Mutant Superoxide Dismutase-1-caused pathogenesis in Amyotrophic Lateral Sclerosis

Daniel Bergemalm
To my beloved children Hanna and Ludvig

Man's conquest of Nature turns out, in the moment of its consummation, to be Nature's conquest of Man

- C.S Lewis
Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating disease that affects people in their late mid-life, with fatal outcome usually within a few years. The progressive degeneration of neurons responsible for muscle movement (motor neurons) throughout the central nervous system (CNS) leads to muscle wasting and paralysis, and eventually affects respiratory function. Most cases have no familial background (sporadic) whereas about 10% of cases have relatives affected by the disease. A substantial number of familial cases are caused by mutations in the gene encoding superoxide dismutase-1 (SOD1). Since the initial discovery of this relationship about 17 years ago, numerous workers have tried to identify the pathogenicity of mutant SOD1 but without any final agreement or consensus regarding mechanism. The experiments in this thesis have been aimed at finding common pathogenic mechanisms by analyzing transgenic mouse models expressing mutant SOD1s with widely different properties.

Mitochondrial pathology and dysfunction have been reported in both ALS patients and murine models. We used density gradient ultracentrifugation for comparison of mitochondrial partitioning of SOD1 in our transgenic models. It was found that models with high levels of mutant protein, overloaded mitochondria with high levels of SOD1-protein whereas models with wild type-like levels of mutant protein did not. No significant association of the truncation mutant G127X with mitochondria was found. Thus, if mitochondrial dysfunction and pathology are fundamental for ALS pathogenesis this is unlikely to be caused by physical association of mutant SOD1 with mitochondria.

Density gradient ultracentrifugation was used to study SOD1 inclusions in tissues from an ALS patient with a mutant SOD1 (G127X). We found large amounts in the ventral horns of the spinal cord but also in the liver and kidney, although at lower levels. This showed that such signs of the disease can also be found outside the CNS.

This method was used further to characterize SOD1 inclusions with regard to the properties of mutant SOD1 and the presence of other proteins. The inclusions were found to be complex detergent-sensitive structures with mutant SOD1 reduced at disulfide C57-C146 being the major inclusion protein, constituting at least 50% of the protein content. Ten co-aggregating proteins were isolated, some of which were already known to be present in cellular inclusions. Of great interest was the presence of several proteins that normally reside in the endoplasmic reticulum (ER), which is in accordance with recent data suggesting that the unfolded protein response (UPR) has a role in ALS.

To obtain unbiased information on the pathogenesis of mutant SOD1, we performed a total proteome study on spinal cords from ALS transgenic mice. By multivariate analysis of the 1,800 protein spots detected, 420 (23%) were found to significantly contribute to the difference between transgenic and control mice. From 53 proteins finally identified, we found pathways such as mitochondrial function, oxidative stress, and protein degradation to be affected by the disease. We also identified a previously uncharacterized covalent SOD1 dimer.

In conclusion, the work described in this thesis suggests that mutant SOD1 affects the function of mitochondria, but not mainly through direct accumulation of SOD1 protein. It also suggests that SOD1 inclusions, present in both the CNS and peripheral tissues, mainly consist of SOD1 but they also trap proteins involved in the UPR. This might be deleterious as motor neurons, unable to renew themselves, are dependent on proper protein folding and degradation.

Keywords: ALS, SOD1, mitochondria, proteome, transgenic mice, inclusion.
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Abbreviations

ALS  Amyotrophic lateral sclerosis
CCS  Copper chaperone for SOD1
CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (1)
CNS  Central nervous system
DIGE  Differential in-gel electrophoresis
DTT  Dithiothreitol
EDTA  Ethylenediaminetetraacetic acid
EGTA  Ethylene glycol-bis(2-aminoethylether)-N,N',N'' tetraacetic acid
ER  Endoplasmic reticulum
FALS  Familial amyotrophic lateral sclerosis
GWAS  Genome-wide association study
HEPES  4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hSOD1  Human superoxide dismutase-1
HSP  Heat shock protein
ir  immunoreactive
IMS  Intermembrane space (mitochondria)
IPKB  Ingenuity pathway knowledge base
LBHI  Lewy body-like hyaline inclusion
LC-MS  Liquid chromatography mass spectrometry
LMN  Lower motor neuron
MALDI-TOF  Matrix-assisted laser desorption ionization time of flight
MND  Motor neuron disease
MOWSE  Molecular weight search
mRNA  Messenger RNA
mSOD1  Mouse superoxide dismutase-1
NRF2  Nuclear factor erythroid 2-related factor 2
NP-40  Nonidet P-40 (octyl phenoxypolyethoxylethanol)
OPLS-DA  Orthogonal projections to latent structures discriminant analysis
PAGE  Polyacrylamide gel electrophoresis
PLS  Primary lateral sclerosis
PMA  Progressive muscular atrophy
ROS  Reactive oxygen species
SALS  Sporadic amyotrophic lateral sclerosis
SDH  Succinate dehydrogenase
SDS  Sodium dodecylsulfate
SLI  Skein-like inclusion
SMA  Spinal muscular atrophy
SOD1  CuZn superoxide dismutase
SOD2  Mn superoxide dismutase
SOD3  Extracellular superoxide dismutase
UI  Ubiquitinated inclusion
UMN  Upper motor neuron
UPR  Unfolded protein response
UPS  Ubiquitin proteasome system
wt  Wild type
Preface

In this thesis, I have tried to target the pathogenic events taking place when mutant superoxide dismutase-1 (SOD1) causes amyotrophic lateral sclerosis (ALS). Although more than 17 years have passed since the original report on this subject, no consensus on the disease mechanism has been reached. In the Introduction I will review the main characteristics of the disease and the most common theories of pathogenesis. The focus is, however, on previous and current work relevant to the experiments described here, including mitochondrial toxicity, SOD1 aggregates, and the use of proteomic methods in ALS. The four papers that constitute this thesis are listed below.


III. Daniel Bergemalm, Karin Forsberg, Vaibhav Srivastava, Karin S Graffmo, Peter M. Andersen, Thomas Brännström, Gunnar Wingsle and Stefan L. Marklund. Superoxide dismutase-1 and other proteins in inclusions of transgenic amyotrophic lateral sclerosis model mice. (Submitted)

Introduction

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is invariably fatal. The progressive loss of motor neurons in the spinal cord, the brain stem, and the motor cortex leads to muscle wasting, paralysis, and eventually respiratory failure. As originally described in the 19th century by Charcot and others, the symptoms are derived from loss of upper and lower motor neurons (UMNs and LMNs) (1;2). Modern criteria are still based on signs of degeneration from both of these compartments in combination with progression and spread of symptoms (the so-called El Escorial criteria) (3;4). The control of muscle movement involves both UMNs and LMNs, and symptoms reflect their functional characteristics. UMNs are involved in voluntary movements and regulation of reflexes. Degeneration of UMNs and the corticospinal tract (descending nerve fibers from UMNs in the spinal cord) therefore leads to weakness in groups of coordinated muscles, symptoms of increased muscular tonus (spasticity), hyperreflexia and repetitive reflexes (clonus). LMNs are responsible for the direct innervations of individual muscle fibers. Depending on the requirements of fine motor ability, one LMN can innervate from a few up to hundreds of muscle fibers. Degeneration of LMNs therefore leads to loss of reflexes, muscle paralysis, spontaneous muscle contractions (fasciculations), and muscle atrophy due to loss of signaling.

Despite the fact that there are clearly defined diagnostic criteria, the clinical situation is often more complex and establishing an ALS diagnosis takes a lot of time and resources; it is sometimes never established (5). This complexity is partly due to motor neuronal diseases that affect only UMNs (primary lateral sclerosis, PLS) or LMNs (progressive muscular atrophy, PMA) but which sometimes eventually develop the criteria of ALS (6;7). As the long-term prognosis is different, for example, for PLS, PMA, and classical ALS, clinical studies have recently been carried out to establish the criteria for and prognosis of these diseases and similar intermediate syndromes with accentuation of UMN or LMN symptoms (6;8-10).

Epidemiology

The incidence, prevalence, and mortality of ALS have been shown to have increased slightly over the past 50 years (11). Most studies have been performed in Europe and the United States, where the incidences have been quite similar with a rough mean of about 2 per 100,000 person-years (variation depending on study ~0.6–3.2 per 100,000) (12-16). In Sweden, the incidence has been shown to have increased from 2.3 per 100,000 person-years in 1991 to 3 per 100,000 person-years in 2005 (17). This corresponds to an approximate lifetime risk of developing ALS of 1 in 400 for the
population studied. Cronin and colleagues performed a review of global epidemiology and concluded that few studies have been performed outwith Caucasian populations but that the incidence is most probably lower in other ethnic groups (18). The mortality rates follow the incidence with marginally lower counts, probably due to under-reporting of ALS on death certificates (19). Historically, it has been shown that males have an increased risk of developing ALS. In the 1960s, a male-to-female risk ratio of about 2:1 was reported whereas this ratio was reported to average 1.3 in the 1990s (11). The decrease in male preponderance (or increase in incidence in women) now appears to have stabilized and recent reports have shown similar numbers (13;15;17). However, a small study recently reported a higher incidence in women than in men in an Italian population (14). It has been speculated that the gender difference may be attributed to protective female hormones and increased exposure to potential risk factors in males, such as smoking (20;21).

**Age of onset and survival**

Most ALS patients are diagnosed at ages ranging between 55 and 85, with a peak incidence at 68 years of age (17;19;20). The variation is, however, great and in a recent report the patients included were between 20 and 90 years of age—which is typical of studies in large populations (15). Though juvenile forms exist, with very early onset of disease, it is nevertheless unusual to be diagnosed before the age of 30 (22). The mean survival time of ALS patients has been estimated to be 32.6 months (range 26.3–43) (11) and this is partly dependent on the site of onset, with the bulbar form being more aggressive (23). Between 5% and 10% of patients have a disease duration of more than 10 years (24;25). Familial ALS (FALS, see below) shows a significantly lower age of onset and higher variability of disease duration (26;27).

**Pathology**

The main characteristics of ALS pathology are due to selective loss of motor neurons in affected areas of the motor cortex, the brain stem, and the anterior horns of the spinal cord (28;29). This affects the fibers that are involved in connecting these different cellular populations, the corticospinal tract, and efferent axons in the anterior roots. Macroscopically, atrophy of the spinal cord and anterior roots is often quite obvious, while atrophy of the precentral gyrus (motor cortex) can only be seen in patients with a very long disease duration (28). Microscopically, the loss of motor neurons is followed by astrocytic gliosis and shrinkage of the few remaining motor neurons. Few of these findings are, however, specific for ALS even though their simultaneous presence clearly strengthens the diagnosis. As a further aid to diagnosis, some characteristic cellular inclusions are visible with routine staining, and these are more or less specific for ALS.
Inclusions

Neurodegenerative diseases are often characterized by the presence of intracellular and extracellular inclusions in the CNS. In Alzheimer’s disease and Parkinson’s disease, inclusions such as senile plaques, neurofibrillary tangles, and Lewy bodies are fundamental for the pathological diagnosis and they have been studied extensively. The mechanisms that give rise to the different types of inclusions are as yet unknown. Studies in cell lines have involved modeling of different entities that arise due to, for example, high expression of mutant proteins or inhibition of protein degradation (e.g. the ubiquitin-proteasome pathway). Examples of such entities are aggresomes, JUNQ/IPOD, and ERAC (30-32). A series of different inclusions are known in ALS; some are more common in SALS or FALS, but there is often overlap (29).

_Bunina bodies_. Bunina bodies are almost pathognomonic for ALS and are generally found in the cytoplasm of surviving motor neurons. They are round or irregular in shape, with a diameter ranging from 1 to 6 µm. When stained with hematoxylin and eosin (H&E), they are eosinophilic hyaline inclusions occasionally surrounded by a thin halo. Bunina bodies stain with antibodies to cystatin C and transferrin, and they have been suggested to be derived from the endoplasmic reticulum or Golgi apparatus (28;29;33).

_Ubiquitinated inclusions (UIs)_.

UIs are very common in ALS patients (close to 100%) and can be further subdivided according to morphology into _Skein-like inclusions (SLIs)_ and _Lewy-body like hyaline inclusions (LBHIs)_.

It has recently been shown that one of the major proteins in UIs from SALS and non-SOD1 FALS patients is TAR DNA binding protein 43 (TDP-43) (34) (discussed further below). SLIs range from 5 to 25 µm and have a filamentous morphology of aggregated fibrils. Similar to SLIs, LBHIs are composed of filaments and are coated with small granules. LBHIs are seen with routine H&E staining and are rounder and denser than SLIs. LBHIs have been found in both astrocytes and neurons from SALS and mutant SOD1-associated FALS, and are the most frequently found inclusions in these patients as well as in SOD1 transgenic mouse models. In both SOD1 FALS and SOD1 transgenic mice, the LBHIs stain intensively for the SOD1 protein (35;36). A range of other proteins have been reported to be present in LBHIs such as neurofilaments, peripherin, and ER chaperones (28;29;37).

_Spheroids_. Spheroids are mainly composed of accumulated phosphorylated neurofilaments, found in the proximal part of motor neuronal axons. They are variable in size, eosinophilic on H&E staining, and are clearly seen in silver impregnations. Spheroids are non-specific and can be found in tissue from both controls and patients with other neurological diseases, but the numbers are far greater in ALS patients (28;29).
Other inclusions. Several other inclusions have been described in ALS patients. One is hyaline conglomerate (neurofilament) inclusions (HCIs), which appear to be rather specific for FALS rather than SALS but have been found in other neurodegenerative diseases (20). Another is basophilic inclusions, which are found in juvenile ALS (28).

Etiology

Genetics of ALS

Most cases of ALS develop without any familial history whereas about 5–10% are regarded as inherited (FALS) (38). The majority of these families have an autosomal dominant disease with variable penetrance, but recessive inheritance also exists (39;40). To be regarded as “familial”, the proband must have at least one or two first- or second-degree relatives that are also affected (41). As the clinical and pathological presentations between SALS and FALS are often similar (42), it is obviously of great importance to characterize familial forms and identify the gene or genes that are involved. So far, mutations in ten genes have been coupled to typical or atypical ALS, and there are six more loci whose genes are as yet unidentified (Table 1).

Table 1. Loci and genes identified in familial ALS

<table>
<thead>
<tr>
<th>Disease Type</th>
<th>Gene</th>
<th>Locus</th>
<th>Inheritance</th>
<th>Clinical Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS1</td>
<td>SOD1</td>
<td>21q22.11</td>
<td>AD (AR)</td>
<td>Classical</td>
</tr>
<tr>
<td>ALS2</td>
<td>ALSIN</td>
<td>2q33</td>
<td>AR</td>
<td>Young onset</td>
</tr>
<tr>
<td>ALS4</td>
<td>SETX</td>
<td>9q34</td>
<td>AD</td>
<td>Young onset</td>
</tr>
<tr>
<td>ALS6</td>
<td>FUS</td>
<td>16q21</td>
<td>AD</td>
<td>Classical</td>
</tr>
<tr>
<td>ALS8</td>
<td>VAPB</td>
<td>20q13.3</td>
<td>AD</td>
<td>Varied</td>
</tr>
<tr>
<td>LMND</td>
<td>DCTN1</td>
<td>2p13</td>
<td>AD</td>
<td>LMND</td>
</tr>
<tr>
<td>ALS</td>
<td>Angiogenin</td>
<td>14q11.2</td>
<td>AD</td>
<td>Classical</td>
</tr>
<tr>
<td>ALS</td>
<td>TARDP</td>
<td>1p36</td>
<td>AD</td>
<td>Classical</td>
</tr>
<tr>
<td>ALS-FTD</td>
<td>CHMP2B</td>
<td>3p11.2</td>
<td>AD</td>
<td>FTD and ALS</td>
</tr>
<tr>
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<td>PRGN</td>
<td>17q21.32</td>
<td>AD</td>
<td>FTD and ALS</td>
</tr>
<tr>
<td>ALS3</td>
<td>?</td>
<td>18q21</td>
<td>AD</td>
<td>Classical</td>
</tr>
<tr>
<td>ALS5</td>
<td>?</td>
<td>15q15</td>
<td>AR</td>
<td>Young onset</td>
</tr>
<tr>
<td>ALS7</td>
<td>?</td>
<td>20p13</td>
<td>AD</td>
<td>Classical</td>
</tr>
<tr>
<td>ALS-X</td>
<td>?</td>
<td>Xp11-q12</td>
<td>XD</td>
<td>Classical</td>
</tr>
<tr>
<td>ALS-FTD</td>
<td>?</td>
<td>9q21-q22</td>
<td>AD</td>
<td>ALS and FTD</td>
</tr>
<tr>
<td>ALS-FTD</td>
<td>?</td>
<td>9p21.3</td>
<td>AD</td>
<td>ALS and FTD</td>
</tr>
</tbody>
</table>

AD, autosomal dominant; AR, autosomal recessive; XD, X-linked dominant; FTD, frontotemporal dementia; LMND, lower motor neuron disease; SETX, senataxin; DCTN1, dynactin.

ALS1/SOD1. The identification of mutations in the gene for superoxide dismutase-1 (SOD1) in 1993 was a real breakthrough in our understanding
of ALS pathogenesis (43). It had been preceded by a publication of a disease-linked locus on chromosome 21, found in families with autosomal dominant ALS (44). The numbers of SOD1 mutations detected in ALS are still increasing, and currently more than 150 different mutations are known (38) (alsod.iop.kcl.ac.uk/als/summary/summary.aspx). As SOD1 toxicity is the main subject of this thesis, a more detailed description follows.

ALS2/Alsin. In 2001, it was shown that mutations in the gene encoding a 184-kDa protein called alsin could cause a slowly progressing atypical ALS mainly with UMN involvement (45;46). Mutations were also found in cases of recessively inherited juvenile ALS. Alsin contains three guanine nucleotide exchange factor (GEF) domains and is involved in the cycling of GDP/GTP in GTPases (46). GTPases are involved in important intracellular signaling such as the RAS pathway, and alsin has been shown to interact with Rab5, a small GTPase involved in endosomal dynamics (47).

There are long and short splice products of the alsin gene transcript. Somewhat surprisingly, Kanekura and colleagues showed that overexpression of the long form of alsin was able to rescue cells from the toxicity of mutant SOD1. The long form of alsin could interact physically with mutant SOD1 but not wild-type (wt) SOD1 (48). Subsequently, alsin has been coupled to glutamate signaling and excitotoxicity (49), and shown to be involved in the survival of motorneurons through its interaction with Rac1 (50;51). As most ALS patients with alsin mutations are homozygous and most mutations are predicted to truncate the protein, it has been suggested that ALS is caused by a loss of function (42). There are, however, no clear signs of motor neuron disease in the four different alsin knock-out models that have been presented (reviewed in (52)).

ALS4/Senataxin. In 2004, mutations in the senataxin gene were shown to be involved in juvenile-onset, autosomal dominant ALS (53). In the same year, it was shown that the autosomal recessive disease ataxia-ocular apraxia 2 was due to mutations in the same gene (54). The phenotype of senataxin-caused ALS is quite atypical, with juvenile onset, distal weakness, and muscle atrophy but normal lifespan (53). From sequence homology, the protein has been suggested to function as a DNA/RNA helicase, and it has been shown to be distributed in both the cytosol and the nucleus in multiple cell types of the CNS (with highest density in the cerebellum and the hippocampus) (55).

ALS6/FUS. Recently, mutations in the gene encoding the protein fused in sarcoma (FUS) were shown to be responsible for the familial ALS variant previously called ALS6 (56;57). A total of 16 mutations were described. FUS is mainly a nuclear protein involved in transcriptional regulation and splicing, with functions that partly overlap with those of TDP-43 (see below) (58). In patients with FUS mutations, similar to TDP-43, the mutant protein was shown to aberrantly accumulate and aggregate in the cytoplasm.
**ALS8/VAPB.** In 2004, Nishimura et al. published the identification of a locus (20q13.3, called *ALS8*) associated with an atypical form of autosomal dominant ALS with slow progression (59). Later that same year, they reported that a mutation in a gene called *VAPB* is responsible for *ALS8* (60). They found the same mutation in several families with different phenotypes including classical rapid-progression ALS, atypical ALS, and late-onset spinal muscular atrophy (SMA)—another motor neuron disease. Since then, screens for mutations other than the P56S mutation originally described have been fruitless (61;62). These studies found polymorphisms present in both ALS patients (e.g. the deletion mutant Delta S160) and healthy controls. From this, it was speculated that the P56S mutation confers a specific toxicity that is not conferred by other mutations (61;63). The normal function of VAPB was initially suggested to involve regulation of the unfolded protein response (UPR) in the endoplasmic reticulum, a function that is lost with the P56S mutation (64). Furthermore, it has been shown that the mutant protein interacts with wt subunits and causes them to co-aggregate (64-66). This results in a dominant negative effect with decrease in UPR even in the presence of wt protein. A reduction in the level of VAPB protein has also been found in both ALS patients and SOD1 transgenic mice without *VAPB* mutations, indicating that there may be a functional link between general ALS pathogenesis, SOD1 toxicity, and VAPB protein (66;67). Apart from actions involving the UPR, Tsuda et al. showed that a domain of VAPB, including the P56S mutation, can be secreted and can act as a trophic factor at Eph receptors. The aggregation of VAPB abolished secretion and resulted in depletion of this non-cell autonomous function (68).

*Dynactin.* Dynactin is a multiprotein complex, required for functioning of the microtubule motor protein dynein. In 2003, a G59S mutation in the largest subunit, *p150*(*glued*), was found to be associated with familial ALS (69). Mutational screening of *p150*(*glued*) has later revealed four other mutations in SALS, FALS, and ALS that are coupled to frontotemporal dementia (ALS-FTD) (70;71). In 2008, Laird and colleagues showed that expression of mutant *p150*(*glued*) protein, but not the wt protein, resulted in a motor neuron disease phenotype in transgenic mouse models (72). The model showed defects in vesicular transport and motor neuron pathology with axonal swelling. Another mouse model, aimed at impairing dynactin/dynein function (through means other than point mutation), was published the same year (73). Although showing neuronal pathology, the mice never developed symptoms of motor neuron disease. Interestingly, the authors showed that inhibition of dynactin/dynein function in mutant SOD1 transgenic mice resulted in extended survival, indicating that there was a functional link. Earlier, two mouse strains, *loa* and *cra1*, with chemically-induced mutations shown to be located in the dynein gene, had been found to show motor neuron disease (74). Co-expression of different mutant SOD1 proteins in these model mice has recently been shown to be beneficial (75-
The result was, however, not uniform for all the SOD1 mutant models tested (77). Furthermore, mutant SOD1 has been shown to interact with the dynactin/dynein complex, which is important for inclusion formation by mutant SOD1 proteins (78). These authors also showed that overexpression of a dynactin subunit can prevent the deposition of large SOD1 inclusions.

**Angiogenin.** Since the original finding of ALS-associated mutations in the angiogenin gene in 2004 (79), several more have been identified in SALS, FALS, and in ALS-FTD (80-86). Angiogenin belongs to the ribonuclease A superfamily and has been shown to induce angiogenesis through four major pathways: degradation of RNA, basement membrane degradation, signal transduction, and nuclear translocation (87). Recent studies have indicated that angiogenin mutations lead to loss of function, affecting several pathways mentioned above, with consequences for neurons dealing with hypoxia (82;88-92).

**TARDBP.** Since the discovery of ubiquitinated TDP-43 protein in inclusions from both FTD and ALS patients in 2006 (34), there has been an explosion of reports regarding this protein and the relationship between the two diseases. The gene TARDBP, encoding the TDP-43 protein, is located on chromosome 1 and is ubiquitously expressed in multiple splice variants (reviewed in (93)). TDP-43 binds RNA/DNA and is involved in diverse functions such as gene transcription, splicing, and mRNA stability. The initial histopathological studies indicated that anti-TDP-43 staining of ubiquitinated inclusions (UIs) was located in both the nucleus and the cytoplasm of neuronal and glial cells, but that TDP-43 was excluded from the nuclei and accumulated in the cytoplasm of affected cells (34;94). TDP-43 was also shown to be cleaved, producing a 25-kDa C-terminal fragment, and to be phosphorylated in extracts from patients but not from controls (34). TDP-43 positive staining of neuronal and glial cell UIs was later shown to be present in SALS patients, FALS patients without SOD1-mutations, and ALS-FTD patients but not in ALS patients with SOD1 mutations (95;96) or in SOD1 transgenic mouse models (97). This has, however, been challenged by a more recent report based on transgenic mice (98).

During the spring of 2008, multiple—almost simultaneous—reports appeared on mutations in the TARDBP gene in SALS and in autosomal dominant FALS (99-103). Since then, reports on findings of new ALS-associated mutations have continued to appear; today, 30 mutations in 22 unrelated families and 29 sporadic cases have been found (reviewed in (58)). Although there are similarities in pathology between FTD and ALS regarding TDP-43 positive ubiquitinated inclusions, only one ALS patient with a TDP-43 mutation has so far been reported to develop cognitive impairment (of Alzheimer type) (104). As to the mechanism(s) of toxicity of mutant TDP-43 in ALS, there is still considerable confusion. Some recent reports have come with possible explanations. Mutated TDP-43 and also its
truncated C-terminal fragment have been shown to sequester the wt protein into cytoplasmic inclusions and thereby deprive the cell of TDP-43 activity (105;106). In contrast, expression of different TDP-43 fragments in a cell line showed that the C-terminal fragment alone was responsible for toxicity without affecting the activity of full-length TDP-43 (107). An antibody specific for the cleaved C-terminal fragment was also shown to selectively stain neuronal cytoplasmic inclusions in tissues from ALS patients (107). Furthermore, TDP-43 has been shown to stabilize neurofilament-light (NFL) mRNA through direct interaction, and it might therefore be involved in the formation of neurofilament inclusions in ALS (108). Iguchi and colleagues studied the effect of siRNA knock-down of TDP43 in Neuro-2a cells (109). This resulted in reduced viability, which was at least partly due to loss of regulation of Rho-family GTPases. As judged by the enormous amount of work invested in such a short time, we are only at the beginning of understanding these mechanisms, and the field will probably become clearer in the near future.

**CHMP2B.** In 2005, mutations in charged multivesicular body protein 2b (CHMP2B) were reported in families with FTD (110). Later, mutations were found in one patient with PMA and in another with ALS-FTD (111). CHMP2B is involved in the endosomal sorting complex (ESCRT) and mutations were suggested to disturb endosome dynamics, as with alsin and dynactin (111). Recently, it has been suggested that mutations in CHMP2B inhibit autophagy, which is an important function for clearance of aggregated proteins (112;113).

**Progranulin.** Mutations in progranulin (PRGN) were initially found in cases of FTD and linked to ALS through one family with a history of both diseases (114). Progranulin is a secreted growth factor with multiple functions and has recently been shown to be involved in caspase-dependent cleavage of TDP-43 (115). In a mutational screen of a spectrum of ALS variants, PRGN mutations were found in one SALS patient and one patient with ALS-FTD (116). In another study in Belgian and Dutch ALS patients, mutations were found but sequence variants were also present in control subjects (117). The authors instead suggested that allelic PRGN variants modify the disease progression and might therefore be more correctly regarded as a genetic risk factor.

**Genetic risk factors**
The distinction between a genetic risk factor and a causative genetic alteration is not always simple, as many findings have been in single SALS patients and not all inheritable mutations have complete penetrance. The number of genes that have been associated with ALS and suggested to modulate the risk in one way or another are numerous, and beyond the scope
of this review (recently reviewed in (118). I will briefly mention a few of the more interesting findings.

**Neurofilament-heavy subunit and peripherin.** Neurofilaments (NFs) have been implicated in ALS from pathological findings of accumulated NFs in inclusion bodies (discussed above) and from NF overexpression and knock-out models in mice that in some cases develop motor neuron deficits (119). Different genetic alterations in the C-terminal domain of the gene encoding the heavy variant of neurofilaments (NF-H) have been found in ALS patients (120-122). Another intermediate filament, peripherin, generates a motor neuron disease phenotype when overexpressed in mice (123). In mutational screening of the peripherin (PRPH) gene in SALS and FALS patients, 20 polymorphisms were found. One gave a truncated protein and was found only in one SALS patient (124). Another homozygous mutation has been found in one patient (125). Although there is not a strong correlation between ALS and neurofilament genes, these proteins appear to be important in the pathogenesis in several ways, including aggregation and axonal transport for example.

**VEGF.** Oosthuyse et al. demonstrated that neuronal knock-down of expression of the gene for vascular endothelial growth factor (VEGF) through deletion of the hypoxia-response element resulted in an adult-onset motor neuronal disease in mice (126). This was followed by the finding that polymorphisms in the promoter sequence of VEGF, causing lower levels of circulating protein, increased the risk of ALS by 80% (127). Later studies have not been able to demonstrate this strong correlation in other populations, but they have found differences in risk attributed to gender (128;129). Like angiogenin, VEGF is involved in response to hypoxia and neovascularisation. The association of both of these molecules with ALS, although not clearly ruled out, indicates that these mechanisms are fundamental for motor neuron survival. Administration of VEGF through injection of viral vectors or as a purified protein has also shown to modify disease in a positive way in ALS transgenic mice (130;131).

**SMN1/2.** The protein survival of motor neuron (SMN) is found in the cytoplasm and nucleus, and is involved in RNA processing through assembly of small ribonucleoproteins into functional complexes (132). Homozygous mutations in SMN1 are responsible for most cases of spinal muscular atrophy (SMA), a motor neuronal disease with juvenile onset with variable survival (but adult forms exist). Another copy of the gene, SMN2, produces a homologous protein that can modulate disease depending on copy number, with more copies being favorable for survival and resulting in less aggressive disease. As SMA is caused by the selective death of motor neurons, similar to ALS, the role of SMN in ALS has been addressed by several studies. Although the results are divergent, there are indications that SMN genetic variants that produce less SMN protein are a risk factor for
ALS (133-135). In co-transfection models with simultaneous overexpression of SMN and mutant SOD1, SMN has been shown to upregulate chaperone activity and to rescue cells from SOD1 toxicity (136). Recently, transgenic mice expressing mutant SOD1 (G93A) were produced in an SMN heterozygote deletion background (SMN +/-) (137). SMN -/- is embryonically lethal but SMN +/- mice show no sign of motor neuron disease. Co-expression of G93A/SMN +/- modulated disease, resulting in a more aggressive ALS phenotype.

**APOE.** Patients with late-onset Alzheimer’s disease have an over-representation of the epsilon 4 allele of the apolipoprotein E (APOE4) gene (138). Several correlation studies in various populations of ALS patients regarding APOE genotype have been conducted (139-145). All such studies have shown that there is no increase in risk of ALS associated with APOE genotype. However, APOE4 carriers have been suggested to have an younger age of onset and a worse prognosis (139;142) while the opposite appears to be true of APOE2/3 (140;145).

**Promoter deletions in SOD1.** In sporadic ALS cases, the homozygous deletion of a 50-bp region of the SOD1 promoter has been shown to be correlated with increased age of onset in cases from British and Irish populations (146). The deletion resulted in a 50% lower promoter activity when expressed in various cell lines, but a reduction in SOD1 protein levels could not be confirmed in brain extracts of SALS patients.

**Genome-wide studies**

The recent advances in genetics have revealed new methods (e.g. microchip technology) for screening of genetic material, which makes it possible to find DNA sequence polymorphisms that are associated with disease in large patient material. Genome-wide association studies (GWAS) use dense maps of single nucleotide polymorphisms (SNPs) to scan the human genome for disease-common alleles (147). This approach has been successful in common diseases such as diabetes and breast cancer where the patient material is huge, and during the last few years they have been performed on different ALS materials (reviewed in (118;148)). The first GWAS performed on ALS in 2007 was conducted in a material of 276 patients. Thirty-four SNPs were found that were potentially associated with disease, but none survived correction for multiple testing (Bonferroni correction) (149). Later that year, a GWAS in a total of 1,337 European patients showed association with a genetic variant in the inositol 1,4,5-triphosphate receptor 2 gene (ITPR2), a protein involved in regulation of intracellular calcium concentration (150). These and other studies in different populations has been conflicting and unable to corroborate each other (151-156). Single studies have reported an association with dipeptidyl-peptidase 6 (DPP6) (154;155) and FGGY (153). These inconclusive results have been suggested
to be due to the problem of sample size in ALS, which will need to be overcome by the use of global multicenter studies in the future (118;148).

In a recent, comparatively large study involving 1,821 SALS patients, Landers and colleagues found an association between survival and alleles in the KIFAP3 gene (157). Homozygosity for the allele was associated with a long survival time but not with risk, site of onset, or age of onset. With a slightly different genetic approach using microsatellite markers, another recent study presented an association between a component of RNA polymerase II (ELP3) and SALS (158). This is interesting in the context of other RNA processing enzymes that have been implicated in ALS pathogenesis, but the association must be confirmed in other studies.

**Other risk factors**

Besides genetic factors, age, and gender, the only risk factor that has been found to be somewhat associated with ALS is smoking (21). This association has been strengthened by a recent prospective study (159). As smoking among women has increased during the last fifty years, this has also been suggested to partly explain the shift in gender ratio towards equality. During the last decade, an unforeseen clustering of ALS cases in professional soccer players has drawn new attention to a possible link between extensive physical activity and motor neuron disease (reviewed in (160)). The studies performed do, however, suffer from small sample sizes and the possible confounding effects of other common exogenous or lifestyle risk factors. In a recent report comparing cohorts of professional soccer players, basketball players, and road cyclists, a higher than expected incidence of ALS was found only in soccer players, indicating that high physical activity per se may not be a risk factor (161).

**Excitotoxicity**

Glutamate is the dominant excitatory neurotransmitter in synapses of the brain and spinal cord. Upon release, glutamate interacts with various presynaptic and postsynaptic receptors (NMDA, AMPA, and KA receptors) and is finally taken up by astrocytes where it is turned into inactive glutamine. This uptake is mediated through glutamate transporters known as EAAT1 to -5, where glial EAAT2 is the predominant species in the CNS and is responsible for removal of glutamate from the synapses. Excessive glutamate results in toxic effects that are mediated by, e.g. Ca²⁺ influx and increased oxidative stress (162). This pathway has been implicated in ALS for a number of reasons. (I) Motor neurons of the ventral horn have been shown to be especially vulnerable to excessive glutamate levels and signaling through AMPA receptors (163-166). It has also been shown that a subset of AMPA receptors, lacking a GluR2 subunit that makes them permeable to Ca²⁺, might be specially toxic for motor neurons (167;168). Activation of AMPA receptors by administration of agonists in vivo has also
resulted in motor deficits in mice (165). (II) An early finding was that
deficits in glutamate transport were specific to ALS patients in affected
regions of the spinal cord and motor cortex (169). This was shown to be due
to a loss of EAAT2 protein in ALS patients (170). Although controversial,
this has been suggested to be caused by aberrant $EAAT2$ mRNAs (171).
These species have also been found in controls, however (172). More
recently, high levels of glutamate have been found in the plasma of ALS
patients (173), as well as reduced glutamate uptake in platelets (174),
suggesting that alterations in glutamate levels are not confined to the CNS.
(III) In experiments on cultured cells from the cerebral cortex and spinal
cord, cerebrospinal fluid (CSF) from ALS patients has been shown to
mediate toxic effects (175-177). One measurable effect was an increase in
cellular Ca$^{2+}$ levels (175) and these studies also showed that administration
of glutamate receptor inhibitors was protective. (IV) The finding that
riluzole, an antiglutamate agent, had an effect on disease progression was a
great breakthrough since no approved drugs existed (178). This effect was
also confirmed in later studies (179;180). How this antiglutamate effect is
produced is not fully known, but it has been suggested that levels of
 glutamate in serum decrease after intake of riluzole (181), a finding that
could not be reproduced in a recent study (182). In cell culture experiments,
it has been shown that riluzole significantly increases the activity of
glutamate transporters such as EAAT2, with increased clearance of
extracellular glutamate (183). It has also been suggested that riluzole
regulates glutamate release and postsynaptic receptor activation, and inhibits
voltage-sensitive channels (162). The riluzole effect appears to be the
strongest evidence for the involvement of glutamate metabolism in ALS
pathogenesis. Whether excitotoxicity is a primary toxic event or just
secondary due to the neurodegenerative disease process requires further
study. How this correlates to other etiological pathways such as mutant
SOD1 toxicity also requires investigation.
Superoxide dismutase-1

The protein

Activity and subcellular distribution. Seventy years ago, Mann and Keilin described a protein that was responsible for binding most of the Cu$^{2+}$ in bovine erythrocytes, which they called haemocuprein (184). It took another thirty years before McCord and Fridovich discovered and reported the function of this copper protein and renamed it superoxide dismutase (SOD, later SOD1) (185). The function described, dismutation of superoxide, is a copper-dependent reaction that dismutes two molecules of superoxide ($O_2^-$) into hydrogen peroxide ($H_2O_2$) in a two-step reaction:

$$O_2^- + SOD-Cu^{2+} \rightarrow O_2 + SOD-Cu^+ \quad \text{(Step 1)}$$
$$2H^+ + O_2^- + SOD-Cu^+ \rightarrow H_2O_2 + SOD-Cu^{2+} \quad \text{(Step 2)}$$

Oxygen, a molecule crucial for all human cells, is used as substrate for energy production mainly in the mitochondrial respiratory chain (MRC). During these reactions, $O_2$ is reduced to water in multistep reactions (186). The superoxide anion is a free radical that can be formed when oxygen picks up one electron in reactions involving the complexes of the MRC. Another major source of superoxide is the group of membrane-bound enzymes known as the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases (several other cellular oxidases that produce superoxide are known). These enzymes are highly expressed by cells of the immune system, such as neutrophils, microglia, and macrophages, and are activated during inflammation for example. Depending on the type of NADPH oxidase (several Nox genes are known), superoxide can be formed both extracellularly and intracellularly. It is of particular interest that recent experiments have shown that massive activation of these enzymes takes place both in SALS and in murine models of ALS (187), and Nox2 knockout (the predominant species in microglia) has been shown to slow disease progression in ALS transgenic mice (187;188). The activation of NOX2 and increased amounts of superoxide have also been shown to be positively regulated by different mutant SOD1s when expressed in cellular models (189). Superoxide is considered to be a moderately reactive molecule but it reacts with FeS clusters, which occur in several enzymes. It can also be highly toxic through its very rapid reaction with NO to form peroxynitrite (ONOO$^-$). Peroxynitrite reacts quickly with CO$_2$ to form nitrosoperoxycarbonate (NOOCO$_2^-$) a highly toxic species that can react with virtually all macromolecules in the cell (e.g. through tyrosine nitration). NO is produced by three different nitric oxide synthetases (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (190). Endothelial NOS produces the NO that is essential for
vascular homeostasis whereas nNOS is expressed in neurons (as well as in other cells), where NO is involved in diverse processes, e.g. neurogenesis and memory. Superoxide can also cause adverse effects by interfering with these normal physiological functions of NO. The activities of eNOS and nNOS are regarded as essentially constitutive whereas the level of iNOS depends on different cellular stimuli such as infection, inflammation, and ischemia. A number of pathological conditions have been shown to cause a significant increase in iNOS, one of them being ALS (191;192), where high NO levels have been speculated to contribute to pathogenesis.

OH⁻, by far the most reactive free radical, can be formed from O₂⁻ and H₂O₂ through reactions involving transition metals such as Fe²⁺ (or Cu²⁺); this is known as the Haber-Weiss reaction.

\[
\begin{align*}
O₂⁻ + Fe^{3+} & \rightarrow O₂ + Fe^{2+} \\
H₂O₂ + Fe^{2+} & \rightarrow OH⁻ + OH⁻ + Fe^{3+}
\end{align*}
\]

(Fenton)

The second reaction is also called the Fenton reaction. Fe³⁺ and Cu²⁺ can also be reduced by other reductants such as ascorbate. As O₂⁻ and H₂O₂ are involved in reactions resulting in the formation of OH⁻, the cell needs efficient systems for handling of these molecules. The oxidation of H₂O₂ into water is catalyzed by several cellular enzymes (e.g. peroxiredoxins, glutathione peroxidases, thioredoxin, and catalase) and is extremely rapid. Superoxide can be scavenged by ascorbate but is mainly taken care of through dismutation via SOD. The importance of this activity is highlighted by the existence of two other SOD enzymes besides SOD1, manganese-SOD (SOD2) (193;194) and EC-SOD (SOD3) (195). These three enzymes are located in different compartments, with SOD1 being responsible for the cytosol (194), the nucleus (193;194;196;197) and the intermembrane space (IMS) of mitochondria (193;198), whereas SOD2 is located in the mitochondrial matrix (194) and SOD3 is secreted into the extracellular space (195;199). The subcellular distribution of SOD1 is, however, controversial and the subject of discussions involving other compartments such as peroxisomes (196;200;201) and the endoplasmic reticulum (ER) (198;202;203). As the mitochondrion is one of the main sites for superoxide production (204) it is not surprising that the matrix has its own enzyme in combination with protection from SOD1 in the IMS. Deletion of SOD2 in mice has also been shown to be lethal during the first days of life (205;206). SOD1 knock-out mice, although not completely healthy (e.g. reduced fertility, axonal repair deficiencies, increased oxidative stress, cataract), do not display any severe phenotype (207-212). Similar to knock-out of SOD1, knock-out of SOD3 has no obvious spontaneous phenotype (212;213). This is apparently caused by adaptions in the germ-line SOD3 knock-outs. Partial conditional knock-out of SOD3 in the lungs of adult mice leads to death from lung failure in just a few days (214). The relatively mild phenotype of
germ-line SOD1 knock-out mice might also be explained by compensatory adaptations.

**Structure and properties.** SOD1 is composed of two equivalent 153-aa long subunits, each containing one Cu$^{2+}$ ion and one Zn$^{2+}$ ion, with a molecular weight of roughly 16 kDa (whereby the enzyme is also known as Cu, Zn-SOD). The gene is located on chromosome 21q22.11. SOD1 is widely expressed, with the highest amounts in the liver and kidneys (215). The core of the SOD1 structure is eight β-strands connected through seven loops and arranged as a β-barrel (216;217). As mentioned, the Cu$^{2+}$ ion is involved in the dismutase reaction but is also important for structure/folding, whereas the Zn$^{2+}$ ion functions as a stabilizer (218). Four histidines (His46, His48, His63, and His120) are the ligands for the binding of copper and His63, His71, His80, and Asp83 are the ligands for Zn (219). Many ligands are present in the Zn-binding loop IV (His48-Asp83). Apart from the binding of the metal ions, the tertiary structure of the monomer is stabilized through an intrasubunit disulfide bond between Cys57 and Cys146. The presence of a structural disulfide bond is an unusual property of a protein located in the reducing environment of the cytosol, and it may be an Achilles heel of SOD1.

There is a specific copper chaperone (copper chaperone for SOD1, CCS), responsible for Cu loading of SOD1 in eukaryotes (220). CCS knock-out mice were shown to display reduced SOD1 activity and a phenotype similar to that seen in SOD1 deficiency (e.g. reduced fertility) (221), but other experiments have shown that human SOD1 can be Cu-loaded to some extent by other means (222). SOD1 is one of the most stable proteins known, and it retains most of its activity in 10 M urea, 4% SDS, and heating to 80°C (218;223). This stability is totally lost in the apo state, when the protein is deprived of metals and upon reduction of the intrasubunit disulfide bond between cysteines 57 and 146. After loss of metals and reduction of the disulfide bond, SOD1 is unable to dimerize and the monomeric form is favored (224-226).

**SOD1 in ALS**

As discussed above, the number of SOD1 mutations found in ALS cases is steadily increasing and today mutations are known in approximately half of the codons (74 out of 153). Most cases are missense mutations, but insertion and deletion mutations exist and for the purposes of this thesis, the truncation mutation G127insTGGG (G127X) serves as an example (discussed below). The mutations confer widely different effects on the SOD1 molecule, with some—such as G127X—causing a severely truncated protein without SOD activity and loss of Cu/Zn binding and normal folding, whereas the common D90A mutant is fully active and properly folded. Despite such diversity, these mutations result in a similar disease phenotype, indicating the existence of an ALS-causing common toxic SOD1 species.
When caused by mutated SOD1, the disease is generally dominantly inherited with varying penetrance. However, the most common of the mutations, D90A, causes mostly recessively inherited ALS (39). Interestingly, this mutation has been found to be inherited as a dominant trait among FALS and SALS cases in populations where the mutation is rare (227). About 20% of FALS has been reported to be caused by mutations in SOD1, which represents about 2% of all cases (223;228). In a study of a mixed population of ALS patients, the frequency of SOD1 mutations was found to be 7.2% (38). This is in accordance with other findings of rather frequent SOD1 mutations in apparently sporadic ALS (229-231).

The phenotype of mutant SOD1 ALS is generally that of typical ALS, although some mutations are known to be associated with atypical features such as long-term survival and bladder control disturbances (e.g. D90A) (231). The similarities between mutant SOD1 ALS and typical ALS have drawn attention to the wild-type SOD1 protein, and recent experiments suggest that SOD1 may be involved in the majority of cases (232-238).

**Transgenic murine SOD1 models**

Since taking CNS tissue samples from living ALS patients is associated with great risks, the need for model systems has been obvious. Different cell lines expressing various mutant SOD1s are routinely used, but since the spinal cord is a very complex tissue with multiple cell types, animal models are often needed. Mice have a highly developed CNS that is in many ways similar to ours, and genetic techniques have been used successfully for many years in this species. Since the discovery of mutant SOD1-induced ALS, various transgenic murine models have emerged. By far the most frequently used one is the G93A mouse, which was first described in 1994 (239). The widespread use of this model is mainly due to the conveniently short survival time (about 125 days, depending on the copy number) and the predictable course of the disease. These characteristics have made it the model of choice for pharmacological trials and virtually all potential therapies have been tested in G93A. The poor reproducibility between preclinical G93A trials and human drug trials, including riluzole, has recently pulled into question the suitability of the model and the relevance of positive drug reports to date (240). From a pathological point of view, there are similarities with human disease, e.g. ubiquitin-staining inclusion bodies, motor neuron loss, and reactive gliosis. During disease progression, the G93A mouse develops overt vacuolar pathology of the CNS (241). These are speculated to be remnants of damaged mitochondria and are common to other “high-level” models (discussed below), but are rarely found in human ALS subjects. The G93A mutation has also been transgenically expressed in rats (*Rattus norvegicus*), a model similar in most ways to the mouse strains used (242). The next mutant mouse model to be published was that with the G85R mutation in 1997 (243). This model develops an ALS-like disease
within approximately one year, despite having a mutant SOD1 level no
greater than the endogenous mSOD1 (i.e. low-level, as discussed below).
Histopathologically these mice differ from the G93A strains, as they lack
vacuoles and show SOD1 inclusions in astrocytes as well as in neurons. The
G127X mouse was generated in our laboratory and the mice are now bred as
homozygotes (35). This mutation was found in a Danish family and is an
insertion of four novel nucleotides after the glycine 127 codon
(Gly127insTGGG) (231). The insertion leads to five novel amino acids
followed by a premature stop codon. This protein is present at very low
levels, about half that in the wt mSOD1 situation. G127X mice live
approximately 216 days and succumb to motor neuron disease after an
extremely rapid disease progression (of less than one week). D90A mice
were also generated in our laboratory and, similarly to G93A, produce high
levels of mutant protein (234). Multiple mutant SOD1 transgenic mouse
models exist, but they will not be discussed further here. Several lines of
mice expressing wt hSOD1 have been generated (239;244;245). None of
them have displayed an ALS phenotype, but pathologically some similar
characteristics are apparent such as loss of neurons in the ventral horn,
vacuolization of mitochondria, and late accumulation of aggregated SOD1
(234;246;247).

There are several other animal models that express mutant SOD1s, e.g.
*Drosophila melanogaster* (248), *Caenorhabditis elegans* (249), zebrafish
(250), and pigs (unpublished). Although not extensively studied, these might
prove to be important in new ways. Recently, canine degenerative
myelopathy, a disease in dogs similar to human ALS, was found to be
caused by a point mutation in the *SOD1* gene (251). Pathological
examination also revealed neuronal SOD1 inclusions similar to those seen in
human mutant SOD1 ALS cases.

**How do mutant SOD1s cause disease?**

**Loss of function**

It soon became obvious from multiple sources that loss of SOD1 function
does not cause ALS.

(i) SOD1 knock-out mice do not present an ALS phenotype.

(ii) Transgenic overexpression of mutant SOD1 leads to ALS
despite there being normal levels of endogenous wild-type
enzyme.

(iii) Transgenic overexpression of most mutations results in several
times more functional SOD1 enzyme.

(iv) Disease is almost always dominantly inherited, and some
mutations do not reduce the total SOD activity (e.g. D90A).
Thus, mutations in SOD1 confer a toxic property to the protein, which is most likely of the same basic character for all mutants.

**A non cell-autonomous toxicity**

Several reports have suggested that the toxic effect of mutant SOD1 is non-cell autonomous. This conclusion is based on the following findings:

- **(I)** Neuron-specific expression of mutant SOD1 does not generate an MND phenotype (252;253). (This was challenged recently; see below).

- **(II)** Astrocyte-specific expression of mutant SOD1 does not generate an MND phenotype (254).

- **(III)** Chimeric mice that have been generated from mice expressing wt and mutant SOD1 (mixed cell-types), develop motor neuron pathology but do not develop a symptomatic MND. In these mice, motor neuron pathology is dependent on a mutant SOD1 context (255). Another recent report has shown that selective expression of mutant SOD1 in motor neurons and oligodendrocytes surrounded by wild-type neurons results in a delayed onset of disease (256).

- **(IV)** Transplantation of wild-type myeloid cells into G93A mice was shown to slow down disease progression (257) and microglia expressing mutant SOD1 were shown to have higher production of ROS and nitric oxide than those expressing WTSOD1 (258).

- **(V)** During disease progression, an increasing amount of lymphocytes migrating into the CNS has been reported (259) and the embryonic knock-out of T-lymphocytes in G93A mice has shown an accelerated disease progression (259;260).

- **(VI)** Extracellular mutant SOD1 has been shown to be toxic to motor neuronal cells only when co-cultured with microglia (192).

- **(VII)** Removal of mutant SOD1 expression in neurons through the Cre/lox system resulted in delayed onset and extended survival but did not abolish MND (261). Using the same technique, mutant SOD1 expression was removed from microglia (261) and astrocytes (262), which slowed disease progression but did not alter the time of onset.

- **(VIII)** Somewhat contradictory, knock-down of mutant SOD1 expression in Schwann cells has been shown to enhance disease progression (263).
(IX) Deletion of mutant SOD1 expression in muscle does not alter the phenotype, suggesting that muscle cells is not involved in the disease (264).

(X) The expression of mutant SOD1 in glial-type cells has been shown to induce apoptotic mechanisms in co-cultured neuroblastoma cells and embryonal spinal motor neurons from mutant SOD1 transgenic mice (265). Co-culture of wt SOD1 motor neurons and G93A glial cells from differentiated embryonic stem cells has resulted in motor neuron damage, indicating a soluble factor (266-268). It has also been shown that mutant SOD1 can interact with chromogranins and thereby be secreted to the extracellular compartment (269).

Recently, it has been shown that neuron-specific expression of high levels of SOD1 is indeed able to produce an MND phenotype in transgenic mice (270). Similar findings have been reported elsewhere (271). These results argue against the initial findings (I) and they probably depend on the level of expression.

In combination, these results argue that multiple cell types present in motor areas modulate disease through different parameters such as disease onset and progression. Although neuron-specific expression of mutant SOD1 might be sufficient, the natural form of the disease is probably dependent in different ways on interactions between most cells of the CNS.

**Aberrant redox chemistry and peroxidase activity**

During the first decade of mutant SOD1 studies, it was speculated whether mutant SOD1 could cause ALS through altered redox chemistry. It was suggested that the active site copper became increasingly exposed and could therefore participate in reactions with peroxynitrite and induce damage to proteins by tyrosine nitration (272-274). Another similar theory suggested that mutant SOD1 could catalyze copper-mediated conversion of hydrogen peroxide to hydroxyl radicals, which—like peroxynitrite—is one of the most reactive ROS (275). Other data contradict these theories. Several SOD1 mutations have either been shown or predicted to be deficient in copper binding and to lack activity (35;243;276;277). An artificial mutant lacking all four copper-binding histidines can still cause disease in transgenic mice (278). In line with this, the knock-out of CCS did not ameliorate the MND phenotype in mutant SOD1 mice despite substantial loss of SOD1 copper binding (279). This theory has not been completely discarded, however, and recently suggestions have been made that metal-deficient SOD1 can bind copper or other transition metals in the zinc site, and thereby take part in aberrant redox reactions (223;280). Recent studies have also suggested that the excessive nitration of proteins, found in the G93A mouse model, may contribute to disease (281).
SOD1 and mitochondria

As already mentioned, SOD1 was shown in the early 1970s to be localized not only in the cytosol, but also in the intermembrane space (IMS) of mitochondria (193). This partitioning was due to an unknown mechanism, as SOD1 does not contain a mitochondrial localization sequence. It was later shown that SOD1 is translated in the cytosol and imported in a disulfide-reduced state, without any metals bound (the apo state) (282;283). It was also shown that CCS is present in the IMS to aid apo-SOD1 turn into mature holo-SOD1, and thereby to get trapped in this compartment (282;284). The mitochondrion is the major cellular site for energy production through oxidative phosphorylation, and therefore also a potential site of generation of oxidative free radicals (204). As mentioned, Mn-SOD is present in the mitochondrial matrix (193) for scavenging of free radicals and it was therefore not surprising that SOD1 was found to be localized in the IMS, probably for the same purpose. It has been shown that knock-out of SOD1 leads to increased carbonyl levels in mitochondria, which can be used as a marker of increased oxidative stress (284).

The first reports on mitochondrial pathology in ALS came from studies on autopsy specimens and from functional measurements on peripheral tissues of patients (reviewed in (285)). Later, similar studies carried out on CNS tissue have revealed aggregated, swollen mitochondria and functional deficits (286-289). This has, however, not been a uniform feature of several other reports (290-292). Autopsy studies are also subject to several drawbacks, such as the issue of post-mortem time, tissue preservation, and the fact that only end-stage disease can be studied (285).

The real take-off in the subject of mitochondrial pathogenesis came with the first reports of severe vacuolization of mitochondria present in the early lines of mutant SOD1 transgenic mice (241;244). These vacuoles were later shown by electron microscopy to be derived from expansion of the IMS evident before the onset of symptoms (293-296). The process of vacuolization was described by Higgins et al. to be distinct from earlier reported vacuole processes (mitochondrial permeability transition (MPT) or autophagy-derived), and it was referred to as Mitochondrial Vacuolation by Intermembrane Space Expansion (293). It was also found that mutant SOD1 accumulates in vacuolated mitochondria and co-localizes with cytochrome C, suggesting a direct toxicity through interaction (293;294). This idea is, however, challenged by the findings that mice overexpressing wt hSOD1 also show vacuolar pathology and accumulation of wt hSOD1 protein without any signs of ALS disease (234;241;244;246;247;294). Other work has shown that not all SOD1 mouse models, e.g. the G85R and H46R (243;297), display this pathology and that it is rarely found in autopsy samples (298). In line with the mitochondrial pathology, several reports have indicated that expression of mutant SOD1 in both cells and transgenic mice
results in alterations in mitochondrial function (299-302) similar to what was originally found in human subjects.

After these initial findings of mitochondrial pathology, dysfunction, and direct association with SOD1, the obvious question was how mutant SOD1 might be toxic to the organelle. Several somewhat divergent mechanisms of toxicity have been suggested. These are not mutually exclusive but are more or less linked.

(I) Mitochondrial accumulation of SOD1 protein aggregates. Several studies have shown that mutant SOD1, and under certain circumstances wt SOD1, can oligomerize and form protein aggregates on the outer surface of the outer mitochondrial membrane or in the IMS (293;303-308). Another report suggested that mutant SOD1 could aggregate in the mitochondrial matrix (309). Although not a well-defined mechanism, the idea of SOD1 that accumulates in mitochondria being toxic was supported by the report that overexpression of CCS along with mutant SOD1 in transgenic mice induced an even heavier mitochondrial load of SOD1 followed by a remarkable decrease in survival time (310). These findings are the subject of some debate due to findings by us (extensively discussed in paper 1) and others showing that mutant SOD1s appear to accumulate (to a relative level higher than mSOD1) in mitochondria in vivo only if the total cellular level of mutant protein is extremely high (e.g. the G93A mouse model) (297).

(II) Mitochondrial release of apoptotic molecules. As mitochondria are important in the regulation of apoptotic pathways, these have been studied in several reports. Cytochrome C is a mitochondrial IMS protein that, upon mitochondrial release, induces apoptosis through activation of cellular caspases. The discovery of mutant SOD1 and cytochrome C co-localization, (293;294) was followed by reports on its cytosolic release (311-313). Cozzolino et al. showed that mutant SOD1 expression caused apoptosis through a pathway involving Apaf1, a scaffold protein of the apoptosome (a protein complex involved in activation of caspases) (311). When Apaf1 was knocked out, apoptosis and mitochondrial pathology was abolished despite similar SOD1 mitochondrial accumulation. Mutant SOD1 has also been shown to interact directly with the anti-apoptotic protein Bcl-2 and, through co-aggregation, to deplete cells of this important function (304). This finding has not, however, been reproduced by another group (314). These and other findings of increase in apoptotic mediators have been partly challenged by experiments in transgenic mice expressing mutant SOD1 in which different important apoptotic mediators have been knocked out: caspase-11 (315) and BAX (314). Both of these mouse models develop an ALS phenotype despite the inhibition of apoptotic pathways. Remarkably, BAX knock-out mice were found to develop motor neuronal disease without loss of motor neuronal cell bodies in the spinal cord. If apoptosis is involved in motor neuronal death in ALS, clearly apoptotic routes other than these must be involved.
(III) Increased production of reactive oxygen species (ROS). There have been several reports on increase in oxidative stress and ROS produced from mitochondria following expression of mutant SOD1s (300-302;306;316-318). The precise source of ROS has not been established, but mutant SOD1 expression appears to inhibit the normal function of the electron transport chain, either through direct interaction/aggregation or through secondary effects, generating a higher leakage of free electrons and thereby more ROS (discussed above). Increase in ROS triggers several pathways such as cytochrome C release/apoptosis, induces damage to mitochondrial DNA, causes protein modifications/alterations, and stimulates nuclear transcription of genes involved in protection and inflammation (285;287;318-323).

(IV) Mitochondrial damage through cytosolic/non-cell autonomous events. It has been suggested that binding of mutant SOD1 to proteins in the cytosol, such as heat shock proteins (HSPs), causes depletion of and toxicity to mitochondria (283;285;324). SOD1 has been shown to interact with a lysyl-tRNA synthetase (mitoKARS) in the cytosol and to co-aggregate at the cytoplasmic face of the mitochondrial outer membrane, thus contributing to mitochondrial dysfunction (325). Recently, it has been shown that selective expression of mutant SOD1 in astrocytes leads to mitochondrial pathology and dysfunction in motor neurons, indicating that the toxicity can be transferred from one cell to another (non-cell autonomous), possibly through oxidative stress (326;327). Cassina et al. also showed that inhibition of mitochondrial function in astrocytes caused by means other than mutant SOD1 resulted in similar toxicity to motor neurons.

(V) Excitotoxicity. Excitotoxicity through extracellular glutamate signaling (discussed above) is a well-studied pathogenic mechanism in both in vivo and in vitro models (285). Mitochondrial dysfunction may enhance the sensitivity to this assault even more in motor neurons than in other cells (285;328). Mitochondria are the main storage site of intracellular Ca$^{2+}$, and dysfunction of Ca$^{2+}$ regulation seems to be important for excitotoxicity (329). Defects in intracellular Ca$^{2+}$ regulation have been found in SOD1 mutant mice (330) and cell models (299;331).

(VI) Impairment of axonal transport. In motor neurons, adequate anterograde and retrograde transport of mitochondria is necessary for cellular function and energy metabolism all through their extremely long axons. This is achieved through interaction with motor proteins such as kinesin/dynein and movement along microtubules (332). There is recent evidence to suggest that these movements are inhibited either by mitochondrial dysfunction or through dysfunction of the transport systems (reviewed in (332)). Disturbance of mitochondrial transport has been reported in SOD1 transgenic mice (333;334) as well as in SALS patients (289). This also suggests functional links between SOD1 toxicity and mutations in dynactin (as discussed above). Furthermore, mutant SOD1 has
recently been shown to co-aggregate with and possibly cause depletion of components of the anterograde transport system (335).

SOD1 aggregation and inclusions

As mentioned above, CNS tissues from FALS patients with mutant SOD1 and transgenic murine models show inclusions with SOD1 immunoreactivity (36;336). The formation of cellular inclusions could be toxic as they may, for example, trap essential proteins and block or interfere with cellular functions (283;335). There is no consensus on how mutant SOD1 ends up in inclusions, but most of the evidence points to impaired degradation, misfolding, and aggregation (32;337;338).

SOD1 degradation. Although the routes of protein degradation in cells of the CNS have not been fully worked out, proteasomal degradation and autophagic/lysosomal degradation appear to be the two main pathways (339). These systems are partly complementary, but the ubiquitin-proteasome system (UPS) is mainly responsible for degradation of ubiquitin-tagged soluble cytosolic proteins and the lysosome for endocytosed or autophagocytosed proteins (339).

Wild-type and mutant SOD1 has been shown to be degraded by the UPS (337;340-343). Important mediators in this degradation have been found to be the chaperones HSP70/HSC70 and the ubiquitin E3-ligases CHIP, dorfin, and NEDL1 (342;344-347). Inhibition of the UPS accelerates aggregation of mutant SOD1 and formation of cellular SOD1 immunoreactive (ir) inclusions in cell models and tissue cultures with similarities to those found in transgenic mice and FALS patients (337;343;348-350). Further on, SOD1ir inclusions co-stain for ubiquitin, chaperones, and proteasomal components (336;342), and SOD1 has been shown to be oligo-ubiquitinated in cell models (351) and in vivo (35;352). Apart from the constitutive proteasome, there is an inducible form called the immunoproteasome (353). The activities of these two entities have been measured in tissues from mutant SOD1 transgenic mice, and it appears that at least the constitutive proteasome is inhibited (354-356). Reduced proteasomal activity has also been found in cells expressing mutant SOD1 (351) as well as in mice overexpressing wild-type protein (357).

Although the proteasomal route to SOD1 degradation seems important, its involvement in ALS pathogenesis is not clear. Transgenic mice expressing G93A SOD1 have been crossed with mice that are deficient in the immunoproteasome (LMP2 -/-) (358). This model did not show a significant alteration in phenotype. Furthermore, inhibition of proteasomal function in organo-typical spinal cord cultures showed no difference in survival of motor neurons between those expressing mutant SOD1 and those from normal tissue (359).
Our knowledge of the role of autophagy in degradation of proteins of the CNS was given a remarkable turnabout when mice deficient in this pathway \((atg7, atg5 -/-)\) were found to show severe neurodegeneration \((360;361)\). The disease was associated with neuronal pathology at several levels, with protein aggregates and inclusion bodies. It was later shown that SOD1 is partly degraded by macroautophagy \((362)\) and that this route is altered in G93A mice \((363;364)\). Ligation of ubiquitin monomers to proteins targeted for autophagic degradation is mediated by lysine 63 (K63) on ubiquitin. When a ubiquitin K63 mutant was co-expressed with mutant SOD1, it resulted in generation of more cellular inclusions \((365)\).

**Misfolding/aggregation.** The formation of cellular inclusions and SOD1 aggregates could thus be caused by deficiencies in degradation and cellular overloading. Other theories and complementary ones suggest that SOD1 aggregation is caused by misfolding of mutant SOD1 monomers either in combination with deficient degradation, loss of folding of chaperones, or by intrinsic aggregation—through aberrant disulfide coupling, for example. *In vitro* studies of wt apo-SOD1 have shown that it can aggregate even under physiological conditions \((366;367)\), although the wt holo protein is regarded as an extremely stable protein. These propensities to misfold may be aggravated in mutant SOD1 \((368-370)\). For nucleation of aggregation, the least common denominator is disulfide-reduced, non-metallated SOD1 \((367;367;367;371;372)\). Similar species have been identified *in vivo*, in a series of transgenic mouse models \((247;373)\). Although intermolecular disulfide bonding exists in SOD1 aggregates both *in vitro* and *in vivo* \((247;366;374;375)\), resulting in SOD1 oligomers, mutation of all four cysteines does not prevent SOD1 aggregation \((371;376)\). C57-C146 disulfide-reduced SOD1 has also recently been shown to be the predominant species in detergent-resistant aggregates \((375)\). This highlights the importance of this *in vivo* phenomenon and indicates that nucleation and building of SOD1 aggregates derives from the constant pool of soluble, disulfide-reduced apo-SOD1 (discussed in paper III).

The composition of SOD1ir inclusions has mainly been explored by histochemical approaches in transgenic mice and FALS patients. Co-staining of inclusions has been found for a number of proteins such as CCS, heat shock proteins, ER chaperones, neurofilaments, kinesin-associated protein 3, p38MAPK \((202;298;335;336;377;378)\), and parts of the UPS mentioned above (discussed in paper III). Analysis of detergent-resistant aggregates has revealed similar proteins such as different heat shock proteins \((278;281;324;379)\). Proteomic approaches have recently emerged as a promising tool for identification of aggregate composition, and they will be discussed below.

Although it is clear that SOD1 aggregates/inclusions are present in most murine models and ALS cases carrying SOD1 mutations, it is not clear whether they are a toxic prerequisite. In G93A mice, overexpression of CCS
has resulted in a more aggressive phenotype without any signs of SOD1 ir
inclusions (310). Other experiments, on inhibition of proteasomal function in
mutant SOD1 cell models for example, have induced SOD1 aggregation but
have not resulted in more pronounced cell death than in controls (380).

SOD1-interacting heat shock proteins. Apart from the proteins found to be
co-aggregated with SOD1 in inclusions, a number of proteins have been
found to interact with SOD1, through co-immunoprecipitation experiments
for example. These are mainly proteins from the family of heat shock
proteins (HSPs), a group of proteins involved in stress responses and protein
folding. The most frequently reported SOD1 interaction partner has been
HSP70/HSC70 (283;324;344;345). As mentioned above, HSP70 has been
proposed to bind misfolded SOD1 and mediate degradation or possibly
refolding. The initial reports stated that this interaction was indeed confined
to mutant SOD1, but later experiments have shown that oxidized wild-type
enzyme acquires mutant-like properties and binds HSP70 (232). Other HSPs
found to interact with SOD1 are HSP105, HSP90, HSP40, HSP25, and αβ-
-crystallin (283;324;344;381). The heat shock response is crucial for the
cellular response to misfolded proteins, and it has been reported that these
pathways may be deficient in SOD1 transgenic mice (382). Furthermore, the
interaction of HSPs and SOD1 has been suggested to be the reason for
cellular depletion of chaperones, as they may be trapped in inclusions (283).
In this way, the initially beneficial SOD1/HSP interaction eventually leads to
loss of important functions and a vicious circle arises through generation of
more misfolded proteins. From the above-mentioned experiments, it was
suggested that induction, overexpression, or exogenous delivery of HSPs
could be beneficial in ALS. Overexpression of HSP70 in cultured cells has
been shown to reduce the amount of insoluble SOD1 aggregates (349) but
not to ameliorate lifespan in SOD1 transgenic mice (383). Administration of
recombinant HSP70 intraperitoneally in SOD1 transgenic mice has been
shown to be beneficial when treatment is started before the onset of disease
(384). In 2004, Kieran and colleagues reported that treatment with
arimoclomol, a co-inducer of heat shock proteins, significantly ameliorated
the disease in SOD1 transgenic mice (385). Although arimoclomol induces
expression of several HSPs, increases in HSP70 and 90 were the most
pronounced. This treatment has also been shown to reduce the number of
ubiquitin-positive inclusions (386). To be effective in survival, treatment has
to be started before or very early after onset of disease, which of course is
difficult to achieve in human ALS subjects (385;386). Still, this is one of the
most promising therapies and human trials are ongoing (387).
The “–omics” in ALS/SOD1 models

The expanding field of proteomic and genomic experimental methods has introduced new ways of exploring pathogenic events without input of prior knowledge. In genomics, microarrays confer the possibility of exploring gene expression patterns in practically the whole genome—during progression of a disease, for example. Proteomic techniques are still more limited, but they may be more reliable since mRNA levels do not always reflect the amount of protein finally produced. Genomics also misses the whole area of post-translational modification of proteins. This and other technical discrepancies have led to partly conflicting results between genomic and proteomic findings. These issues will probably be solved some time in the future and the different approaches shown to complement each other. Here, will follow a brief review of “global” genomic and proteomic experiments performed in the context of ALS, i.e. those involving total changes in a tissue or cell.

Genomics

In recent years, several gene-expression studies using the microarray technique have been performed both in transgenic mice (321;388-392) and on autopsy material from human ALS cases (393-397). One serious drawback of autopsy material is the difficulties associated with post-mortem time and major cellular/mRNA alterations that take place after death. While the early studies were performed on whole spinal cord tissue or parts thereof (motor cortex in (396;397)) with a wide range of cell types, more recent genomic studies have been specifically performed on motor neurons collected by laser-capture techniques. As all of these studies have been highly individual in terms of the techniques and models used, the findings have been quite diverse regarding alterations in individual genes. The focus here must be to find similarities in expressional regulation of pathways and functional classes of genes. Most studies have found alterations in mitochondria, in oxidative stress, in the citric acid cycle, and in neurotransmission (GABA and glutamate metabolism). The effect on energy metabolism appears to be a common denominator.

Proteomics

A series of global proteomic studies have been performed in slightly different murine models of ALS. In a study on pooled homogenates from terminal G93A mice, Strey et al. found seven proteins to be differentially regulated compared to non-transgenic mice and mice expressing wt hSOD1 (398). These protein alterations were not present in asymptomatic mice. Atkin et al. performed a study at disease onset in the G93A rat model (202). Out of 15 proteins that were altered, 12 were identified of which some were ER chaperones involved in the unfolded protein response (UPR). In a study
comparing G93A mice to transgenic mice expressing wt hSOD1, Massignan et al. reported a mutant-specific change in 15 proteins in presymptomatic mice (399). Similarly to the study by Atkin et al., they found upregulation of the ER chaperone protein disulfide isomerase (PDI). However, most alterations were in proteins that are involved in metabolism, particularly from mitochondria. Lukas et al. performed a similar study on presymptomatic and terminal G93A mice but also included non-transgenic mice as controls (400). Using fractionation of spinal cord homogenates and LC-MS/MS, they compared the proteome from different fractions on the basis of functional categories (gene ontology analysis). This was preferred, since LC-MS generates semi-quantitative data and the alterations in individual proteins become less reliable. Interestingly, they performed a set of microarray experiments for comparison of proteome and gene expression data in the same model. As in other reports, the most obvious alterations were found in mitochondrial pathways, results that were not, however, corroborated by gene expression analysis. A study looking for differentially expressed proteins in the hippocampus of mice overexpressing wt hSOD1 found changes in 41 out of 157 proteins identified (401). This model has a high steady-state level of SOD1, much like the G93A model, and does develop loss of motor neurons but not overt symptoms. Several of the differentially expressed proteins involved mitochondria and the cytoskeleton.

Using a cell model expressing different mutant and wt hSOD1s, with proteomic techniques Di Poto et al. found 17 proteins to be differentially regulated (402). Most proteins were involved in cellular metabolism and antioxidant defense. The most serious drawback of studies with cell lines may be the lack of a diverse cellular context, which in recent years has been shown to be important for motor neuronal disease (403) (discussed above). Proteomic methods have also been used for screening of potential biomarkers in cerebrospinal fluid and plasma (404-409). Although no consensus biomarker has been found, cystatin C may be a candidate as its potential as a biomarker has been able to replicate in the CSF of ALS patients.

**Proteomics on SOD1-interacting proteins**

Different methods have been used to try to identify SOD1 interaction partners. Proteomic methods have recently given new insights in this area.

With the addition of a chemical cross-linker in lysates from mutant SOD1 expressing cells, Zhang et al. were able to immunoprecipitate and—by proteomic methods (LC-MS/MS)—identify SOD1 interaction partners (410). Interestingly, apart from known partners such as CCS and HSP70, the heavy chain of dynein was implicated, thus linking mutant SOD1 with dysfunction of motor proteins as suggested previously. Using a similar strategy, Watanabe et al. used transgenic mice expressing FLAG-tagged truncated
SOD1 for immunoprecipitation and identification of SOD1 interaction partners by LC-MS/MS (411). Thirty proteins were found to co-precipitate with SOD1 in mutant mice, whereas only a few were present in homogenates from wild-type expressing or non-transgenic mice. Wang and colleagues immunoprecipitated soluble and insoluble fractions containing mutant SOD1 (G85R coupled to yellow fluorescent protein) from murine spinal cord (379). The soluble fractions contained CCS and various HSPs, whereas the insoluble fractions consisted mainly of cytoskeletal components. In a similar study on spinal cords from terminal G93A mice, proteins in detergent-resistant (insoluble) pellets were analyzed with 2-dimensional gels and MALDI-TOF (281). They found 42 protein spots selectively accumulated in insoluble pellets from G93A mice, as compared to a strain expressing wt hSOD1. Shaw et al. performed a study on detergent-insoluble aggregates and immunoprecipitated SOD1 aggregates for identification of co-aggregation proteins in mutant SOD1 transgenic mice with different characteristics (412). The only other protein found with reasonable consistency was vimentin, suggesting that SOD1 was the main constituent of these species.

Metabolomics

In the search for possible biomarkers of ALS disease, the expanding field of metabolomics may be more promising than genomic and proteomic methods. These new methods deal with quantification and analysis of small molecules (of 60–1,000 Da), such as peptides and degradation products, derived from accessible extracellular fluids. Work in identifying biomarkers in ALS is in progress (413).
Aims

- To determine whether ALS can be ascribed to toxic effects of mutant SOD1 accumulating in mitochondria. Are different mutant SOD1 species, like the truncated G127X protein, uniformly present in mitochondria? (paper I)

- To relate tissue vulnerability to distribution of the defective, truncated G127X mutant SOD1. Are the toxic properties of mutant SOD1 expression confined to the CNS in human ALS? (paper II)

- To determine the composition of SOD1 inclusions in the spinal cord of mutant SOD1 transgenic mice and the properties of SOD1 protein trapped in them (paper III)

- To gain insight into ALS pathogenesis from analysis of changes in individual protein levels in the spinal cord of SOD1 transgenic mice at onset of disease. (paper IV).
Methods

SOD1 transgenic mice

A variety of transgenic mouse models of ALS were used in the different experiments discussed here. The high-expressing G93A mutant line (G93A Gur) was obtained from the Jackson laboratory, as were the mice expressing wt hSOD1. The mouse strain expressing the G85R mutant was obtained from Dr D.W. Cleveland. G127X and D90A mice were generated in our laboratory (discussed above). Mice were deemed terminally ill when, owing to limb weakness, they could no longer reach for food in the cages. This occurred for the D90A, G85R, G127X, and G93A mice at an average age of 407, 345, 216, and 124 days, respectively. The presymptomatic D90A, G85R, and G127X mice used were around 100 days old, whereas the G93A mice were around 50 days old. For the experiments in paper IV, mice were collected at their peak body weight. This occurred at day 195 ± 14 for the G127X mice and at day 325 ± 22 for G85R mice. As controls, we used non-transgenic littermates from the G93A and D90A breeding program, or occasionally pure C57BL/6J BomTac (B6) mice that were used for all breeding.

G127X patient

With a post-mortem delay of only 10 h, CNS and peripheral tissue from a Danish patient carrying the G127X mutation were quick-frozen at –80°C or stored in formalin after the post-mortem examination. This patient had showed rapidly progressing disease with truncal-thoracic onset and a survival time of 6 months.

Homogenization of tissue

Generally, tissues were homogenized in 25 volumes of appropriate buffer. In all experiments on organelles and inclusions with density gradient separations, an iso-osmotic buffer was used (mitochondrial buffer, consisting of 0.21 M mannitol, 0.11 M sucrose, 10 mM K HEPES, pH 7.2, 1 mM EGTA, and the Complete antiproteolytic cocktail without EDTA (Roche Diagnostics, Basel, Switzerland)). Tissues for immunoblot were supplied with 25 volumes of PBS (10 mM K phosphate, pH 7.0, 0.15 M NaCl and EDTA-free Complete antiproteolytic cocktail). For discrimination between disulfide-reduced and oxidized SOD1, the homogenization buffer was
supplied with 40 mM iodoacetamide. For 2-D gels, the spinal cords were homogenized in 10 volumes of lysis buffer (1 M Tris-HCl, pH 8.5, 2 M thiourea, 7 M urea, and 4% CHAPS). For 2-D gels and immunoblot, tissue was homogenized using an Ultraturrax (IKA, Stufen, Germany) followed by sonication using a Sonifier Cell Disruptor (Branson, Danbury, CT) for 30 s. For the preservation of organelles and separation of inclusions, a more gentle approach using dounce glass homogenizers was used.

**Density gradient separation**

Separation of organelles from tissue extracts can be performed in different ways, although density gradient centrifugation is the most commonly used method. Other methods involve gel filtration and cell sorting/flow cytometry. For density gradient separations, sucrose solutions have been the medium that is traditionally used. The advantage has mainly been low cost and availability, but for the separation of organelles the hypertonic characteristics of sucrose gradients have been a great disadvantage. This causes organelles to shrink due to osmotic water loss and affects their density (414;415). As organelles such as lysosomes, mitochondria, and peroxisomes exhibit very similar densities, hypertonic gradients make them inseparable. Furthermore, the high viscosity of sucrose solutions requires long centrifugation times at high speed. The introduction of non-ionic, iodinated, high-density substances developed as contrast media for X-ray purposes, such as metrizoic acid (415;416), brought a great improvement to organelle separation. The latest addition, iodixanol (5,5’-[(2-hydroxy-1-3-propanediyl)-bis (acetylamino)] bis [N,N’bis (2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenecarboxamide]) has a molecular weight of 1,550 Da and a density of 2.08 g/ml combined with a relatively low viscosity and low osmolarity (417). These characteristics preserve organelle morphology and increase the resolution of particles of similar density. Also, iodixanol is compatible with antibody-based and spectrophotometric assays. Density gradients are generally used as discontinuous gradients or continuous gradients. Discontinuous gradients are made with a series of different concentrations (at least two) of the medium that are put on top of each other. In this way, particles of similar density are trapped at the interfaces between gradient steps and can be easily identified. The major drawback in organelle separation is the overlap in density distribution between different organelles or other subcellular particles. A well-known example is the similarity in buoyant density between mitochondria and lysosomes from liver tissue. The problem can be partially avoided by the use of continuous gradients. In this case, the gradients are premade, more or less linearly, depending on the method used. We used the “two-chamber” technique, which is simple and highly reproducible (418). The two chambers are loaded with iodixanol diluted to the two appropriate densities representing the higher and lower
ends of the density range; here we used 12% and 38%. The chambers are connected at the bottom, which allows passage of the denser medium into the chamber of the lighter. A peristaltic pump draws the mixed medium from the chamber of the lighter medium and delivers it to the bottom of a centrifuge tube. During this process, the denser medium is mixed consecutively with the lighter medium, bringing a denser mix to underlay the medium already present (Figure 1).

![Figure 1. Two-chamber set-up used for making linear gradients.](image)

The preformed gradient is close to linear and the centrifugation will not affect the slope in any significant way, at least during the short centrifugation times used here. Although vertical and fixed-angle rotors are efficient for gradient separation, swinging-bucket rotors are traditionally used as the effect on the preformed gradient is minimal. These also minimize the risk of material adhering to the walls of the tube, and they were therefore used for all gradient separations.

For our separations, a 9-ml gradient was produced with a linear density covering 1.07–1.21 g/ml using 12–38% iodixanol in mitochondrial buffer. Homogenates were passed through a nylon filter of 5-µm pore size by centrifugation at 20 g for 5–10 min. Filtered homogenates were supplied with iodixanol to a final concentration of 8% to minimize the formation of a thick lipid layer at the homogenate/gradient interface, and loaded on top of the gradient. Centrifugation was carried out at 100,000 g for 90 min at 4°C and fractions were collected from the bottom of the tube.

To separate mitochondria from peroxisomes, the homogenate was supplied with 6 mM CaCl₂, 5 mM succinate, and 5 mM K₂HPO₄ prior to separation in order to cause permeability transition pore opening and swelling of the mitochondria.

**Marker enzyme assays**

For determination of organelle separation in density gradients, enzymatic activities or protein content (by immunoblot) of certain compartment-specific markers were measured. For mitochondria, the activity of succinate
dehydrogenase (SDH) was determined. Samples were incubated with 100 mM Na HEPES, pH 7.4, 1 mM EDTA, 10 mM succinic acid, and 0.08% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) for 90 min. To stop the reaction, three volumes of 2-propanol were added, followed by centrifugation at 14,000 g for 10 min. The absorbance of the supernatants at 550 nm was then measured. For detection of lysosomes, alkaline phosphatase activity was determined with p-nitrophenyl phosphate as substrate. The activity of lactate dehydrogenase (LD) was measured using the CytoTox96 kit (Promega, Madison, WI) and used as a cytosolic marker. The distribution of other organelles was determined by immunoblotting.

**Dot-blot assay**

A filter-trap assay for aggregated proteins was used in papers I and III, somewhat modified from that originally described by Wang et al. (419). Fractions from separations were filtered through 0.15- or 0.2-µm cellulose acetate membranes (Schleicher and Schuell, Dassel, Germany) in a dot-blot apparatus. In paper I, the homogenates and separated fractions were supplied with 0.1% SDS and gently sonicated before centrifugation and filtration. This increased the sensitivity of the assay 25-fold. After filtration, the membranes were subjected to immunoblotting. In paper III, the fractions from density separations were subjected to filtration without any prior treatment. Individual wells with trapped aggregates were then punched out and incubated in 1 × SDS-PAGE sample buffer for immunoblotting or DeStreak rehydration buffer (GE Healthcare) for 2-dimensional (2-D) electrophoresis.

**Antibody-based assays**

**Immunoblotting**

Immunoblotting (western blots) were performed according to standard procedures. Samples were diluted with the same amount of 2 × SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 20% glycerol, 4% SDS, and bromophenol blue) and separated on SDS-PAGE (Criterion™ Cell, Bio-Rad). When discrimination between C57-C146 disulfide-reduced and oxidized SOD1 was required (paper III), β-mercaptoethanol was omitted from the sample buffer. Gels were blotted onto polyvinylidene difluoride (PVDF) membranes and probed with primary antibody. All secondary antibodies were conjugated with horseradish peroxidase and chemiluminescence was generated with the addition of ECL Advance substrate (GE Healthcare). Separated bands were visualized using film and digital imaging (Chemidoc XRS, Bio-Rad). For quantification of
hSOD1, wt hSOD1 with its concentration determined by quantitative amino acid analysis was used as original standard (420).

**Histopathology**

Tissues were fixed with 4% paraformaldehyde in phosphate-buffered salt solution (10 mM K phosphate, pH 7.4, 0.15 M NaCl), dehydrated and embedded in paraffin. Transverse paraffin sections were prepared for immunohistochemistry or stained with hematoxylin/eosin. In paper II, liver and kidney sections were stained with periodic acid-Schiff, van Gieson, and reticulin stains and liver also with periodic acid-Schiff diastase, Fe, and Faucet’s stain. For immunohistochemistry, enhancement using microwave-heating for 20 min in citrate buffer, pH 6.0, was performed and the sections were blocked for 30 min in goat sera prior to the staining procedures. Single immunohistochemistry was performed on the sections using the Ventana AEC (Ventana Medical Systems, Tucson, AZ) and alkaline phosphatase red immunohistochemistry system, according to the protocol of the manufacturer.

**Antibodies**

*SOD1 antibodies*. The primary antibodies used for detection of human and murine SOD1 were mainly polyclonal rabbit antibodies raised against keyhole limpet hemocyanin-coupled peptides corresponding to amino acids (aa) 24–39 (hSOD1-specific) or 24–36 (mSOD1-specific) (35). For analysis of the G127X protein in human tissue, an antibody raised against the novel C-terminus of the truncated protein was used (ADDLGGGQRWK, with new amino acids highlighted) (35).

*Commercial antibodies*. The antibodies to lamin A/C, Tim23, and β-COP (Golgi β-coatomer protein) were obtained from BD Biosciences (Franklin Lakes, NJ), anti-PMP70 (70-kDa peroxisomal membrane protein) was from Affinity BioReagents (Golden, CO) or Zymed (San Francisco, CA), and the anti-synaptotagmin antibody was from Stressgen (Victoria, British Columbia, Canada). Antibodies to HSC70, isocitrate dehydrogenase, UCH-L1, isopeptide, and CNP were from Abcam (Cambridge, UK), antibodies to Cop9s8 were from PTGlab (Chicago, IL), antibodies to FABP were from R&D Systems (Minneapolis, MN), and antibodies to FIS1 were from Biosite (Täby, Sweden). Anti-rabbit or anti-mouse IgG secondary antibodies were from GE Healthcare (Uppsala, Sweden), anti-goat IgG was from Santa Cruz (Santa Cruz, CA), anti-sheep IgG was from DakoCytomation (Glostrup, Denmark), and anti-rat IgG secondary antibodies were from Abcam (Cambridge, UK).
Size-exclusion chromatography

Tissues were homogenized after the addition of 20 mM iodoacetamide using an Ultraturrax and sonication as described above. The iodoacetamide was added to alkylate free thiol groups in proteins and low-molecular-weight compounds. After centrifugation at 20,000 g for 30 min, the supernatants were subjected to chromatography in 1 cm × 30 cm Superdex 75 columns (GE Healthcare, Uppsala, Sweden) using PBS with 5 mM iodoacetamide. The elution rate was 45 ml/h, and the eluate was collected in 0.3-ml fractions. The SOD activities (421) and hSOD1 protein content of the fractions were determined.

Proteomic techniques

Differential in-gel electrophoresis (DIGE)

DIGE uses the properties of different protein-binding fluorophores with similar mobilities on SDS-PAGE. At present, three different fluors are available, which makes it possible to separate two different samples along with an internal standard on every gel in a 2-D gel experiment. This minimizes the number of gels needed and the internal standard makes it possible to compare a great number of different samples from different gels. The procedure was performed according to personal communications and the handbook of the manufacturer (GE Healthcare).

For each new DIGE experiment, the internal standard was generated by mixing the same amount of protein from all the animals included. Quantified homogenates were mixed with Cy2 (internal standard), Cy3, or Cy5 (0.25 mM in dimethyl formamide) and incubated on ice for 30 min in the dark. Samples from transgenic or control mice were labeled with either Cy3 or Cy5 in a random way, as suggested by the manufacturer. Reactions were quenched by addition of 1 μl of 10 mM lysine (Riedel-De Haën AG, Seelze, Germany) and incubated on ice. Cy2-, Cy3-, and Cy5-labeled samples were mixed, to be run on a single gel, and incubated with the same amount of 2 × sample buffer for DIGE (1 M Tris-HCl, pH 8.5, 2 M thiourea, 7 M urea, 4% CHAPS, and 40 mM DTT).

2-D electrophoresis

DIGE samples were run on 24-cm pH 3-11NL or pH 4-7 strips (GE Healthcare, Uppsala, Sweden). The pooled samples were supplied with IPG buffer (GE Healthcare) and DeStreak rehydration buffer (GE Healthcare), and rehydrated into IPG strips for 12–16 h. Isoelectric focusing (IEF) was performed on a Protean IEF cell (Bio-Rad) at 20°C and maximum current was set to 50 μA/strip, with increase of the voltage stepwise until 35,000 or 45,000 Vh was reached (3-11NL and 4-7, respectively). Following IEF, the
strips were equilibrated for 15 min in equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 30% (v/v) glycerol, 2% SDS, and 0.002% bromophenol blue) containing 1% DTT and then for 15 min with 2.5% iodoacetamide in the same buffer. The second dimension was run using the Ettan DALTsix electrophoresis system (GE Healthcare) with low-fluorescence glass plates.

In paper III, unlabeled samples were run on 11-cm pI 3-11NL strips and the Criterion™ Cell electrophoresis system (Bio-Rad) was used for the second dimension. For analysis, gels were post-stained with a silver stain (422). Briefly, the gels were fixed for 30 min in 40% methanol/10% acetic acid, sensitized (with 30% methanol, 0.1% sodium thiosulfate, and 6.8% sodium acetate), stained with silver nitrate (0.25% silver nitrate and 0.015% formaldehyde), developed (2.5% sodium carbonate and 0.03% formaldehyde), and the reaction was stopped in 1.46% disodium EDTA. For Coomassie staining, the gels were fixed in 10% methanol/7% acetic acid for 1 h. Staining was performed overnight with G-250 staining solution (0.8% phosphoric acid, 8% ammonium sulfate, 20% methanol, and 0.08% CBB-G250), briefly washed with 25% methanol, and stored in deionised water.

Computer analysis

CyDye-labeled DIGE gels were scanned in a Typhoon 9410 imager (GE Healthcare) using excitation and emission filters specified by the manufacturer (Cy2, Cy3, and Cy5; excitation 480 nm, 540 nm, and 620 nm, and emission 530 nm, 590 nm, and 680 nm, respectively). Further analysis was done using DeCyder 6.5 software. Spot detection was done using the differential in-gel analysis (DIA) module and data from this application were then entered into the biological variation analysis (BVA) software for spot matching between gels and variation analysis. Statistical significance was calculated using Student’s t-test.

MALDI-TOF

Selected spots were picked manually and dehydrated with acetonitrile, dried in a Speed-vac (Savant; GMI, Ramsey, MN) and finally incubated with trypsin (Promega, Madison, WI). The protein digest was applied to a MALDI-TOF target plate (Applied Biosystems) with α-cyana-4 hydroxy-cinnamic acid solution (Agilent Technologies, Santa Clara, CA). Calibrant was spotted onto the plate and used for external calibration. The plate was put in a Voyager DE-STR mass spectrometer (Applied Biosystems) and the peptide spectra recorded. Peptide peak lists were generated using Data Explorer software version 4 (Applied Biosystems). Proteins were identified by searching the Swissprot and IPI databases using an in-house Mascot server (Matrix Science Inc., Boston, MA). To obtain a Mowse score for the G127X dimer, this sequence was added to the Swissprot database searched. Search parameters were set to allow mass deviations of ± 50 ppm and one or two missed cleavage sites, as well as fixed modifications such as
carbamidomethylation of cysteine and oxidation of methionine. Identifications that were statistically significant (with a Mowse score in the range of at least 60–66, p-value < 0.05), and that showed a gel position close to the theoretical mass and isoelectric point, were regarded as positive.

**LC-ESI MS/MS and data analysis**

Samples were incubated in 10 mM dithiothreitol (DTT) and 6 M guanidine hydrochloride with Tris, pH 9.0. Iodoacetamide was added and the samples were kept at 37°C in the dark. The reaction mixture was filtered and washed twice with 0.2 M ammonium bicarbonate and trypsin added. The resulting peptides were collected by centrifugation, lyophilized, and resuspended in 0.1% formic acid.

Peptide analysis was done by reverse-phase liquid chromatography electrospray ionization-tandem mass spectrometry as described by Srivastava et al. (423) using a capillary HPLC system coupled to a quadrupole time-of-flight mass spectrometer (CapLC Q-TOF Ultima™, Waters Corporation). ProteinLynx Global Server software version 2.2.5 was used to convert raw data to peak lists for database searching. Proteins were identified by a local version of the MASCOT search program and the Mascot Daemon application (version 2.1.6) using the Swissprot protein sequence database with mouse taxonomy. Peptides with Mascot ion scores exceeding the threshold for statistical significance (p < 0.05) were selected. Proteins were classified as having been identified either if at least two peptides with a Mascot score above the statistically relevant threshold (p < 0.05) were found or if only one peptide achieved the required Mascot score with at least four consecutive y- or b-ions and with a significant signal-to-background ratio.

**Network analysis**

Proteomic studies generate huge amounts of data. To find common themes among altered proteins, network analysis can be used. Ingenuity Pathways Knowledge Base (IPKB) uses published information on interactions between proteins and clusters the data into networks, and identifies cellular pathways that may be significantly altered ([www.ingenuity.com](http://www.ingenuity.com)). The data set containing protein identifiers (Swissprot) and corresponding expression values was uploaded to the IPKB. Networks of the identified proteins were then generated algorithmically along with identification of functional and canonical pathways, and diseases in which the uploaded proteins are known to be involved. Fisher’s exact test was used by the software to calculate a p-value for biological functions and diseases. The significance of canonical pathways was measured in 2 ways: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway, and (2) Fisher’s exact test.
Statistics

Means and standard deviations were calculated using Microsoft Excel (paper I). In paper IV, DeCyder 6.5 used Student’s $t$-test for calculation of p-values. To minimize the risk of false positives due to multiple comparisons in paper IV, orthogonal projections to latent structures discriminant analysis (OPLS-DA) was used for multivariate statistical evaluation in the SIMCA-P software version 11.5 (Umetrics AB, Umeå, Sweden).
Results and Discussion

Paper I

Experiments in our laboratory had shown that the extremely high level of SOD1 protein present in G93A, D90A, and wtSOD1 transgenic mouse models of ALS was associated with incomplete Cu\(^{2+}\)-charging of 30–50% of the hSOD1. This was probably caused by CCS insufficiency (247). In contrast to these models (called high-level models), other models expressed SOD1 at similar rates (in terms of mRNA levels) but accumulated mutant protein at levels that were similar to the normal level of mSOD1 or even less (G85R, G127X) (low-level models). This is probably due to rapid recognition for degradation, as these two mutant SOD1s are highly destabilized by their mutations. At that point in time, one line of evidence had suggested that mutant SOD1 preferentially localizes to mitochondria, where it interferes with normal function and may be responsible for vacuole pathology (293;294;303;304;309) (discussed above). It had also been shown that SOD1 is imported to the mitochondrial IMS in the apo state, where it is loaded with metals and disulfide-oxidized—at least partly due to activities of CCS (282;283). As high-level models accumulate comparable high levels of metal-deficient, C57-C146 disulfide-reduced subunits (247), we hypothesized that this might lead to a propensity for excessive mitochondrial accumulation. Furthermore, we wanted to explore whether destabilized mutants like G85R and G127X would be retained inside the mitochondrial IMS as they, at least in the G127X case, cannot be disulfide-oxidized and are unlikely to bind the prosthetic metals. Ultracentrifugation in density gradients is the method of choice for separation of organelles (as discussed in Methods). We chose to work with continuous iodixanol gradients, as this system offers the advantages of high resolution and iso-osmotic properties. From these gradients, we were able to look at patterns of segregation rather than accumulation of similar-density particles at interfaces.

As also shown by others, we found an extremely high association of mutant SOD1s with mitochondria in the high-level models, and they reached levels 100-fold higher than that of endogenous mSOD1. The quantity of this association was similar for wild-type enzyme and mutant enzymes, with only slightly higher levels for mutants, and there was no difference between spinal cord and brain—or between time points (presymptomatic stage and terminal stage). This showed that the mitochondrial association is due to an intrinsic property of SOD1 that is not qualitively different between the wt protein and mutant proteins. In the low-level G85R model, the relative levels that appeared to co-fractionate with mitochondria were similar to what was
found for the wild-type murine enzyme in control mice (~2%). This contrasted with the relative levels found in high-level models (> 9% of all mutant SOD1) and suggested that the level of SOD1 protein and also the disulfide-reduced/oxidized state are major determinants of mitochondrial association. In presymptomatic G127X mice, very low levels were found at the position of mitochondria (0.6%) and the pattern of gradient distribution suggested that there was no co-fractionation. When the cytosolic marker lactate dehydrogenase (LD) was measured, the contamination from this compartment was at a similar level. In terminal G127X mice, the levels of aggregated SOD1 distributed all over the gradient (showed by a filter-trap assay) made it impossible to quantify any mitochondrial association but there were no signs of apparent co-fractionation. These results did indeed indicate that the truncated G127X protein does not accumulate in mitochondria, probably due to its inability to achieve a holo state (i.e. to bind the metals and form the disulfide bridge). We also explored the association between murine wt SOD1 and mitochondria. In the controls and low-level models, this was quantified to be approximately 2%, with no difference between tissue (brain and spinal cord) and time point (presymptomatic or terminal). In the high-level models, however, the relative associations were similar to that of the mutant human enzyme: ~8–9%. In the study by Jonsson et al., it was shown that the Cu\(^{2+}\) and CCS deficiency caused by overexpression of mutant enzyme cause a similar amount of murine SOD1 to be trapped in the apo state (at least disulfide-reduced) (247). These results suggested that the accumulation of disulfide-reduced protein in these models is responsible for the high association between SOD1s and mitochondria.

These findings were somewhat different to those in the report by Liu and colleagues, who studied a similar set of transgenic animals (303). They found preferential association of mutant SOD1 (but not wt hSOD1) with mitochondria and an age-dependent accumulation and aggregation at the cytoplasmic surface of the mitochondrial outer membrane. The discrepancies are mainly due to different methods of separation. Liu et al. used discontinuous gradients, which collect particles of similar density at interfaces, in combination with differential centrifugations that are likely to co-pellet heavy aggregates and mitochondria, resulting in artificial association. As shown by us in this work, and as explored further in paper III, all of these models accumulate high levels of aggregated SOD1 material with age. These aggregates are trapped in inclusions that show overlapping densities with all organelles when subjected to gradient separation in the absence of detergent (paper III). In a recent study by the same group, these authors tried to overcome the issue of aggregate co-fractionation by flotation of mitochondria through loading of the sample at the bottom of the tube (305). This is unlikely to overcome the problem, however, since such SOD1 inclusions behave like organelles, in terms of density, when not exposed to detergents. A weakness of our study is that, despite gentle treatment, a
A recent study has shown that physiological association of wt SOD1 with mitochondria is dependent on intramolecular disulfide bonding and interaction with CCS, but that this regulation is absent in mutant SOD1s—causing aggregation and intramitochondrial accumulation (308). Another similar study has indicated the importance of misfolded/aggregated SOD1 in mitochondrial toxicity and that the aggregation is partly dependent on SOD1 cysteines (424). As the association between mitochondria and aggregated SOD1 is impossible to explore in terminal mice by density separation methods, we cannot rule out the possibility that such interaction takes place even in the context of G127X. In recent unpublished experiments, we have been able to concentrate mitochondria by a filter-trap assay but we are still unable to find any mitochondrial association in presymptomatic G127X.

Is the mitochondrial pathogenic hypothesis, then, a simple artifact in transgenic mouse models? This is unlikely, since there is overwhelming evidence from patients and transgenic mouse models that there are signs of dysfunction and pathology in mitochondria (discussed above). In paper IV, we also found evidence of mitochondrial dysfunction in the G127X mice. Similarly to our own findings, in a recent immunohistochemical study on H46R mice—another model with low-levels of SOD1 devoid of mitochondrial vacuolization (but with other mitochondrial pathology)—the authors were unable to find any association between SOD1 and mitochondria (297). This strengthens the conclusion that models with extreme levels of mutant protein are those that display mitochondrial vacuoles. From my point of view, mitochondria are, according to these findings, most probably involved in the pathogenic process of ALS but might also suffer from unspecific damage due to high accumulation of mutant SOD1. The main damage is not related to direct interaction/accumulation of mutant SOD1 and mitochondria, but is due to other mechanisms (as discussed above). In the G93A model where extreme amounts of mutant protein accumulate in the IMS, this probably causes overt pathology (e.g. vacuoles) and modulates disease with an effect on the survival of motor neurons. Depletion of mitochondrial S-nitrosothiol, caused exclusively by mutant SOD1s with intact copper binding, has been suggested to be responsible for vacuole pathology in these kinds of models (425). The toxic effect of G93A SOD1 overloading was recently shown to be a disease modulator by simultaneous overexpression of CCS and G93A in transgenic mice, leading to even higher loads of mitochondrial SOD1 and a marked shortening of survival (310). This could be the explanation for why certain
therapeutic interventions in this model have had an effect on the course of disease in mice but lack any effect in human trials.

**Paper II**

As discussed above, the G127X mutation was first described in a Danish pedigree in 1997 (231). The insertion of TGGG after codon 127 leads to a terminal stop codon preceded by five novel amino acids. This allows generation of mutant-specific antibodies and analysis of mutant SOD1 in the presence of wild-type SOD1 from the non-mutated allele. In control humans, SOD1 levels are by far the highest in the liver and the kidneys (215). When mutant SOD1 levels are measured from different tissues in low-level transgenic mice such as G127X, the highest protein levels accumulate in the spinal cord (247). As the spinal cord is the main tissue affected by ALS, the vulnerability may be caused by inadequate recognition and degradation of misfolded SOD1. From studies of CNS tissues from a G127X patient, it had previously been shown that mutant protein accumulates at very low levels and is mainly seen in inclusions (35). In paper II, we studied both CNS tissue and peripheral tissues from a second G127X patient who succumbed to ALS.

Similar to what has been found in G127X mice, we found the highest levels of mutant protein to be in the CNS. By far the highest levels were found in the vulnerable ventral horns. The amount was estimated to be about 8% of the total amount of SOD1 protein. The wild-type SOD1 levels were highest in liver and kidney, as expected. To explore the properties of mutant and wild-type protein further, tissue homogenates were subjected to density gradient ultracentrifugation as described in paper I. As in brain tissue of terminal G127X mice, we did not find detectable levels of dense SOD1 material in tissue from the frontal lobe. From ventral horns, about 50% of the mutant protein entered the gradient with a pattern identical to that seen in spinal cord of terminal G127X mice. Interestingly, similar to spinal cord, a substantial amount of the G127X mutant protein from homogenates of liver and kidney entered the gradient. Thus, aggregated mutant SOD1 can also appear in peripheral tissues. From histopathological examinations using the G127X-specific antibody, we found SOD1 inclusions in motor areas of the ventral horn as well as in hepatocytes and kidney tubular epithelium.

These results suggest that degradation of G127X protein is subject to difficulties, not only in the CNS but in peripheral tissues. In quantitative terms, this accumulation is by far the most abundant in the ventral horns of the spinal cord. Whether aggregated/inclusion SOD1 reflects the disease process or actually causes it is, however, unclear (discussed above). If defined as a marker of disease, the ALS pathogenesis might also involve peripheral tissues but at a much slower rate and obvious organ dysfunction might not be prominent during the lifespan of a human. This particular
subject had signs of chronic liver disease, for which we found no definite explanation. Future studies in peripheral tissues will be able to tell us whether ALS is a disease that is restricted to the CNS alone or if some signs of damage appear elsewhere.

**Paper III**

In the density gradient ultracentrifugation studies of spinal cord homogenates from terminal transgenic mice, we had found that dense SOD1 material was distributed all over the gradient (paper I). This material was not present in presymptomatic mice and was aggregated, as it could be captured in a filter-trap assay. Aggregated SOD1 is known to accumulate in large inclusions in both ALS transgenic mice and human ALS patients carrying SOD1 mutations and it has been suggested that this process could be deleterious, e.g. through depletion of essential proteins. Pure protein aggregates have a very high density and had been shown to accumulate in the bottom of the tubes when the homogenate was subjected to detergent (paper I). The composition and properties of such detergent-resistant aggregates have been extensively analyzed before (discussed in introduction) and the difference between such entities and what we refer to as “inclusions” are described in figure 2.

Firstly, we wanted to address the physical character of SOD1 inclusions from murine models expressing mutant SOD1 of widely different character (G93A, D90A, G85R, and G127X). The inclusions were shown to be distributed all over the gradient at densities lower than expected from pure protein aggregates, and to be disrupted upon addition of weak detergent. SOD1 protein in inclusions was not accessible to antibodies without the addition of detergent, but it was sensitive to digestion by a proteinase. This suggested that the SOD1 protein is not present on the surface of inclusions and that it is not completely covered by a continuous lipid membrane, for example.

In presymptomatic high-level models G93A and D90A, large amounts of SOD1 protein enter the gradient. This material was shown in paper I to accumulate mainly in mitochondria. To characterize the properties of SOD1 in the gradient of these high-level models in greater detail, non-reducing immunoblotting was used to discriminate between SOD1 species with the C57-C146 disulfide bond oxidized and reduced. It was shown that SOD1 with the disulfide bond intact closely followed the distribution of mitochondria. In presymptomatic G85R and G127X models, only minute amounts entered the gradient.

In gradients from terminally ill mice of all models, large amounts of mutant protein entered the gradient, some at higher densities than in gradients from presymptomatic animals. When subjected to weak detergent for disruption of organelles and filter-trapping followed by non-reducing
immunoblotting, only disulfide-reduced SOD1 and, in some cases, another SOD1-species with a non-native disulfide bond, were trapped, showing that these are the predominant species in aggregates. A minute portion of the aggregated SOD1 was found to be ubiquitininated and about 30% was shown to oligomerize or bind to other proteins through disulfide coupling.

**Figure 2.** Biochemical isolation of inclusions. There are different routes for isolation of aggregated materials. The standard procedure involves different detergents and sequential centrifugations producing a detergent-resistant pellet (middle). In contrast to simple centrifugations (left), more or less stringent protocols produce a reproducible pellet containing detergent-resistant material free from detergent-soluble cellular components. In contrast, density gradient centrifugations on homogenates free from detergents (right), enables the separation of in vivo particles containing both soluble and detergent-resistant materials with much less contamination than though a pure centrifugation protocol.
In G85R and G127X mice, a proportion of the inclusions were denser than most organelles. These dense SOD1 inclusions gave us the opportunity to study their composition without any major contamination from organelles and cytosolic proteins. When separated on 2-D gels, apart from SOD1, 10 proteins not present in the controls could subsequently be identified using MALDI-TOF MS. The most abundant was mutant SOD1, representing at least 50% of the total protein. Two were cytoplasmic chaperones, 4 were cytoskeletal proteins, and 4 were proteins that normally reside in the endoplasmic reticulum. Most of these proteins had been found to interact or co-aggregate with SOD1 before, but the ER chaperone calreticulin had not previously been found to associate with SOD1.

When tissue sections are stained for mutant SOD1s, inclusions are clearly visible in motor neurons, astrocytes, and in the neuropil. The entities isolated here most probably represent a mixture of such inclusions. The set of proteins found to co-fractionate with SOD1 also shows that the inclusions are not only derived from motor neurons, but also from glial cells. The fact that the pathology induced by mutant SOD1s involves not only motor neurons but also glial cells is well established (non-cell autonomous), and the presence of inclusions in other cell types has been reported before (35;243;336). It is possible that there may be different inclusions, with high densities that overlap, formed in the terminal phase of the disease—some containing SOD1 and some not. Although they might possibly be different cellular entities, they might all include aggregated material that could deplete the cell of important functions.

Several immunohistochemical studies have explored the composition of ALS inclusions. The most frequently described inclusions from SOD1 transgenic mice have been ubiquitinated inclusions (UIs, described in introduction) in motor neurons and astrocytic hyaline inclusions (AST-His). These are also present in both sporadic and familial ALS patients. In mice, UIs are frequently SOD1-positive but they also stain for proteins such as CCS, heat shock proteins (HSPs), GRP78, neurofilaments, NEDL1, dorioin, proteasome 20s, EAAC1, and p38MAPK with varying frequency (336;342;347;377;378). AST-HIs contain similar proteins, but with the astrocytic markers αβ-crystallin and GFAP at high frequency (243;426). The relationship between such histological entities and the biochemical isolates from these experiments is not easy to determine, however.

**Paper IV**

Proteomic techniques allow screening for pathogenic alterations with minimal input of prior knowledge. Use of the latest techniques makes it possible to look at thousands of proteins at the same time. Differential in-gel electrophoresis (DIGE) enables the separation of two different samples and an internal standard on the same 2-D gel using pre-labeling with
fluorophores of similar molecular weight (discussed in more detail in Methods). We hypothesized that expression of mutant SOD1 in transgenic mice affects several different pathways in affected cells. One way of exploring these events simultaneously is to subject tissue homogenates from suitable time points to DIGE. We chose to work with G127X mice, as their mutant protein level is only half the level of the endogenous mSOD1, thus reducing the risk of overexpression artifacts (discussed in paper I). As time point, we choose their peak body weight, as this has been shown to reflect the onset of symptoms where the systemic effect of motor neuron disease is still small (383).

The DIGE gels revealed about 1,800 protein spots that could be processed further. From multivariate analysis, we found that about 420 protein spots contributed significantly to the difference between controls and transgenics. This is more than expected and suggests that 20–25% of proteins are altered by pathogenesis in affected tissue. We can only speculate that the majority of these alterations probably reflect compensatory actions taken for the survival of the cell. From another angle, this suggests that protein alterations are taking place in most cells of the spinal cord and not only in motor neurons. This is in line with recent reports of SOD1 pathogenesis being non-cell autonomous.

Owing to limitations of 2-D gels and MALDI-TOF, 53 significantly altered protein spots could be reliably identified. As it is inevitable to encounter false-positive hits in multiple comparisons such as this, we used stringent exclusion criteria. The DIGE handbook suggests that differences down to 10% can be viewed as reliable, but we chose 25%. The use of stringent statistics with a univariate p-value of 0.01 and a multivariate correlation of $p_{corr} > 0.07$ reduced the risk of false positives significantly. In fact, when the different samples were assigned randomly (controls and transgenics mixed), virtually no significant spots appeared using these criteria.

For interpretation of the 53 protein alterations identified, the material was subjected to analysis using Ingenuity pathway knowledge base (IPKB, described in Methods). One of the main findings was the representation of altered mitochondrial proteins. We had shown in paper I that the G127X protein does not associate with mitochondria (a finding strengthened in paper III), but still mitochondrial functions are clearly altered in this model. This suggests that the mitochondrial involvement may be secondary to primary noxious action of mutant SOD1s elsewhere. Furthermore, we found alterations in the NRF2-mediated stress response, a pathway involved in protection from oxidative stress. Regulation of proteins involved in oxidative stress has been reported previously (427,428). It is particularly interesting that all proteins found in this pathway were upregulated. A previous gene-expression study on NSC34 cells expressing mutant G93A found NRF2 and some downstream targets to be downregulated (429). This was also reported
in a study on isolated motor neurons of G93A rats (430). As our study only
detected downstream targets of this oxidative response, we can only
speculate that (I) this response might be heavily upregulated in other cells of
the spinal cord but still be downregulated in motor neurons, or that (II) the
isolated system of motor neuronal/NSC34 cells does not adequately reflect
the *in vivo* situation, or that (III) the upregulation of the proteins in this study
is due to stimulation of another pathway similar to NRF2. In a study by Kraft
et al. (431), double transgenic mice expressing mutant SOD1 with a reporter
for NRF2 activation were generated. When these authors followed NRF2
activation in different cell types during the course of the disease, they found
a clear activation of this antioxidant response in several cell types of the
spinal cord. This correlates well with our finding, and might indicate that the
NRF2-mediated response activation in transgenic mice is dependent on an
accurate cellular context. One consequence of increased oxidative stress is
protein oxidation, which has been shown to be elevated in G93A mice (432).

Protein degradation was another pathway that appeared to be affected by
mutant SOD1 expression. Proper degradation of protein is essential to cells,
particularly for neurons as they lack the ability to regenerate. The UPS is one
of the major degradation pathways (discussed in introduction), and here it
was found to be differentially regulated. Oxidation and altered expression of
UCH-L1 has been found in brains from both Alzheimer’s and Parkinson’s
disease cases by proteomic methods (433). The UCH-L1 upregulation found
here is supported by an earlier report from our group showing intense
immunostaining in motor neurons and astrocytes of a G127X patient (35).
The COP9 signalosome complex is a multifunctional complex involved in
apoptosis, protein degradation, and hypoxia (reviewed in(434)). An
important function of the signalosome is to inhibit ubiquitination through
deneddylatation of cullin-type E3 ubiquitin ligases. As subunits 4 and 8, which
are scaffold proteins in this complex, were found to be upregulated, this
could indicate inhibition of ubiquitin ligase activity and of proper protein
degradation.

In immunoblots of samples from G127X patients and transgenic mice, a
SOD1 band is present at double the molecular weight of the monomeric
species (35). A similar band has been seen in other SOD1 transgenic mouse
models, and also in ventral horn extracts from SALS cases (233;247). This
species was identified in the DIGE gels, and MALDI-TOF analysis revealed
a spectrum including only G127X SOD1 peptides. This indicates that the 32-
kDa band seen in immunoblots is a covalently-coupled SOD1 dimer,
previously uncharacterized.

In conclusion, we found alterations in cellular pathways such as oxidative
stress, mitochondrial function, and protein degradation. This is in agreement
with earlier proteomic studies on the G93A model, although few specific
proteins overlap (399;400). We also found evidence for a novel dimeric
SOD1-species that might be common in ALS regardless of genetic background.
Conclusions

Paper I

- High-level mutant SOD1 models accumulate more than 100 times more SOD1 protein in mitochondria than non-transgenic mice, probably due to high levels of metal-deficient, disulfide-reduced SOD1 species. There is no obvious quantitative difference between brain and spinal cord or between presymptomatic stage and terminal stage.

- The truncated G127X protein is not retained in the mitochondria of presymptomatic animals.

- The difference in mitochondrial accumulation of SOD1 between different mouse models correlates with differences in mitochondrial pathology (such as vacuoles) and indicates that these may represent overexpression artifacts.

- If mitochondrial deficits are universally present in mutant SOD1 ALS, this is apparently not caused by mitochondrial accumulation of SOD1 protein.

Paper II

- Aggregation of mutant SOD1 is not confined to the CNS of a human ALS patient, but is also evident in peripheral tissues such as the liver and kidney.

- Defects in recognition and degradation of misfolded SOD1 might explain the vulnerability of motor areas.

Paper III

- SOD1 inclusions are complex entities containing both detergent-resistant and detergent-susceptible protein aggregates, and they are not enclosed by a continuous membrane.

- Disulfide-reduced SOD1 protein is the major species in SOD1 inclusions from the spinal cord of transgenic mice.

- Besides cytoskeletal proteins, the main constituents of SOD1 inclusions are protein chaperones, normally residing in the ER. This is in line with recent reports on the
involvement of the ER-controlled unfolded protein response in the pathogenesis of ALS.

Paper IV

- Expression of the G127X mutant of SOD1 in transgenic mice affects the expression of up to 25% of other proteins in the spinal cord. Alterations in important mitochondrial pathways, response to oxidative stress, and protein degradation are most prominent.

- The G127X protein gives rise to a previously uncharacterized, covalently-coupled dimer. This species may be formed by other SOD1 mutants and wild-type SOD1 and might be a common modification in both ALS patients and transgenic mouse models.
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