conditions, however, the radical is much more stable and can be measured using an ordinary spectrophotometer at 250 nm. In this assay, an excess of O₂⁻ is produced by KO₂ at pH 9.5 and the decay of the superoxide radical is continuously measured using a spectrophotometer. The addition of SODs catalyzes the decay of O₂⁻ to hydrogen peroxide and oxygen in a first-degree order and, by measuring the speed of the decay and after correction for the very slow second-degree spontaneous disproportionation of O₂⁻, the SOD activity can be calculated. In the assay, one unit is defined as the enzymatic activity that yields a decay of O₂⁻ at a rate of 0.1 s⁻¹ in a 3 ml buffer\(^{501,502}\). One unit in the assay corresponds to about 4.3 ng wild-type hSOD\(^1\)\(^{503}\) or 8.6 ng human SOD\(^3\)\(^{504}\) or 65 ng bovine SOD\(^2\)\(^{501}\).

For the differentiation between SOD2 and the other two SODs, 3 mM of cyanide is added to the buffer which almost completely inactivates Cu-containing SODs. Currently, discrimination between SOD1 and SOD3 activity in this assay is not possible. The assay is very sensitive for the activity of CuZn-SODs, but, for the
detection of SOD2, the assay is much less sensitive and only about 10% of that for CuZn-SODs.

**Pyrogallol method**: Since the alkali conditions used in the direct spectrophotometric method could inactivate unstable SOD1 mutants (such as G127X SOD1), a second method was used to measure the SOD activity in G127X mice (Paper IV). This method is based on the auto-oxidation of pyrogallol, at pH 7.8, which is dependent on $O_2^{•−}$ and the auto-oxidation is thus inhibited by SODs. The process is recorded using a spectrophotometer at 420 nm and one unit in the assay is defined as the amount of SOD that inhibits the auto-oxidation by 50%. Like the direct spectrophotometric method, SOD2 is measured by adding 1 mM cyanide. One unit in this assay corresponds to about 650 ng hSOD1 or 700 ng bovine SOD2.

**Nitroblue tetrazolium gel assay**: The third method that was used for measuring SOD activity was a gel-based method. One advantage of this method is that the migration pattern or the isoelectric point of the native enzyme is directly seen. The method was first developed by Beauchamp and Fridovich, but to enhance the sensitivity some minor modifications were made. The assay is a negative assay where SODs inhibit the reduction of nitroblue tetrazolium to formazan by $O_2^{•−}$ in the gel, causing an achromatic zone in an otherwise blue gel. The superoxide radical is generated by the auto-oxidation of riboflavin by light. To increase the sensitivity of the assay, the concentration of nitroblue tetrazolium was lowered from 2.45 mM to 0.25 mM and a 200 mM TRIS-cacodylate buffer pH 7.8 was used.

Here we used isoelectric focusing using Immobiline (Amersham) gels to separate the proteins. In Paper I, a pH gradient of 4.5-5.4 was used and equal amounts of hemolysate SOD activity were applied for all cases. In Paper IV, a gradient of pH 3.5-9 was used and brains from 100-day-old mice were homogenized in 10 volumes of pH 7.0 PBS and centrifuged at 20,000 g for 30 minutes. Twenty, 40 and 60 µl of the supernatant were then separated by isoelectric focusing. Focusing conditions were according to the manufacturer’s descriptions.

Gels were then scanned in a Fluor-S Multiimager (Bio-Rad) and quantified using Quantity One software (Bio-Rad). The quantification of SOD activity using this negative method has limited accuracy, but the values in Paper I are in reasonably good agreement with the expected values.

**SOD ELISAs**

A hSOD1 enzyme-linked immunosorbent assay (ELISA) (Papers II-V) was used to quantify hSOD1, mainly for mouse genotyping purposes. This assay has been previously described by Behndig et al. The hSOD1 ELISA is highly specific for native wt-hSOD1 and the ELISA therefore shows no reactivity to G127X hSOD1 and very low reactivity to G85R hSOD1. The reactivity to G93A hSOD1 is, however, high. Initially, D90A hSOD1 had a somewhat lower reactivity in the ELISA. The use of other secondary antibodies, however, circumvented this problem and the ELISA is now just as reactive to wt-hSOD1 as D90A hSOD1. The ELISA shows no cross-reactivity towards mSOD1.
To detect SOD3, the ELISA described by Karlsson and Marklund was used\(^\text{509}\) (Papers \(\text{II, IV}\)). The SOD3 ELISA shows no cross-reactivity to hSOD1. For conversions to activity units, 8.6 ng/U was assumed\(^\text{504}\).

**Blotting procedures**

**Immunoblotting**

Samples were solubilized 1:1 in 2 × SDS-PAGE (SDS-polyacrylamide gel electrophoresis) sample buffer (100 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 20% glycerol, 4% SDS and bromphenol blue), heated for 5 min at 95°C and then separated on SDS-PAGE gels (Bio-Rad). The gels were then electroblotted onto nitrocellulose membranes (Paper I) or polyvinylidene difluoride (PVDF) membranes (Amersham) (Papers \(\text{II-V}\)). Blots were probed with the SOD1 and CCS antibodies described above. In Paper I \(\text{125I-labeled protein A was used to detect primary antibodies and the bands were visualized using a Bio-Rad GS-525 phosphorimager and quantified using Molecular Analyst software (Bio-Rad). In Papers II-V, horseradish peroxidase-conjugated anti-rabbit IgG antibodies or biotinylated anti-rabbit IgG antibody and horseradish peroxidase-conjugated streptavidin (Amersham) were used for detection and chemiluminescence was generated using substrate from Pierce or Amersham. Bands were visualized on film or by using a Fluor-S Multimager or Chemidoc imager and Quantity One software (Bio-Rad).**

For the quantification of SOD1 by immunoblotting (Paper \(\text{III, IV, V}\)), wild-type hSOD1 with the concentration determined by quantitative amino acid analysis\(^\text{281}\) was used as the original standard. For the quantification of mSOD1, the 24-36 mSOD1 specific antibody was used and in G127X mice the 131-153 antibody.

In the CNS from the human G127X ALS patient, the mutant-specific Inger antibody was used for the determination of the mutant enzyme (Paper IV). In this case, G127X mutant SOD1 in a transgenic mouse brain homogenate previously quantified against the original standard was used as standard.

In general, quantifications by immunoblots were run at least in duplicate.

**Northern blotting**

Total RNA was prepared from mouse brain using the Trizol reagent (Invitrogen), according to the manufacturer’s description. Electrophoresis was performed using the NorthernMax-Gly kit (Ambion) and RNA was blotted onto Hybond XL nylon membranes (Amersham). Blots were probed using ULTRAhyb hybridization buffer (Ambion) and the MegaPrime random labeling kit (Amersham). Bands were visualized with a Bio-Rad GS-525 Molecular Imager and quantified using Molecular Analyst software (Bio-Rad). Samples were normalized against β-actin (Clontech). SOD1 mRNA was detected using a purified PCR fragment as a probe. Primers were 5’-GCG TGG CCT AGC GAG TTA TG-3’ and 5’-ATC CTT TGG CCC ACC GTG TTT TCT G-3’ yielding a 248-bp amplicon of exons 1-3. The PCR amplicon was purified using High Pure PCR Product Purification kit (Roche Diagnostics) (Papers \(\text{III, IV, V}\)).
Southern blotting
Mouse DNA was prepared from tails using standard methods and cleaved with *Bgl*I. DNA was then separated on 0.7% agarose gels and blotted onto Hybond XL nylon membranes (Amersham). The *EcoRI*-BamHI fragment of the *SOD1* gene described above was used as the probe. The labeling and visualization of bands were as described for northern blots. The copy number was determined by quantification against known amounts of the *EcoRI*-BamHI fragment (Papers III, IV).

2D-gel electrophoresis
Spinal cords from 100-day-old and terminal G127X mice were homogenized in 8 M urea, 4% CHAPS and 2% Bio-Lyte 3-10, swollen into 7-cm non-linear pH 3-10 immobilized pH-gradient strips (Bio-Rad) and electrofocused according to the manufacturer’s instructions. The strips were then top loaded onto 12.5% SDS-PAGE gels, electrophoresed and finally electroblotted onto polyvinylidine difluoride membranes and immunoblotted as described above with the 24-39 anti-hSOD1 antibody and an anti-ubiquitin antibody (Sigma) (Paper IV).

Immunohistochemistry

**Human histopathology**

Pieces from the frontal, temporal and precentral cortical regions of the brain and the cervical, thoracic and lumbar spinal cord were dissected from the G127X patient. The pieces were then immersion fixed in paraformaldehyde as described above and embedded in paraffin wax and sectioned for histopathological analysis (Paper IV).

**Mouse histopathology**

For histopathological analysis, five mice of each age and genotype were studied. After fixation, the CNS was dissected in situ and the cervico-thoracic and thoraco-lumbar borders, as well as the junction between the brain stem and spinal cord, were defined with guidance by the ventral roots. The spinal cord was then divided into three parts corresponding to the cervical, thoracic and lumbosacral levels respectively. Each of these levels was then equally divided into five pieces which were separately embedded in paraffin wax. The uppermost piece of each level and the second lowermost piece of the lumbosacral level were sectioned and used for the histopathological investigations (Papers III-V).

**Staining procedure**

Single- and double-labeling immunohistochemistry was performed using the Ventana AEC and alkaline phosphatase red immunohistochemistry system, using the protocol of the manufacturer, and was preceded by microwave irradiation of the sections in citric acid buffer for 3x5 minutes. Antibodies used for SOD1 staining (Papers III-V) were mainly the 4-20 and 131-153 anti-SOD1 peptide antibodies described above. Tests were performed with the other anti-hSOD1 peptide antibodies on one terminal mouse of each strain, which gave similar results as the 4-20 and 131-153 anti-hSOD1 peptide antibodies. In studies of the G127X protein, the 131-153 hSOD1 antibody
Superoxide Dismutase 1 and Amyotrophic Lateral Sclerosis

was used as a negative control, since this antibody does not recognize G127X. For
the detection of the G127X protein, the two G127X-specific antibodies were used.
These antibodies do not stain non-G127X tissue. Antibodies to the following proteins
were also used:

- GFAP Dako (Papers III-V)
- Ubiquitin Dako (Paper IV)
- UCH-L1 Ultraclone (Paper IV)
- αB-crystallin Novocastra (Paper IV)

Sections were also stained with hematoxylin/eosin, Klüver-Barerra, modified
Bielschowsky and periodic acid-Schiff (Papers III-V).

Other experiments

Effect of postmortem time

To mimic the effect of the storage/postmortem time of a deceased human body on the
various assays carried out on the patient (Paper IV), pieces of brain from G127X
transgenic mice were put aseptically into small tubes and kept at 37°C for 6 h,
followed by room temperature for 6 h, and then at 4°C. Immediately thereafter and
after different times, tubes were snap frozen to -80°C for subsequent thawing and
analysis.

Fibroblast culture experiments

Fibroblasts were grown in RPMI 1640 (Roswell Park Memorial Institute) containing
10% fetal calf serum, 70 μg/ml bensylpenicillin and 2 mM L-glutamine. To inhibit
the proteasome, cells were incubated with 10 or 20 μM of two different irreversible
proteasome inhibitors, lactacystin or 3-nitro-4-hydroxy-5-iodophenylacetyl-LLL-
vinylsulfone (NLVS) (Calbiochem) for 48 hours\textsuperscript{510}. Cultures without proteasome
inhibitor served as controls. Cells were then lysed by sonication in 50 mM K
phosphate, pH 7.4, 3 mM DTPA, 0.3 M KBr and Complete with EDTA and
immunoblotted using the Agaz antibody. Equal amounts of fibroblast lysate SOD
activity were applied to the gels. For analysis of aggregates, fibroblasts lysed in PBS
pH 7.0 without detergent were centrifuged once at 20,000 g for 30 minutes and then
analyzed by immunoblotting using the Agaz and the 131-153 hSOD1 antibodies
(Paper IV).

Analysis of SOD1 aggregates

Analysis of detergent-resistant aggregates: Brain and spinal cord samples from the
mice were homogenized in 25 volumes of PBS (pH 7.0) with 0.1% of the detergent
Nonidet P40 (NP40) added (Roche Diagnostics). Homogenized samples were then
centrifuged at 20,000 g for 30 minutes at 4°C. The supernatants were removed and
the pellets were resuspended and sonicated in double the original volume of
homogenizing solution, followed by centrifugation. This washing step was repeated 5
times. Following the last wash, the pellets were resuspended and sonicated in 1 × SDS-PAGE sample buffer. The samples were then analyzed by immunoblotting, using the 24–39 hSOD1 specific antibody (Papers III-V).

**Analysis of detergent-soluble aggregates:** Tissues from G127X transgenic mice were homogenized in 25 volumes of PBS (pH 7.0) without detergent added and centrifuged at 20,000 g for 30 minutes at 4ºC. The supernatants were carefully removed and the pellets were resuspended in PBS pH 7.0 with 0.1% NP40. Resuspended pellets were then analyzed with immunoblotting using the 24-39 and 131-153 hSOD1 antibodies. The relative amounts of G127X SOD1 and mSOD1 respectively in the pellets were quantified against dilutions of the original homogenate in immunoblots. The proportion (percentage) of pelleted mSOD1 (processomes) is then subtracted from the proportion (percentage) of pelleted G127X and this difference (Fig. 6) is termed detergent-soluble aggregates (Paper IV).

![Figure 6](image)

**Stabilities of human and murine SOD1s**

Pools of packed erythrocytes from three D90A homozygous individuals, three control individuals and three control C57Bl/6J mice were mixed with 1.6 volumes of 37.5/62.5 vol/vol chloroform/ethanol at -20ºC to precipitate and remove the hemoglobin of erythrocytes. After vortexing and centrifugation (2,500 g, 10 min), 200 μl of the SOD1-containing upper phase was added to 400 μl 3.75 M guanidinium chloride in 0.1 M Na-HEPES, pH 7.4, with 3 mM DTPA and incubated at 37ºC. The denaturation was stopped after different times by adding 50 μl aliquots of the mixture to 400 μl 50 mM Na-HEPES pH 7.4 with 0.25% bovine serum albumin, followed by analysis of the SOD activity.
For analysis of the thermal stabilities, 100 μl of the chloroform/ethanol upper phase was added to 200 μl Na-HEPES, pH 7.4, with 3 mM DTPA and the tube was placed in a 70ºC water bath. After different times, 50 μl aliquots were added to the HEPES buffer at room temperature and analyzed as described above (Paper III).

**Supplementation of homogenates with Cu**

The tissues were homogenized in 25 volumes of the PBS as described above. To the homogenates, CuSO₄ (1 mM) or an equal volume of PBS was added. Homogenates were then incubated overnight at 4ºC, followed by the analysis of SOD activity. Three mice of each strain were analyzed. Non-transgenic mice and SOD1 knockout mice were used as controls (Paper V).

**Cytochrome oxidase assay**

The activity of cytochrome oxidase (Complex IV) was measured in spinal cord homogenates of mice from the high-expressing SOD1 transgenic strain and non-transgenic mice of different ages according to Birch-Machin et al. 511. In this method, the oxidation of cytochrome C by adding potassium ferricyanide was followed with a spectrophotometer at 550 nm (Paper V).

**Inductively coupled plasma atomic emission spectrometry**

Tissues were dissected with stainless steel tools with powder-free gloves and stored at -80ºC until further analyzed. Test tubes used for analysis were rinsed with nitric acid and MilliQ water (Millipore). Weighed tissue pieces were put in the test tubes and then solubilized using 50% nitric acid, 50% hydrogen peroxide and finally diluted 5 times with MilliQ water. Samples were then analyzed using inductively coupled plasma atomic emission spectrometry (ICP) on a PE Optima 3000XL (PerkinElmer) (Paper V).

**Recombinant hSOD1s**

Recombinant human SOD1 variants were co-expressed with the copper chaperone for superoxide dismutase (CCS) in *E. coli* and purified as previously described 512. CuSO₄ (3 mM) and ZnSO₄ (30 μM) were added to the culture medium. The metal contents were determined by graphite furnace atomic absorption (Paper V).

**Total protein analysis**

Total protein was measured using a Coomassie Brilliant Blue G-250 assay (Bio-Rad), standardized with human serum albumin 513 (Papers I-V).
**Hemoglobin analysis**

Total hemoglobin was determined with a standard cyanomethemoglobin assay (Bioreagens) using a spectrophotometer at 540 nm (Papers I, III-V).

**Statistical calculations**

For statistical calculations SPSS or Microsoft Excel software were used. As a measure of variance of means, standard deviation (SD) (Papers I-V) was used. When comparing two groups (Papers I, II, V), the non-parametric Mann-Whitney U test was used and, when comparing multiple groups (Paper II), one-way ANOVA (analysis of variance) was used and, if significant, Tukey’s post-hoc test was performed to determine differences between groups. The significance of correlation (Paper II) was calculated with Spearman’s rho test.
RESULTS AND DISCUSSION

Paper I

No alteration in D90A mutant hSOD1 levels in recessive pedigrees

It has been suggested that the recessive inheritance pattern seen for D90A mutant SOD1 is due to a tightly linked protective factor that could act by downregulating the mutant protein. To investigate this and to further explore the effects of a putative protective factor, erythrocytes from heterozygous individuals from recessive and dominant D90A families and sporadic heterozygous D90A ALS patients were analyzed. Families were considered recessive if no D90A heterozygote in the family had developed ALS and dominant if heterozygous individuals, including family members, had contracted the disease. In all, erythrocytes from 21 heterozygous individuals from 17 different Scandinavian, German and US recessive families were examined. The dominant cases were eight individuals from six different families from Belgium, Great Britain and Russia. Six of these individuals had ALS. As controls, 13 healthy individuals were analyzed simultaneously. There was no difference in erythrocyte SOD1 activity between “recessive” and dominant individuals (56.4 ± 5.4 U/mg Hb and 56.6 ± 2.7 U/mg Hb respectively). The heterozygous individuals had a somewhat lower activity than the controls (59.5 ± 3.4 U/mg/Hb p < 0.05). The D90A to wild-type ratio was measured with immunoblots that are able to distinguish between the wild-type and the D90A allele. Here, too, there was no difference (0.78 ± 0.10 vs. 0.81 ± 0.09). These data suggest a specific activity of D90A that is 114% of the wild-type enzyme which is in perfect agreement with previous findings in homozygous D90A patients that also reported a 14% increase in the specific activity for the D90A protein. The turnover of hSOD1 in homozygous and heterozygous individuals therefore appears to be similar.

To analyze whether the protective factor altered the quaternary structure of SOD1, isoelectric focusing and SOD activity staining were performed. In homozygous D90A and wt-hSOD1 patients, multiple bands were seen, in agreement with previous published results. The reason for this banding pattern is not known, but there are several possibilities, such as (de)methallation, carboxylation, (de)acetylation, phosphorylation, or oxidation of cysteines to cysteine-sulfenic acid or by disulfide-linked glutathione. However, some of these post-translational modifications appear less likely. For example, copper deficiency inactivates the protein and other modifications do not necessarily confer charge differences.

To aid in the analysis of the banding pattern, we analyzed the available fractions of previously ion-exchange purified enzyme from homozygous D90A patients and controls. Fractions I and III were of equal molecular weight according to mass spectrometry, while fraction II showed a heterodimeric pattern and was suggested to be partly glutathionylated. On zymograms, fraction I only displayed one band, while the other two fractions contained several bands. Thus it could not be concluded that glutathionylation was responsible for the multiple banding patterns. The D90A
mutations confer the protein a more positive charge, which is also reflected on the zymograms. As expected, the heterozygous individuals displayed a heterodimeric formation between the wild-type and D90A subunits. After inspecting and quantifying the zymograms, we were not able to discern any difference in the banding pattern between recessive and dominant cases.

There was therefore no evidence that the putative factor in recessive D90A cases acts by downregulating synthesis, influencing degradation or by altering the quaternary structure of the D90A protein.

One caveat when it comes to this study is of course that only two of the dominant cases were documented familial cases. It may be that the sporadic ALS cases carried the recessive allele and for unknown reasons contracted the disease. However, Scandinavia aside, the recessive D90A allele is very rare. In the United Kingdom, which is in the proximity of Scandinavia, aberrant hSOD1 patterning (such as that seen for the D90A mutation) was found in 7 individuals out of 11,237 tested (corresponding to a frequency of 62 per 100,000 people)\textsuperscript{521}. In Russia outside the Kola Peninsula, 12 of 3,093 tested (corresponding to a frequency of 388 per 100,000 people) had aberrant hSOD1 patterning. This mutation is therefore very rare, although the possibility of it occurring in ALS from other causes could not be excluded. The two Belgian dominant FALS individuals were also very similar to the other D90A individuals in the amount and structure of the D90A protein.

Another concern may be that here we have investigated erythrocytes not CNS tissue. The transcriptional regulation of SOD1 is poorly understood but may be different in the CNS. To conduct a similar study on CNS tissue would, however, be practically impossible due to difficulties obtaining CNS tissue from individuals with hSOD1 mutations. So far, we have analyzed CNS tissue from six homozygous D90A ALS patients and the preliminary results suggest that the hSOD1 levels in the ventral horns of these patients are very similar to those of controls (K.S. Graffmo et al., unpublished results). Additionally, our studies of CSF from homozygous D90A patients showed that SOD1 levels were similar to those of controls (see Paper II below).

**Paper II**

*CSF hSOD1 levels are unaltered in ALS*

In other neurodegenerative diseases, such as Alzheimer’s and Parkinson’s disease, the proteins that are found to be mutated in familial cases are clearly also involved in sporadic cases. For example, in Alzheimer’s disease, accumulations of Aβ are seen in the amyloid plaques characteristic of the disease and, in certain familial cases, the disease is linked to mutations in the amyloid precursor protein (APP)\textsuperscript{238}. Curiously, Alzheimer’s disease patients have been associated with a paradoxical decrease in Aβ1-42 in CSF\textsuperscript{522}. It is conceivable that similar alterations may also pertain to ALS and SOD1. This notion was investigated by analyzing CSF from controls (n=37), SALS (n=54), FALS (n=12) and mutSOD1-FALS (n=14) patients in terms of SOD content and molecular forms of hSOD1.
SOD content was analyzed by measuring both cyanide-sensitive and -insensitive activity and the levels of hSOD1 and SOD3 were evaluated with ELISAs. FALS and SALS cases had somewhat higher hSOD1 protein levels compared with controls. However, the mean age was also lower in the control group and there was a significant correlation between age and hSOD1 levels (p<0.001 for all individuals without mutSOD1). Assuming a linear correlation between hSOD1 activity and age in non-mutant hSOD1 individuals, the hSOD1 activity can be roughly described by the equation:

\[
\text{Activity} = 0.33 \times \text{Age} + 13.8 \quad \text{(U/ml)}
\]

For controls with a mean age of 52.1, this corresponds to a value of 31.0 U/ml, for SALS 33.9 U/ml (mean age 60.8) and for FALS 35.4 U/ml (mean age 65.6). These values are all reasonably close to the measured values and suggest that the difference seen between controls and ALS patients only reflects the age of the individuals. An age dependent increase in SOD activity in CSF has also previously been reported. The levels of SOD3, cyanide-insensitive SOD (SOD2) and total protein were unremarkable. Homozygous D90A patients had hSOD1 levels that were very close to those of controls. The hSOD1 levels in CSF samples from patients heterozygous for A89V, S105L and G114A mutSOD1 were clearly lower.

The levels of the CNS intracellular protein content are mirrored in the CSF protein content. Factors contributing to CSF content can, for example, be the levels of protein expression, the degree of cell damage and the turnover rate of proteins. There was no evidence of a general efflux of protein to the CSF, since protein levels were similar between groups. This does not of course exclude the possibility that individual proteins are altered. Previous studies have in some instances reported such differences in ALS, although the results have not been clear cut. For example, the levels of VEGF have been reported to be both lower and unchanged (with widely differing values), whereas tau levels have been reported to be both higher and unchanged. Others have reported an increase in NF-L and PEDF and a decrease in Aβ1-42.

In our study, we were not able to find any evidence that the levels of hSOD1, SOD2, or SOD3 were altered in ALS. This is different from what has been reported from more acute conditions, such as stroke, meningitis and encephalitis, where hSOD1 levels can be several times higher in the CSF. This probably reflects the slow progression and chronic nature of ALS.

As has been discussed and investigated in Paper I, it has been suggested that the putative protective factor in recessively inherited D90A ALS acts by downregulating the D90A protein. Similar to the study of erythrocytes (Paper I), we found no indication that this is the case, since D90A levels in the CSF of homozygous D90A patients is very similar to the SOD1 levels seen in controls.

The levels of SOD in ALS/MND CSF have been investigated in four other studies. Three smaller studies (18, 11 and 10 ALS patients) used different methods to measure total SOD activity, cyanide-sensitive SOD (SOD1 and SOD3) and SOD1 protein and found that ALS correlated with a decrease in SOD activity. In the somewhat larger study by Yoshida et al. consisting of 25 MND patients, there was no
difference in the amount of SOD1 protein compared with controls, which is in agreement with our study, the largest so far.

**Increased levels of cleaved hSOD1 in D90A ALS patients**

The main finding in this paper was the occurrence of a second, lower molecular weight, hSOD1 reactive band on CSF immunoblot. The molecular weight difference (~2.5 kDa) and the lack of reactivity with an N-terminal antibody suggest that this band represents the remains of a ~20 aa N-terminal cleavage of hSOD1. This band was also seen in CNS homogenates but at much lower levels. It was not a storage artifact, since CSF snap frozen in liquid nitrogen was no different from samples stored over-night at 4°C or at room temperature. The relative amount of the cleaved subunit was significantly higher for homozygous D90A patients than for controls, SALS and FALS cases (e.g. 32% for homozygous D90A patients vs. 24% for controls). Heterozygous carriers of the other hSOD1 mutations (A89V, S105L and G114A) also showed an increase in cleaved hSOD1, but it is difficult to draw any safe conclusions based on single cases. There were no other differences on immunoblots.

As has been discussed above, little is known about the degradation of hSOD1. It is possible that the N-terminal cleaved hSOD1s represent an initial step in the route of degradation of hSOD1 (a proteolytic fragment corresponding of amino acids 1-20 have been observed after proteasomal digestion in vitro). There are several possibilities why this band is increased in homozygous D90A patients. Previous studies and Paper III demonstrate that, although D90A is very stable, it is somewhat less stable than wt-hSOD1, suggesting that this mutant is more rapidly degraded than wt-hSOD1, which could contribute to an increase in the amount of proteolytically cleaved hSOD1. An alternative explanation is that the degradation of the mutant hSOD1, for unknown reasons, is slower than that of the wild-type protein or that the proteolytic machinery in these patients is compromised, leading to increased levels of partly digested hSOD1.

The reason why this band is selectively accumulated in CSF is not known. It is possible that the cleaved hSOD1 more easily leaks to the CSF from the CNS tissue or that in cells with a high proportion of the cleaved specie, this fragment is toxic and result in cell death.

C-terminally truncated hSOD1s, like the more common hSOD1 missense mutations, have previously been reported to cause ALS (see above and Paper IV). The shortest ALS associated mutSOD1 reported so far is the K118TGP-Stop mutation that results in a 32 amino acid truncation. This part of the protein cannot therefore be toxic per se. The toxicity of an N-terminally cleaved protein is, however, not known. Interestingly, in transfected cell cultures, hSOD1 fragments consisting of amino acids 1-75, 38-153 and 49-153 were all highly prone to aggregate and, in chicken embryo spinal cords, a YFP-tagged hSOD1 peptide consisting of only amino acids 1-36 induced aggregates and TUNEL positive cells. The situation may thus be similar to Alzheimer’s disease where fragments of the protein are highly amyloidogenic (such as the Aβ1-42). One can speculate that the N-terminal proteolytic cleavage of hSOD1 may represent such a fragment that could act as a seed for aggregation.
Papers III-V

In these three papers, the generation of D90A (Paper III) and G127X (Paper IV) mutSOD1 transgenic mice is described and the two strains are compared with other mutSOD1 transgenic strains and a mouse strain expressing wt-hSOD1 (Table 5). Additionally, mutSOD1 in G127X transgenic mice is compared with an ALS patient carrying the same mutation (Paper IV).

Table 5 Umeå data on the survival of hSOD1 transgenic mouse strains

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Line</th>
<th>Survival ± SD (days)</th>
<th>Expression of hSOD1. Percentage of G93AGur ± SD (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D90A</td>
<td>134 mm</td>
<td>407 ± 46 (n = 65)</td>
<td>51% ± 8%</td>
</tr>
<tr>
<td>D90A</td>
<td>154 mm</td>
<td>492 ± 84 (n = 6)</td>
<td>ND</td>
</tr>
<tr>
<td>D90A</td>
<td>134 / 154</td>
<td>495 ± 46 (n = 3)</td>
<td>ND</td>
</tr>
<tr>
<td>G127X</td>
<td>716 mc</td>
<td>477 ± 88 (n = 39)</td>
<td>ND</td>
</tr>
<tr>
<td>G127X</td>
<td>716 mm</td>
<td>250 ± 38 (n = 202)</td>
<td>63% ± 8%</td>
</tr>
<tr>
<td>G127X</td>
<td>832 mc</td>
<td>213 ± 18 (n = 6)</td>
<td>ND</td>
</tr>
<tr>
<td>G127X</td>
<td>832 mm</td>
<td>126 ± 4 (n = 10)</td>
<td>ND</td>
</tr>
<tr>
<td>G85R</td>
<td>mc</td>
<td>345 ± 41 (n = 77)</td>
<td>43% ± 6%</td>
</tr>
<tr>
<td>G93A</td>
<td>Gur mc</td>
<td>124 ± 12 (n = 267)</td>
<td>100%</td>
</tr>
<tr>
<td>G93A</td>
<td>Gur&lt;sup&gt;δ&lt;/sup&gt; mc</td>
<td>253 ± 31 (n = 113)</td>
<td>50% ± 10%</td>
</tr>
<tr>
<td>wt-hSOD1</td>
<td>mc</td>
<td>–</td>
<td>60% ± 4%</td>
</tr>
</tbody>
</table>

mm denotes homozygous mice and mc mice hemizygous for the transgene insertion
ND = Not determined

Phenotypes of mutant hSOD1 transgenic mice

As described above, the D90A and G127X protein is two very different hSOD1 mutations. In actual fact, these two mouse strains also develop somewhat different MND phenotypes. The D90A transgenic mice develop a more slowly progressing MND with progressive paralysis and muscle wasting that usually starts in one hind limb and then slowly progresses over weeks. The disease duration is approximately 50 days. An unusual feature with this strain, which is not observed in other mutSOD1 transgenic mice strains, is the occurrence of a urinary bladder distended by urine in terminal mice. This feature resembles the problems with micturition from which some D90A patients suffer<sup>488</sup>, which suggests that this feature may be specific to D90A mutSOD1.

The G127X transgenic mice, on the other hand, develop a much more rapidly progressing MND, where the disease duration could be as short as two days. Fore limb onset is common. G93A and G85R mice fall between these two models, as G85R are closer to the G127X mice, while G93A are more similar to the D90A transgenic mice. This possibly reflects the stabilities and the levels of these two proteins, as discussed below. For a given hSOD1 mutation, survival is inversely proportional to the levels of mutSOD1 expression (e.g. homozygotes compared to...
heterozygotes). But between different mutations the fit is not optimal, suggesting that the toxic potential of the mutSOD1 might differ (Table 5 and Fig. 7).

![Graph showing the association between hSOD1 expression and survival](image)

**Fig. 7** The association between the expression levels and the survival of different mutSOD1 transgenic strains, n = 3.

**Similar mRNA levels – widely differing protein levels**
In Paper V, the levels of hSOD1 mRNA in the CNS are compared with the “steady-state” levels of hSOD1 protein. Despite similar levels of expression (Table 5), which should be proportional to the protein synthesis rate, the amounts of hSOD1 protein in the CNS vary widely between strains. The different strains can be divided into two groups, one high hSOD1 level group (wt-hSOD1, D90A, and G93A) with hSOD1 levels of ~500-2,000 µg/g ww and one group (G85R and G127X) with much lower levels, ~50-100 µg/g ww. Interestingly, in the spinal cords of G85R and G127X transgenic mice, a surge in hSOD1 was seen in terminal mice, as previously noted for G85R mice. Compared with non-CNS tissue, hSOD1 was selectively accumulated in the CNS, which was especially evident in the low-level strains. Even more conspicuously, in the patient carrying G127X mutSOD1, only minute amounts of the mutant protein were detected (Paper IV), but here too mutSOD1 was selectively accumulated in disease susceptible tissues (Fig. 8).
These findings suggest that the degradation of misfolded hSOD1 in the CNS is impaired. In fibroblasts from the G127X patient, the G127X protein was not detected (Paper IV), but, if treated with a proteasome inhibitor (NLVS or lactacystin), the mutant protein was readily seen (without signs of ubiquitination). Although proteasome inhibitors are not 100% specific for proteasomal proteolysis, this suggest that at least a portion of the mutant protein is rapidly degraded by the proteasome in agreement with previous findings (see above).

**Inactive SOD1 in the CNS of transgenic mice – CCS deficiency?**

In the CNS of the high-level hSOD1 transgenic mice, SOD1 activity is smaller than might be expected from the amount of protein (Paper V). By incubating the homogenates with copper ions, a large proportion of the activity could be restored. The hSOD1s in these strains are therefore copper-deficient. The reason for this is not completely understood, but it appears as if this is not due to deficient uptake in the intestine, since copper supplementation to mice did not augment activity. Nor did it appear as if the transport of copper into CNS cells was deficient, since the copper-dependent COX enzyme was unaffected. The levels of CCS were upregulated in the CNS of transgenic mice but only modestly (3-5 times the levels of non-transgenic mice). In the liver of transgenic mice, where the hSOD1s is almost fully active, CCS levels were relatively higher (5-10 times the levels of non-transgenic mice) than in...
the CNS, thereby partly providing an explanation for the lack of copper charging in CNS tissue. The reason for this discrepancy is not known and overall, the regulation of CCS is poorly understood.

The two low-level mutSOD1 transgenic strains were also both inactive, but for other reasons. The G127X protein lacks the C-terminal part of the protein and thereby the disulfide bond and part of the active site cleft\(^{258}\). This severe protein defect is probably enough to completely inactivate the protein (Paper \(\text{IV}\)). G85R mutSOD1 has previously been shown to have very low activity \textit{in vitro} and in cells\(^{337,338,535,536}\), as well as in G85R transgenic mice\(^{360}\). The low activity has been attributed to the low copper affinity of this mutSOD1\(^{337,535,536}\). Previous findings are in agreement with the 30% activity of G85R seen when supplemented with copper ions. Interestingly, recombinant G85R was found to be fully active relative to its copper content (Paper \(\text{V}\) and \(^{537}\)). Consequently, in the spinal cord of transgenic mice, G85R is probably not copper charged at all at the active site.

The zinc status of SOD1 in the transgenic mice is very difficult to evaluate and it is not known how SOD1 acquires the zinc ion.

\textbf{Disulfide-reduced SOD1 in high-level hSOD1 transgenic mice}

In the strongly reducing intracellular environment, SOD1 has a unique intrasubunit disulfide bond that is of structural importance for the protein (see above). The amount of disulfide-reduced SOD1 was measured using immunoblots on homogenates where the cysteines had been quenched with the alkylating substance iodoacetamide to prevent re-oxidation (Paper \(\text{V}\)). Two bands, that appeared to differ by \(~2\) kDa in size, were then seen on non-reducing SDS-PAGE gels. The higher mobility band was referred to as oxidized and the lower mobility band as reduced. Similar differences in mobility have previously been observed for prokaryotic\(^{538}\), yeast\(^{301}\) and human SOD1s\(^{288}\), but the identity of the bands was further ascertained by analyzing recombinant proteins. This was done on recombinant wild-type or G85R hSOD1 with all four cysteines mutated to alanine (and thus disulfide-reduced) or only the cysteines not involved in disulfide formation (Cys6/Cys111) and thus disulfide-oxidized in the oxidizing environment. The amount of disulfide-reduced SOD1 was quantified versus dilutions of homogenate disulfide-reduced with \(\beta\)-mercaptoethanol.

In the spinal cords of high-level hSOD1 transgenic strains, between 8% and 14% of the hSOD1 was disulfide-reduced. The relative amounts of disulfide-reduced hSOD1 in peripheral tissues (liver and kidney) were lower. In low-level lines, “all” hSOD1 was disulfide reduced, but some of the disulfide-reduced hSOD1 had apparently engaged in disulfide coupling with other proteins or with SOD1, as has previously been noted \textit{in vitro}\(^{288}\). The endogenous mSOD1 showed a reduction pattern similar to that of hSOD1 in the high-level transgenic strains. However, in the low-level strains, all mSOD1 appeared to be disulfide-oxidized. This suggests that disulfide reduction is partly related to the copper content which is probably not limiting in the G127X and G85R mutSOD1 strains. As discussed above, G85R probably has a very low copper affinity, which would explain the propensity of this mutant protein to be disulfide-reduced, although copper deficiency \textit{per se} does not necessarily confer disulfide reduction. The propensity for disulfide reduction of G85R has also been noted \textit{in vitro}\(^{344}\).
**Similar amounts of detergent-resistant aggregates in terminal mice**

Despite the widely differing levels of mutSOD1 protein in the transgenic strains, similar amounts of hSOD1 detergent-resistant aggregates, associated with heavy smearing, accumulated in terminal mice (Papers III-V). In the G127X patient, almost all the mutant enzyme was aggregated, but it was also shown that this mutant protein aggregated rapidly when tissues were stored non-frozen (Paper IV). The occurrence of the detergent-resistant aggregates late in disease makes it unlikely that they are the direct cause of disease, since mutSOD1 transgenic mice show pathological alterations long before. It is, however, possible that aggregates also occur earlier but are solubilized by the detergent used in the assay. This was investigated in G127X mice (Paper IV), as described in the methodology section. Indeed, throughout the life of these mice, there is a burden of detergent-soluble aggregates (or protoaggregates) of about 20% of the total amount of the G127X protein.

Another conspicuous finding is the occurrence of HMW species of mutant but also of wild-type hSOD1 enriched in the spinal cords of transgenic mice (see above and Fig. 9).

![Fig. 9](image)

*Fig. 9* Immunoblot of spinal cord homogenates from 100-day-old hSOD1 transgenic mice using a human SOD1 specific antibody. Equal amounts of homogenate are loaded in each lane. Note that the prominent HMW band has a relative mobility similar to that of the monomeric hSOD1 band.

One band, 30-35 kDa in size, is especially prominent. The amounts of this band are similar between strains, despite widely differing levels of hSOD1 protein. The intensity of this band increases slowly with age. Similar species of mutSOD1 are also
seen in the G127X patient. Under oxidative conditions, this banding pattern on immunoblots is even more striking (see above). It has therefore been proposed that these species represent SOD1 cysteines linked with cysteines on other molecules or other SOD1 subunits\textsuperscript{288}. The exact nature of these species is, however, not known. In Paper IV, investigations were conducted to determine whether ubiquitin was covalently attached to the HMW species of G127X. This was in fact the case, but only in terminal mice and not in the major band of the HMW species. Interestingly, on these 2D immunoblots, the HMW species was readily seen, but, when gels were stained with silver, the spots could not be seen, suggesting that the amount of these species is overestimated (D. Bergemalm personal communication).

**Histopathology**

The spinal cord histopathology was investigated in all transgenic strains at 50, 100, 200, 400, 600 days of age or at the terminal stage (Paper III-V). Similar to the data on hSOD1 protein levels, the strains divided into two groups. The high-level hSOD1 group (wt-hSOD1, D90A, G93AGur and G93AGur\textsuperscript{36}) demonstrated vacuolization, SOD1 staining in the ventral roots and SOD1-positive inclusions that increase with age with a terminal surge in occurrence. The low-level strains (G85R and G127X) demonstrated fewer large SOD1-positive inclusions, no vacuolization and no SOD1 staining in the ventral roots. There was, however, no obvious difference in motor neuron loss in the two groups at terminal disease and all strains developed similar amounts of small dense granular SOD1-positive inclusions, mainly in the neuropil. The degree of astrocytosis (GFAP-positive cells) was also similar between strains and increased time-dependently. In G127X mice, astrocytes were positive for α\textsubscript{B}-crystalline (other strains were not investigated).

These results suggest that the vacuolization pathology seen in the high-level strains, but not in the low-level strains, is not required for the ALS-causing toxicity of mutSOD1s. However, a negative influence of the vacuoles on motor neuron survival cannot be excluded. Likewise it appears as if the presence of mutSOD1 in motor neuron axons is not a requirement for toxicity (G85R and G127X mice and also noted in L126Z mice\textsuperscript{365}). It is difficult to discern the type of inclusion corresponding to the detergent-resistant aggregates. The large SOD1-positive inclusions seen in all strains are one candidate, but the amounts of these inclusions differed between the high- and low-level strains whereas the detergent-resistant aggregates demonstrate similar amounts in the spinal cords of terminal mice of all the transgenic strains. Possibly, these inclusions are more amorphous and are easier solubilized in the high-level strains. The occurrence of the small dense granular inclusions was similar between strains and the alteration that differed least between the different strains.

In the G127X patient, motor neuron loss was seen at all levels of the spinal cord. Bunina bodies, LBHIs and skein like inclusions were seen in remaining motor neurons. Both LBHIs and skeins stained with the G127X-specific Inger antibody. There were also small round inclusions in motor neuron somata and the neuropil staining for Inger. No staining with Inger was seen in motor neuron axons. Astrogliosis was especially prominent in the corticospinal tract and astrocytes also often stained with α\textsubscript{B}-crystalline and some also had LBHIs. Many similarities in the
histopathological picture were thus noted between the G127X patient and the G127X mice.

**SOD1 aggregates and motor neuron toxicity**

The way mutSOD1 aggregates is far from completely understood. As has been described above, the form of the protein that is most likely to aggregate is the monomeric, disulfide-reduced, metal-deficient protein. In the hSOD1 transgenic mice, disulfide-reduced, copper-deficient hSOD1 exists (Fig. 10), constituting subfractions of the high-level hSOD1s and larger proportions of the low-level hSOD1s. These modifications of hSOD1 transform the protein from an unusually stable enzyme capable of withstanding 10 M urea and 4% SDS, as well as high temperature and long-term storage, to a protein that could (simplistically) unfold when we develop a high fever (melting point 42.9°C)\(^{288}\). Mutations in the hSOD1 protein further destabilize the protein, causing even more protein to un/misfold (see above). A recent report from the Valentine Lab, in which some mutSOD1s, e.g. H46R, H48Q, D101N and D124V, had thermal stabilities similar to the wt-hSOD1, contradicts this last statement\(^{347}\). Like previous results, metal deficiency and disulfide reduction made the protein less stable. In this study (and others), the results relate to studies of the folded protein. Little is known about what happens *in vivo* when the protein folds and if folding fails.

The *in vitro* stabilities of the different mutSOD1s have been associated with disease duration\(^{349}\). It is not known why the stability correlates to disease duration and not age at onset, for example. One possibility is that an unknown factor triggers the seeding of an aggregated hSOD1 species and initiates disease. Subsequently, the seed sequesters additional unfolded hSOD1 species. Onset would therefore be more dependent on the putative trigger, while disease progression would be dependent on the recruitment of additional hSOD1 to aggregates. One can only speculate about the nature of this putative trigger, but environmental, life-style related and genetic factors may come into play. The existence of a trigger may also provide an explanation for the differences in penetrance observed for different mutSOD1s. Some mutSOD1 may be less sensitive to the trigger (wt-hSOD1?, see below), which could result in a reduced penetrance. The sensitivity for this trigger may not be related in any way to the stability of the protein.

The reasons for the particular susceptibility of motor neurons are far from clear. It is not a matter of proteins levels, since several organs have higher levels of hSOD1 than CNS, even though it could be argued that motor neurons have higher levels than most other CNS cells (see above). This argument, however, appears not to be valid since ventral horns in humans, in which motor neurons account for ~30% of the volume (somas, axons and dendrites) (T. Brännström personal communication), do not have higher levels of hSOD1 than other parts of the CNS (Paper IV, unpublished observations). One clue is that the CNS accumulates misfolded hSOD1 in transgenic mice and that the spinal cord develops detergent-resistant aggregates (see above). This implicates the proteasome and the recognition of substrates to be degraded as a vulnerability factor (Fig. 10). Another possibility is that the environment in the CNS is such that the aggregation of proteins is favored. CCS levels may be another vulnerability factor, since the regulation of this protein does not appear to be as dynamic in the CNS as in other organs (see above), which could make a larger
proportion of the SOD1 protein more copper-deficient and disulfide-reduced in the CNS than in other organs. It is possible that the CCS could also have more classical chaperoning properties that could aid in the handling of misfolded SOD1, although this has not yet been shown (this is perhaps also less likely since mice expressing mutSOD1 on a CCS null background do not have a different phenotype\textsuperscript{404}).

In spite of this, it has not yet been convincingly shown that aggregates of hSOD1 are toxic to motor neurons or how they might be toxic. What has been shown is that mutSOD1 readily aggregates in motor neurons. Nevertheless, several downstream toxic mechanisms have been proposed. For example, interactions between aggregates/inclusions of mutSOD1 and the mitochondrial matrix\textsuperscript{539} and the mitochondrial intermembrane space\textsuperscript{403} have been shown and it has also been suggested that the mutSOD1 could aggregate on the cytoplasmic surface of mitochondria\textsuperscript{376}. Possibly these accumulations of mutSOD1 could be harmful to the mitochondria. In astrocytes of G85R mice, hSOD1 containing inclusions were seen together with a decrease in astrocytic EAAT2\textsuperscript{360}. In mutSOD1 transgenic mice, accumulations of hSOD1 and neurofilaments (spheroids)\textsuperscript{540} are seen in perikarya and the proximal axon. Possibly this could lead to cytoskeletal disruption resulting in the impaired axonal transport previously reported in mutSOD1 transgenic mice\textsuperscript{497,541,542}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{sod1-01.png}
\caption{Turnover of SOD1 and hypothetical mechanism of disease. After transcription, multiple forms of SOD1 are formed, disulfide-reduced/oxidized, metallated/unmetallated and monomeric/dimeric. These forms are also in equilibrium with each other. Mutations in the SOD1 protein would shift the equilibrium to the more immature forms that are more likely to misfold. The degradation of the mature protein is most likely to occur via the phagocytic/lysosomal system. The degradation of a misfolded protein would be mediated by the proteasomal system. It is possible that the recognition and degradation of the misfolded SOD1s via the proteasome is slow in the CNS. This could cause the protein to aggregate, leading to cytotoxicity.}
\end{figure}
The aggregation of hSOD1 can also lead to proteasome inhibition and a decrease in HSPs (the “quality control machinery”) that could result in an accumulation of misfolded proteins, thereby starting a cascade of aggregation leading to a vicious circle. Possibly, the terminal surge in the hSOD1 aggregates in transgenic mice represents the final breakdown of the “quality control” machinery in the cells.

Little is also known about the form of the aggregate that would confer cytotoxicity. It has been suggested that the large inclusions seen in older hSOD1 transgenic mice are non-toxic aggregates possibly representing aggresomes. Aggresomes are deposits of aggregates that are gathered by retrograde transport along the microtubules. This could be a way for the cells to handle misfolded and aggregated proteins, possibly by degradation via the autophagic route. The putative toxic aggregated hSOD1 species could therefore be misfolded oligomers of hSOD1 similar to the protoaggregates seen in G127X mice.

Could wt-hSOD1 cause ALS?

The similarities between wt-hSOD1 and D90A mutSOD1 led us to investigate the properties of this mutant hSOD1 and to compare it with wt-hSOD1 to search for a clue to the toxicity of this mutant hSOD1 (Paper III). These experiments were performed on homozygous D90A mice and wt-hSOD1 transgenic mice with similar levels of hSOD1 expression and hemizygous D90A mice with lower levels of expression (Table 5). In erythrocytes from transgenic mice, the D90A mutant protein was nearly as stable as the wild-type protein, but both were much less stable than mSOD1 when exposed to denaturants or high temperature. These data relating to the high stability of mSOD1 confirm previous observations.

Interestingly, wt-hSOD1 transgenic mice also developed detergent-resistant aggregates, although less and later than in terminal mutSOD1 transgenic mice. In this respect, hemizygous wt-hSOD1 transgenic mice fell between homozygous D90A mice, that develop MND, and hemizygous D90A mice, that do not develop MND. The histopathological picture of these three mouse strains supported this view and appeared in the following order with regard to the degree of pathology observed: homozygous D90A mice > wt-hSOD1 transgenic mice > hemizygous D90A mice. This suggests that the aggregation potential of wt-hSOD1 is less than equal to, but more than half of the D90A mutant enzyme, raising the possibility that hSOD1 could be involved in SALS without hSOD1 mutations. This would also be in analogy to what is seen in Alzheimer’s and Parkinson’s diseases (as discussed above).

This interpretation is supported by the paper from Jaarsma and co-workers, who noted that wt-hSOD1 transgenic mice suffered from mild motor deficits and a 20-30% motor neuron loss in two year old mice (when detergent-resistant aggregates start to develop) compared with non-transgenic mice. Inclusions containing hSOD1 have also been observed in ALS patients without hSOD1 mutations (T. Brännström personal communication and above).
CONCLUSIONS

I. In erythrocytes, there was no evidence that the putative protective factor in recessive D90A cases acts by downregulating synthesis, influencing degradation or by altering the quaternary structure of D90A hSOD1.

II. In CSF, there was no difference in SOD isoenzyme content between ALS patients lacking hSOD1 mutations and controls.

There was no evidence that the putative protective factor in recessive D90A cases acts by downregulating synthesis the mutant enzyme.

CNS contains N-terminally cleaved hSOD1 that is enriched in the CSF.

ALS patients who are homozygous for D90A mutSOD1 have higher levels of N-terminally cleaved hSOD1 in the CSF compared with controls. Could the N-terminally cleaved fragment be involved in ALS pathogenesis?

III. D90A hSOD1 transgenic mice develop an ALS-like phenotype similar to that of ALS patients homozygous for D90A mutSOD1.

The in vivo and in vitro properties of D90A hSOD1 are almost indistinguishable from those of wt-hSOD1.

Mice overexpressing wt-hSOD1 develop detergent-resistant aggregates similar to those seen in D90A transgenic mice, although less and later. This suggests that wt-hSOD1 could be involved in ALS cases without hSOD1 mutations.

IV. G127X hSOD1 transgenic mice develop an ALS-like phenotype similar to that of ALS patients heterozygous for G127X mutSOD1.

Similar to what is found in a G127X patient, detergent-resistant aggregates and high molecular weight hSOD1 species develop in affected motor areas of the mice.

In mice and humans, minute amounts of G127X mutSOD1 are sufficient to cause motor neuron disease.

V. Disulfide-reduced, copper-deficient and aggregation-prone hSOD1 is enriched in the CNS of hSOD1 transgenic mice. The motor neuron degeneration caused by mutSOD1s may therefore be attributable to long-term exposure to misfolded, aggregation-prone, disulfide-reduced SOD1. Such hSOD1 species should constitute minute subfractions of the stable mutants and larger proportions of the unstable mutants.
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APPENDIX – PAPERS I-V