Of mice and men

SOD1 associated
Human Amyotrophic Lateral Sclerosis and
Transgenic Mouse Models

by

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Cover illustration:
α-motor neuron, kindly provided by Dr Thomas Brännström.
I am not young enough to know everything
Oscar Wilde (1854 – 1900)

I refuse to answer that question as I don't know the answer.
Douglas Adams (1952 – 2001)
ABSTRACT
Amyotrophic lateral sclerosis, ALS, is a progressive fatal neurodegenerative disorder affecting motor neurones in motor cortex, brain stem and spinal cord. This inevitably leads to paralysis, respiratory failure and death. In about 5% of patients with ALS there is an association with mutations in gene for the abundant intracellular scavenging enzyme superoxide dismutase1, SOD1. The noxious property of SOD1 is proposed to be due to gain of function. In familial cases the inheritance is most commonly dominant.

This study focus on two disparate SOD1 mutations occurring in Scandinavia. The recessive D90A mutation which has properties similar to that of the normal wild-type human SOD1. The dominantly inherited G127insTGGG mutation, G127X, causes a C-terminal truncation of the last 21 amino acids and is a highly unstable protein.

Transgenic mice were created expressing D90A and G127X mutated human SOD1. Results from studies of tissue from the central nervous system of patients carrying either of these mutations were compared with similar tissue collected from transgenic mice generated with the same mutations. Tissue from the mice were also compared to central nervous tissue from several other transgenic mouse strains expressing human wild type SOD1 as well as other ALS associated human SOD1 mutations.

The transgenic mice expressing D90A respectively G127X mutated human SOD1 develop motor neurone disease. Microscopic studies of central nervous tissues from G127X transgenic mice reveals inclusions of aggregated misfolded SOD1 in motor neurones and adjacent supporting cells. These inclusions are composed of detergent resistant aggregates and preceded by accumulations of minute quantities of detergent-soluble aggregates. The inclusions mimic those found in G127X patients.

In D90A transgenic mice the progression, as in the humans, was slower and the mice, as the patients, showed bladder disturbance. In the D90A patients, the SOD1 inclusions mimic those found in sporadic ALS patients.

Aggregation of SOD1 in central nervous tissue appears to be related to severity of disease. Degenerative features as vacuolization and gliosis precedes phenotypic alterations. Changes are seen not only in motor areas but also in higher centres of the telencephalon.

Keywords: aggregates, ALS, amyotrophic lateral sclerosis, inclusions, misfolded, vacuolisation, SOD1, transgenic
# TABLE OF CONTENT

<table>
<thead>
<tr>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
</tr>
<tr>
<td>TABLE OF CONTENT</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
</tr>
<tr>
<td>PREFACE</td>
</tr>
</tbody>
</table>

## 1: AMYOTROPHIC LATERAL SCLEROSIS

- **1:1 HISTORICAL NOTES** | 11 |
- **1:2 MOTOR SYSTEM** | 11 |
- **1:3 TYPES OF MOTOR NEURON DISEASE** | 13 |
  - **1:3:1 Amyotrophic lateral sclerosis** | 13 |
  - **1:3:1:1 Sporadic amyotrophic lateral sclerosis** | 13 |
  - **1:3:1:2 Familial amyotrophic lateral sclerosis** | 14 |
  - **1:3:2 Primary lateral sclerosis** | 14 |
  - **1:3:3 Progressive muscular atrophy** | 15 |
  - **1:3:4 Progressive bulbar paresis** | 15 |
- **1:4 DIAGNOSTIC CRITERIA** | 15 |
- **1:5 SUPEROXIDE DISMUTASE 1** | 16 |
  - **1:5:1 D90A** | 16 |
  - **1:5:2 G127insTGGG (G127X)** | 17 |
- **1:6 TRANSGENIC ANIMAL MODELS** | 17 |
  - **1:6:1 TRANSGENIC MICE** | 17 |
  - **1:6:2 OTHER TRANSGENIC ANIMAL MODELS OF MOTOR NEURON DISEASE** | 18 |
- **1:7 INCLUSIONS** | 19 |
  - **1:7:1 Bunina bodies (Fig. 2A)** | 19 |
  - **1:7:2 Lewy body like hyaline inclusions (Fig. 2B)** | 19 |
  - **1:7:3 Skein like inclusions (Fig. 2C)** | 19 |
  - **1:7:4 Other entities** | 19 |

## 2: AIMS

## 3: MATERIALS AND METHODS

### 3:1 MATERIALS

- **3:1:1 HUMANS** | 21 |
  - **3:1:1:1 D90A** | 21 |
  - **3:1:1:2 G127X** | 21 |
  - **3:1:1:3 CONTROLS** | 22 |
- **3:1:2 MICE** | 22 |
  - **3:1:2:1 Making of a transgenic mouse** | 22 |
  - **3:1:2:2 C57BL/6J inbred mouse strain** | 23 |
  - **3:1:2:3 Termination** | 23 |
  - **3:1:2:4 Mice histologically investigated in paper IV** | 24 |
  - **3:1:2:5 Genotyping of mice** | 24 |
  - **3:1:2:6 D90A** | 24 |
- **3:1:2:7 G127X** | 24 |
- **3:1:2:8 OTHER MICE STRAINS** | 25 |
  - **3:1:2:8:1 G93A mice and G93A<sup>dl</sup> mice** | 25 |
  - **3:1:2:8:2 Human wtSOD11 mice** | 25 |
  - **3:1:2:8:3 G85R mice** | 25 |
  - **3:1:2:8:4 C57BL/6JBonTac mice** | 26 |
  - **3:1:2:8:5 Control mice** | 26 |
3:1:3 ANTIbODIES .............................................................................................................................. 26
  3:1:3:1 SOD1 antibodies .................................................................................................................. 26
  3:1:3:2 Commercial available antibodies ...................................................................................... 26
3:1:4 STAININGS USED TO VISUALISE CELLS AND TISSUE .......................................................... 29
3:2 METHODS ................................................................................................................................... 31
  3:2:1 HISTOLOGY .......................................................................................................................... 31
    3:2:1:1 Preparations of tissues .................................................................................................... 31
    3:2:1:2 Microscopy slides ........................................................................................................... 31
    3:2:1:3 Immunohistochemistry .................................................................................................. 31
      3:2:1:3:1 Grading of immunohistochemistry ........................................................................... 32
    3:2:1:4 Stereology of mice spinal cords .................................................................................... 32
  3:2:2 CHEMISTRY .......................................................................................................................... 33
    3:2:2:1 Preparation of tissues .................................................................................................... 33
    3:2:2:2 Southern blotting ........................................................................................................... 33
    3:2:2:3 Northern blotting .......................................................................................................... 33
    3:2:2:4 Immunoblotting [Western blotting] .............................................................................. 33
    3:2:2:5 2-D gel electrophoresis ................................................................................................. 34
    3:2:2:6 Quantification of SOD1 .................................................................................................. 34
    3:2:2:7 Centrifugations .............................................................................................................. 34
    3:2:2:8 Analysis of detergent-resistant aggregates ............................................................... 34
    3:2:2:9 Analysis of detergent-soluble aggregates ................................................................. 35
    3:2:2:10 SOD activity analysis .................................................................................................. 35
    3:2:2:11 Stabilities of human and murine SOD1s ................................................................. 35
    3:2:2:11 Isoelectric focusing and SOD staining ...................................................................... 36
    3:2:2:12 Effects of buffer composition, pH and chelators on SOD activity ......................... 36
    3:2:2:13 Effects of post-mortem time ..................................................................................... 36
  3:2:3 STATISTICAL ANALYSIS ...................................................................................................... 36
4 RESULTS ........................................................................................................................................ 38
4:1 PAPER I ...................................................................................................................................... 38
  4:1:1 The G127X patient ................................................................................................................. 38
    4:1:1:1 Histopathology .............................................................................................................. 38
    4:1:1:2 Biochemistry .................................................................................................................. 38
    4:1:1:3 Fibroblast cultures .......................................................................................................... 39
  4:1:2 Mice ...................................................................................................................................... 39
    4:1:2:1 Histopathology .............................................................................................................. 40
      4:1:2:1:1 SOD stainings and SOD1 positive inclusions .......................................................... 40
      4:1:2:1:2 αβ-crystallin,UCHL1 and ubiquitin stainings ............................................................ 40
      4:1:2:1:3 Giosis ........................................................................................................................ 41
      4:1:2:1:4 Similarities between mice and patient ................................................................. 41
    4:1:2:2 Biochemistry .................................................................................................................. 41
      4:1:2:2:1 Immunoblots ........................................................................................................... 41
      4:1:2:2:2 Ubiquitin ................................................................................................................... 42
      4:1:2:2:3 SOD activity .............................................................................................................. 42
      4:1:2:2:4 Comparison with the G93A mice ........................................................................... 43
      4:1:2:2:5 Detergent soluble aggregates .............................................................................. 43
      4:1:2:2:6 Detergent resistant aggregates ............................................................................ 44
      4:1:2:2:7 Stability ..................................................................................................................... 45
      4:1:2:2:8 Post mortem time effects ....................................................................................... 45
4:2 PAPER II ..................................................................................................................................... 45
  4:2:1 Mice ...................................................................................................................................... 45
<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEC</td>
<td>3-Amino, 9-Ethyl Carbazole</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FALS</td>
<td>familial amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>FTD</td>
<td>fronto-temporal dementia</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>H2G2</td>
<td>hitchhiker’s guide to the galaxy</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>hCG</td>
<td>human choriogonadotropin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HFE</td>
<td>hemochromatosis</td>
</tr>
<tr>
<td>HSP</td>
<td>hereditary spastic paraplegia</td>
</tr>
<tr>
<td>JALS</td>
<td>juvenile amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LBHI</td>
<td>Lewy body like hyaline inclusions</td>
</tr>
<tr>
<td>LMN</td>
<td>lower motor neuron</td>
</tr>
<tr>
<td>MND</td>
<td>motor neurone disease</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NLVS</td>
<td>3-nitro-4-hydroxy-5-iodophenylacetyl-LLL- vinylsulfone</td>
</tr>
<tr>
<td>PBP</td>
<td>progressive bulbar paresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>protein gene product 9.5</td>
</tr>
<tr>
<td>PLS</td>
<td>primary lateral sclerosis</td>
</tr>
<tr>
<td>PMA</td>
<td>progressive muscular atrophy</td>
</tr>
<tr>
<td>PMS</td>
<td>pregnant mare serumgonadotropin</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SALS</td>
<td>sporadic amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNARE</td>
<td>SNAP Receptor (protein involved in membrane fusion)</td>
</tr>
<tr>
<td>SOD1</td>
<td>superoxide dismutase 1; copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA binding protein</td>
</tr>
<tr>
<td>UCHL1</td>
<td>ubiquitin carboxyl-terminal hydrolase isoenzyme 1</td>
</tr>
<tr>
<td>UMN</td>
<td>upper motor neuron</td>
</tr>
<tr>
<td>wtSOD1</td>
<td>wildtype SOD1</td>
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</table>
The topic of this thesis is superoxide dismutase 1 associated amyotrophic lateral sclerosis and transgenic mouse models of this infirmity. This study focus on two disparate SOD1 mutations occurring in Scandinavia. The recessive D90A mutation which has properties similar to that of the normal wild-type human SOD1. The dominantly inherited G127insTGGG mutation, G127X, which causes a C-terminal truncation of the last 21 amino acids and is a highly unstable protein.

Tissues from patients with these two SOD1 mutations occurring were analysed using histopathological and biochemical methods. During this study novel SOD1 antibodies were generated and used, providing a more accurate tool for evaluating the effects of the enzyme has on neuronal and other tissue.

Transgenic mice carrying the same human SOD1 mutations, D90A and G127X, were generated. The mice were observed thoroughly, examined and analysed using similar methods as used for the human studies. These transgenic SOD1 mice were then compared to several other strains of mice transgenic for human wild-type and mutated SOD1.

This thesis starts with an introduction to the covered fields, after which materials and methods are described. The remaining part of the thesis deals with the results of this study and conclusions that can be made.

Some emphasis is put on the breeding of transgenic mice, since this is essential for getting good and reliable research results.

This thesis is based on the following papers:


III Graffmo KS, Brännström T, Jonsson PA, Marklund SL, Andersen PM. SOD1-positive cytoplasmic and intranuclear inclusions are found in familial amyotrophic lateral sclerosis patients with the D90A superoxide dismutase 1 gene mutation. Manuscript

IV Graffmo KS, Marklund SL, Brännström T. Widespread neuronal and astrocytic superoxide dismutase 1 inclusions in transgenic mouse models of ALS. Manuscript

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1: AMYOTROPHIC LATERAL SCLEROSIS

1:1 HISTORICAL NOTES
Amyotrophic lateral sclerosis, ALS, is an always lethal motor neuron disease affecting approximately 2/100 000 individuals every year in the Western countries. With a mean survival of three years after diagnosis, the prevalence is approximately 6/100 000 individuals [1]. Men are more commonly affected than women, and the mean age of onset is 58 years. The presence might increase since survival in some cases is prolonged due to new and better treatment [2-5].

ALS was described and named by the French neurologists Aran 1848/1850 [6], Jean Marie Charcot and A Joffroy [7, 8] in 1869. Charcot was the person who described adult onset progressive muscular wasting as a disease of the spinal motor neurons. He also realised that the degeneration of the corticospinal tract was of importance in the pathological process.

Later progressive bulbar palsy, PBP, described by Duchenne in 1860, and progressive muscular atrophy, PMA were included into the concept of ALS. PMA is however more heterogenous, but the vast majority of the patients fulfil Charcot’s disease criteria with upper and lower motor neuron symptoms. The term Motor Neuron Disease, MND is often used interchangeably with ALS.

1:2 MOTOR SYSTEM
All movements, regardless if they are voluntary or involuntary, are controlled by signal patterns from the brain and from the spinal cord. Both spatial patterns as well as temporal patterns are generated from neural circuits. The corticospinal tract constitutes the descending motor pathways by which upper motor neurons in the cerebral cortex project to lower motor neurons in the ventral horn of the spinal cord.

At the level of the lower medulla oblongata is the pyramidal decussation where approximately eighty percent of the nerve fibres of the pyramid tract cross to the collateral side. They continue as the lateral corticospinal tract forming part of the lateral funiculus. The remaining twenty percent of the nerve fibres continue uncrossed as the anterior corticospinal tract. Those fibres cross the midline at the level of their final destination.

Muscle cells are controlled by upper and lower motor neurons. The upper motor neurons are found in the motor cortex in the brain. The cells in the motor cortex deal with initiation and planning of movements as well as directing voluntary movements.

The lower motor neurons are found in the anterior horns of the spinal cord and in the brainstem. The lower motor neurons are controlled by local neural circuits within the brainstem and the spinal cord that coordinate the individual groups of skeletal muscles. These local circuits are also influenced by sensory input from the surroundings. The neural circuits are responsible for both the more elementary reflex movements and more complicated motor activities. There is also influence from the upper motor neurons concerning the more complicated movements.

The upper motor neurons are influenced by circuits in the cerebellum and in the basal ganglia which controls the precise orientation temporally and spatially. The axons from the upper motor neurons constitute the descending pathways that influence the lower motor neurons.
Most of the pathways interact with the lower motor neuron circuits, while a few interact directly with the lower motor neurons.

Neurons in the primary motor cortex, the lateral premotor cortex and the medial premotor cortex deals with planning, initiating and directing of voluntary movements. Other centers in the brainstem centres regulate postural control and basic movements.

Neurons in the brainstem are part of the system that regulates the muscle tone, and handles the response to information from sight, hearing, balance and body (proprioception). Thus the eyes, the head and the body are oriented in space.

The basal ganglia are found in the forebrain and act as suppressors of movements, and they also prime the upper motor neuron circuits for initiating movements.

The efferent pathways of the cerebellum detect the difference between planned movements and actually performed movements. That control mechanism is used for correcting errors and can also be considered as a learning tool for acquiring motor skills.

Motor neurons innervating a single skeletal muscle are called a motor neuron pool and are grouped together in clusters. The motor neuron pools innervating adjacent muscles are usually grouped together, i.e. motor neuron pools for arm muscles are found in the cervical enlargement. Neurons innervating postural muscles are found are located medially whereas neurons innervating distal muscles are progressively located more laterally.

In the anterior horns of the spinal cord the α-motor neurons are found. α-motor neurons innervate the extrafusal muscle fibres and also contribute to the muscle tone. An α-motor neuron and the muscle cell fibres it supplies are called a motor unit. The number of muscle fibres innervated by an axon varies depending of the need of how precise a muscle must contract. Motor units innervating eye-muscle supply one or a few muscle fibres, while motor units innervating leg muscles supply some 50 muscle fibres [9, 10]. There are also γ-motor neurons which innervate intrafusal muscle fibres that participate in the regulation of muscle tone. The few β-motor neurons share properties with both α- and γ-motor neurons.

Fig.1 Schematic figure of structures involved in motor control. Adapted from Hall 2004
1:3 TYPES OF MOTOR NEURON DISEASE

1:3:1 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is Greek meaning “scarring due to muscles wasted because of lack of nourishment.” In advanced cases this is seen as pale areas in the lateral parts of the spinal cord.

ALS affects the motor system. The corticospinal tracts, the corticobulbar tracts, the motor neurons in the ventral horn of spinal cord and the cranial motor nuclei in the brainstem are affected.

The main symptoms of ALS are muscle weakness and fasciculations, which is due to the degeneration of lower motor neurons in the brainstem and spinal cord.

Depending of which motor neurons that first are affected the initial symptoms may vary. Common early symptoms are muscle weakness in a hand or a foot, which progressively spread to the rest of the extremities. Fasciculations and cramping are most often seen in the muscles of hands and feet.

If the lumbar motor neurons are primarily affected the patient often starts with diffuse signs as difficulties to climb stairs or stumbling. The destruction of the motor neurons in the ventral horns of the lumbar enlargement leads to distal weakness and foot drop.

Patients with symptoms from cervical motor neurons with proximal weakness may initially have difficulties in moving their shoulder, especially while needing to abduct the shoulder as when combing or washing hair. When the distal muscles are affected the ability to grip small objects is impaired. Upper motor neuron involvement give more pronounced fasciculations and brisk reflexes.

Some patients are initially affected in the bulbar region which gives difficulties in speech and swallowing, but spares the locomotion in the initial stages. Bulbar palsy caused by involvement of brain stem lower motor neurons causes facial weakness, impaired palatal movement as well as wasting, weakness and fasciculation of the tongue. If the patient is affected by upper motor neuron impairment – pseudobulbar palsy – the condition is characterized more of dysarthria, brisk jaw reflex and emotional lability as crying or laughing pathologically.

Later stages of the condition are characterised of difficulties in swallowing and breathing as well as shortness of breath. The final cause of death is usually the weakness of the breathing musculature. The patient often dies of pneumonia or due to an overload of carbon dioxide.

1:3:1:1 Sporadic amyotrophic lateral sclerosis

The vast majority of patients with ALS have no affected first degree relatives or no apparent risk factors for developing the disease. This group of sporadic cases constitutes approximately ninety percent of all ALS patients. They are referred to as sporadic ALS or SALS.

In some apparently sporadic cases, mutations in the SOD1 gene have been described [11].
1:3:1:2 Familial amyotrophic lateral sclerosis

The remaining ALS patients have at least one known affected first degree relative. These cases are referred to as familial ALS or FALS. Patients with FALS usually are younger when affected, the gender distribution is more equal and there might be a higher frequency with co-morbidity such as frontotemporal dementia, parkinsonism and sensory disturbances. Most pedigrees suggest dominant inheritance, but recessive and X-linked modes of inheritance are described. Inheritance might also be somewhat secluded due to incomplete penetrance.

Approximately one fifth of the familial cases are associated with mutations in the oxygen radical scavenging enzyme superoxide dismutase 1, see below.

Ten different gene loci have been described in connection to FALS.

Table 1 Familial ALS loci Adapted with permission from Jonsson 2005 [12]

<table>
<thead>
<tr>
<th>DISEASE TYPE</th>
<th>LOCUS</th>
<th>GENE</th>
<th>INHERITANCE</th>
<th>REF.</th>
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<tbody>
<tr>
<td>ALS1 Classical ALS</td>
<td>21q21.11</td>
<td>SOD1</td>
<td>Dominant/Recessive</td>
<td>[13]</td>
</tr>
<tr>
<td>ALS2 JALS, PLS, HSP</td>
<td>2q33.1</td>
<td>Alsin/ALS2</td>
<td>Recessive</td>
<td>[14, 15]</td>
</tr>
<tr>
<td>ALS3 Classical ALS</td>
<td>18q21</td>
<td>-</td>
<td>Dominant</td>
<td>[16]</td>
</tr>
<tr>
<td>ALS4 JALS, slow progression</td>
<td>9q34.13</td>
<td>SETX</td>
<td>Dominant</td>
<td>[17]</td>
</tr>
<tr>
<td>ALS5 JALS</td>
<td>15q15.1-q21.1</td>
<td>-</td>
<td>Recessive</td>
<td>[18]</td>
</tr>
<tr>
<td>ALS6 Classical (+ FTD?)</td>
<td>16q12.1-21.1</td>
<td>-</td>
<td>Dominant</td>
<td>[19-21]</td>
</tr>
<tr>
<td>ALS7 Classical</td>
<td>20p tel-p13</td>
<td>-</td>
<td>Dominant</td>
<td>[21]</td>
</tr>
<tr>
<td>ALS8 Heterogeneous ALS/SMA</td>
<td>20q13.33</td>
<td>VAPB</td>
<td>Dominant</td>
<td>[22]</td>
</tr>
<tr>
<td>ALS-X Late onset</td>
<td>Xp11-q12</td>
<td>-</td>
<td>X-linked dominant</td>
<td>[23]</td>
</tr>
<tr>
<td>ALS/FTD Classical ALS and/or FTD</td>
<td>9q21-q22</td>
<td>-</td>
<td>Dominant</td>
<td>[24]</td>
</tr>
<tr>
<td>VEGF</td>
<td>6p12</td>
<td>VEGF</td>
<td>Dominant</td>
<td>[25]</td>
</tr>
<tr>
<td>Angiogenin Classical ALS</td>
<td>14q11.2</td>
<td>ANG</td>
<td>Dominant with low penetrance</td>
<td>[26, 27]</td>
</tr>
</tbody>
</table>

FTD = frontotemoral dementia; HSP = hereditary spastic paraplegia

1:3:2 Primary lateral sclerosis

Primary lateral sclerosis, PLS, affects the upper motor neurons. The patients suffer from marked spasticity, which often is symmetrical involving bulbar muscles, axial muscles and all four extremities. The patients also have gait disturbances. Most patients are over 50 years of age at time of onset, and there is a male predominance. The duration from diagnosis until death is longer, survival has been reported to over 15 years. The cause of death is often unrelated to PLS.

Contrary to ALS there is no motor neuron degeneration in the spinal cord. Among the few autopsy studies published loss of Betz’ cells, degeneration of corticospinal tract somewhere
from the internal capsule to the medulla throughout the spinal cord as well as gliosis in precentral gyrus and/or anterior horn have been described. [28-32].

1:3:3 Progressive muscular atrophy
A patient affected by progressive muscular atrophy, PMA, has symptoms due to involvement of the spinal lower motor neurons. Upper motor neurons are unaffected.

Muscle fibre twitching and involuntary contractions are not uncommon as initial symptoms. Disease progression is slow, patients may survive more than 25 years after diagnosis. The muscle weakness is less severe and spasticity, brisk tendon reflexes and emotional lability is rarely if ever present.

1:3:4 Progressive bulbar paresis
A patient affected by progressive bulbar paresis, PBP, has symptoms due to involvement of the lower motor neurons in the brainstem. Initial symptoms involve muscles that control talking, chewing and swallowing. With the inevitable progression of the condition basic functions as eating, drinking and breathing will deteriorate. Difficulties in swallowing easily cause aspiration and thus pneumonia is a not uncommon cause of death. Estimated survival time after diagnosis is usually short; patients often die within a couple of years.

1:4 DIAGNOSTIC CRITERIA

Table 2 Revised El Escorial criteria[33]

<table>
<thead>
<tr>
<th>THE DIAGNOSIS OF AMYOTROPHIC LATERAL SCLEROSIS [ALS] REQUIRES:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A - the presence of:</strong></td>
</tr>
<tr>
<td>(A:1) evidence of lower motor neuron (LMN) degeneration</td>
</tr>
<tr>
<td>by clinical, electrophysiological or neuropathologic examination,</td>
</tr>
<tr>
<td>(A:2) evidence of upper motor neuron (UMN) degeneration</td>
</tr>
<tr>
<td>by clinical examination, and</td>
</tr>
<tr>
<td>(A:3) progressive spread of symptoms or signs within a region or to other regions, as determined by history or examination,</td>
</tr>
<tr>
<td>together with</td>
</tr>
<tr>
<td><strong>B - the absence of:</strong></td>
</tr>
<tr>
<td>(B:1) electrophysiological and pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration, and</td>
</tr>
<tr>
<td>(B:2) neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.</td>
</tr>
</tbody>
</table>
SUPEROXIDE DISMUTASE 1

Superoxide dismutase 1 (SOD1) is an enzyme that converts intracellular superoxide radicals into hydrogen peroxide. Hydrogen peroxide is then removed by other scavenging enzymes such as catalase etc.

\[ 2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \]

SOD1 is a relatively abundant enzyme which is found in various amounts in all of the tissues of the body. SOD1 is primarily located to the cytosol, but is also found in the nucleus and the mitochondrial intermembrane space.

The gene for SOD1 is situated at chromosome 21 and the protein contains 153 amino acids.

SOD1 is present as a homodimer where the subunits are tightly connected to each other with hydrophobic bonds. The copper ions carry out the catalytic function of the enzyme. The zinc ions are important for the stability of the protein. The protein is furthermore stabilised by a disulphide bridge, which is an uncommon feature for an intracellular protein.

The connection between SOD1 and familial ALS was first described 1993 by Rosen et al. [13], since then 139 different ALS associated mutations have been reported in the SOD1 gene (Peter Munch Andersen, personal communication).

Absence of SOD1 does not result in any diminished motor abilities, as shown with the SOD1 knockout mouse model [34]. These mice had a normal growth rate and no signs of increased susceptibility to oxidative stress were reported. Neuropathological changes were sparse, a slight increase of small neurons in the ventral horns of the lumbar part of the spinal cord. The mice were more sensitive to trauma, as shown by exposing them to axotomy.

D90A

Already in 1973 Beckman and Pakarinen [35, 36] reported polymorphisms in the gene for SOD1 in northern Sweden and in northern Finland as well as in areas with people of Scandinavian descent. It was later shown that a variant, later identified as the D90A mutation, had somewhat lower activity than the more common (non-mutated) isoenzyme [37]. In the D90A mutation the amino acid asparagin in position 90 is changed to alanine due to a missense mutation in basepair 1078 where adenine is switched to cytosine. The D90A mutation has shown to have almost normal activity, compared to other mutations which have severely reduced activity or no activity at all.

ALS patients carrying the D90A mutation have signs of both upper and lower motor neuron involvement [38-40]. They live longer; up to 22 years after diagnosis and the disease progression is slow. Patients have a specific EMG pattern when tested neurophysiologically [39, 41, 42].

Positron emission tomography studies using the GABA\(_{\text{A}}\)-receptor ligand flumazenil has revealed changes both in motor as well as in non-motor areas as the left fronto-temporal and anterior cingulate cortices [43]. PET studies using the 5-HT\(_{1A}\)-receptor ligand WAY100635
showed changes in all cortical areas when compared with normal controls with the most pronounced changes seen in the left anteriolateral temporal lobe [44].

The patients also have micturition difficulties [39], unlike other ALS patients. These disturbances might implicate involvement of Ne Onufrowicz, a set of motor neurons in the anterior horn of the sacral cord innervating the bladder and pelvis muscles. Similar micturition disturbances have been detected in transgenic mice carrying the human D90A SOD1 mutation.

Unlike most of the other mutations in the SOD1 gene, patients with this mutation showed recessive inheritance.

1:5:2 G127insTGGG (G127X)

Andersen et al described a Danish family where four generations had been affected by amyotrophic lateral sclerosis [45]. DNA analysis showed an insertion of four base pairs, TGGG, after codon 127 in the gene resulting in a frame shift causing a truncation of the resulting enzyme. The activity of SOD1 in erythrocytes of the heterozygous patients was reduced to 40% of the normal level.

1:6 TRANSGENIC ANIMAL MODELS

1:6:1 TRANSGENIC MICE

Transgenic mouse models are a practical tool to investigate disease mechanisms and to find ways to affect the outcome of disease. The mice can be followed and thoroughly examined from early ages, and thus the even the presymptomatical stages of the disease can be examined and evaluated as well as providing a tool for trials of pharmacological and other treatments. In human patients this approach is not possible since tissue samples can not be taken during life time due to the high risk of damage to the patient e.g. becoming paralysed due to loss of a part of the spinal cord.

For a lethal condition, such as ALS, where pedigrees suggest an inherited condition, finding the gene and then inserting it into the mouse is a way to follow progression of the disease. At the same time analysing tissues at varying stages of the disease can give new and valuable information to understand the pathogenesis and possible ways of interfere with the disease process. Thus possible treatments can be explored and evaluated.

In the early 1980-ies the transgenic technology was developed. The first report of a transgenic mouse with a phenotype relevant to the inserted gene was published 1982 by Palmiter et al [46].

The first transgenic mouse model of ALS was presented in 1994 [47]. The transgenic mouse strain presented in this article, the G93A strain, has since become the standard used for in vivo studies of ALS, albeit being shown to possess several for ALS uncharacterical morphological features. For instance, spinal cord motor neurons of the G93A mouse show extensive vacuolisation both in conventional microscopes as well as electron microscopically, a feature not seen in humans with ALS [48, 49]. Transgenic mice has proven to be a good scientific tool, and several other strains with other SOD1 mutations have been created [50-54].
However, regardless the fact that the G93A mouse is affectionately known as “the ALS mouse” it’s not a good model of human ALS since it differs in several aspects from the human condition.

1:6:2 OTHER TRANSGENIC ANIMAL MODELS OF MOTOR NEURON DISEASE

Transgenic rats was first described by Howland et al 2002 [55]. These rats overexpressed the SOD1 mutation G93A [G93A Cu(2+)/Zn(2+) SOD1]. Their work concentrated on glutamate transport activity in synaptosomes, and also the effect of the pharmaceutical compound riluzole. Rats have the advantage of being larger rodents than mice, meaning they give more raw materials to work with than you can get from mice as well as allowing easier surgical manipulations.

The first invertebrate model of ALS was described 1995 by Phillips et al who used a Drosophila melanogaster model to describe neuropathological changes due to mutated SOD [56]. Drosophila melanogaster is a fruit fly commonly used in genetical and biological developmental research. The generation time is about 14 days and the lifespan is about twice that time, depending on temperature. Drosophila melanogaster has four pairs of chromosomes and the genome is well known.

Nematodes as Caenorhabditis Elegans have been used in ALS studies. The first article of MND in C Elegans was published 2001 by Oeda et al [57]. This soil living worm, approximately 1 mm long, has a short temperature dependent life-span. The generation time is 3.5 – 4 days and the lifespan is approximately 20 days. It’s easily grown on agar plates and self fertilises. Caenorhabditis Elegans has a completely known genome and the hermaphrodite consists of 959 cells whose developmental fate is mapped. By adding fluorescent proteins aggregates of faulty proteins easily can be visualised even in living organisms.

Pigs as model animals are less common. Transgenic pigs were first mentioned in the middle of the 1980-ies [58] as a possible way of creating replacement organs. Fears of transspecies viral contamination diminished the progression rate in this area. Ongoing studies on transgenic pigs with mutated pig SOD1 have according to preliminary results showed a clinical and neurological picture comparable to human disease (press release from Ministeriet for Fødevarer, Landbrug og Fiskeri Danmarks JordbrugsForskning 2006-06-02)

Recently Lemmens et al [59] described a model using zebra fish where introduction of mutant human SOD1 caused axonopathy which was said to be more prominent in motor neurons.
1:7 INCLUSIONS

Fig 2 Inclusions found in patients with ALS

1:7:1 Bunina bodies (Fig. 2A)
Bunina [60] described granular dense inclusions found in the cell soma of motor neurons. These inclusions are often considered to be a histopathological hallmark of ALS. In routine stainings Bunina bodies appear as small, up to 2-5 μm in diameter, eosinophilic inclusions. They have been claimed to be positive for cystatin C, a cystein protease inhibitor [61]. Cystatin C is 120 amino acid long 13,2 kDa protein which has two disulfide bridges [62, 63]. These inclusions can be found single, in clusters or in formations resembling chains. Depending on staining method chosen they appear red (hematoxylin and eosin), dark blue (phosphotungstic haematoxylin) or blue (Luxol fast blue).

1:7:2 Lewy body like hyaline inclusions (Fig. 2B)
Hyaline inclusion bodies are found in motor neurons in the anterior horn of the spinal cord and seen in routine hematoxylin and eosin preparations [64]. They do not usually share the peripheral halo seen in the Lewy bodies found in Parkinson’s disease even though they also are ubiquitin positive. Ultrastructurally, they are composed of arrays of 10-20 nm filaments with a slightly fuzzy profile [65].

1:7:3 Skein like inclusions (Fig. 2C)
Yarn like inclusions which are ubiquitin positive and found in motor neurons of the anterior horn of the spinal cord, brainstem and motor cortex [66]. Skein like inclusions are intracytoplasmatic filamentous structures that are not visible with routine stainings as haematoxylin and eosin. Ultrastructurally, the filaments are composed of arrays of 10-15 nm linear filaments associated with granules [67].

1:7:4 Other entities
Other entities named conglomerate inclusions, basophilic inclusions and Hirano bodies have been linked to ALS. They seem to either represent aging phenomena in general or to be part of degeneration. They may be seen in many different neurodegenerative diseases (Hirano bodies) or are found only in a very small subset of ALS patients. The relationship between the conglomerate inclusions to the Lewy body-like hyaline inclusions is debated.
2 AIMS

The aims of this thesis are to describe and evaluate mutated SOD1 in patients, create corresponding transgenic mouse models and to describe and evaluate mutated SOD1 in these mice.

Describe and map out D90A and G127X mutated SOD1 in patients using histopathological, biochemical and molecular methodologies.

Create mice transgenic for the above mentioned mutated human SOD1s.

Follow disease progress for these transgenic mice.

Describe and map out D90A and G127X mutated SOD1 in mice using histopathological, biochemical and molecular methodologies.

Characterise histological changes during the disease progress in the transgenic mice and to compare these changes with patients carrying the same SOD1 mutation.

Compare the D90A and G127X transgenic mice with other transgenic mouse strains.
3: MATERIALS AND METHODS

3:1 MATERIALS

3:1:1 HUMANS

Patients with ALS were prospectively enrolled at the Department of Neurology, Umeå University Hospital, except for the patient with the G127X mutation who was enrolled at Hvidovre University Hospital, Copenhagen. Non-neurological control patients and control patients with other neurological conditions were enrolled at Umeå University Hospital.

The deceased patients underwent a full post mortem examination, where tissue from brain, spinal cord, peripheral nerves and muscles as well as most internal organs were collected. The collected tissues were either snap frozen in liquid nitrogen and stored in -80° C until analysed; or cut into small pieces three mm thick, which were fixed in 4% phosphate buffered formaldehyde at least overnight until further processed.

A full informed consent was obtained from each of the patients and confirmed by family members, according to Swedish law and hospital principles. The studies followed the code of the Helsinki Declaration and were ethically approved by the ethical committees of the University of Umeå, Sweden and the University of Copenhagen, Denmark.

3:1:1:1 D90A

Tissues were collected from five patients carrying the D90A SOD1 mutation that were selected for this study from a large group of patients prospectively enrolled at Department of Neurology, Umeå University Hospital. Genotyping had shown the D90A mutation.

All patients with the D90A SOD1 mutation had a familial disease. Two of the patients belonged to the same family previously described by Andersen et al as “Family B” [38] All patients in this subgroup as well as in the larger group fulfilled the El Escorial criteria for ALS [33].

3:1:1:2 G127X

A patient carrying the G127X mutation[45] underwent post-mortem examination 20 h after death. The whole brain and spinal cord was removed and frozen at -80° C. For obtaining tissue pieces for analysis, the central nervous tissue was dissected under a microscope at a frozen aluminium plate at -20° C. Tissue pieces for histopathology were then immersion fixed in 4% paraformaldehyde in 0.1 M Na phosphate, pH 7.4 . For biochemical analyses tissue pieces sized 10 – 50 mg wet weight were dissected.

At the time of death the patient was 62 years and had tetraparalysis with signs of upper motor neuron involvement as well as lower motor neuron involvement. She had been diagnosed with left arm onset ALS 32 months prior to her demise.
3:1:1:3 CONTROLS

Corresponding tissues were sampled from deceased patients with other neurodegenerative conditions than ALS and from patients who died from pneumonia or heart disease [68]. The patients were age and gender matched in as much extent as possible.

The patients with other neurodegenerative conditions (n=27) had a mean age of 74 ±19, range 2-92 years. The neurodegenerative conditions included Alzheimer’s disease (n=15), Parkinson’s disease (n=7); multiple sclerosis (n=3); tuberous sclerosis (n=1) and Huntington’s disease (n=1). Non-neurological control patients (n=19) had a mean age of 69 years ±17, range 37 – 91 years.

Control tissue for the G127X patient consisted of tissue from corresponding parts of the central nervous system of five age-matched individuals who died from non-neurological conditions.

Tissues from ALS patients were also used as controls for the D90A patients. These tissues were collected at autopsy from patients prospectively enrolled at the Department of Neurology, Umeå University Hospital, who fulfilled the El Escorial criteria for ALS [69]. A careful history of familial disease was taken from all patients. This group consisted of 16 patients with SALS (mean age 70 ± 9 [49–83] years) and 6 patients with FALS (mean age 63 ± 4 [55–68] years). Genotyping as indicated above found no mutations in SOD1 in these 22 patients.

Furthermore, a retrospective search for clinicopathologically confirmed cases of ALS from the hospital records yielded 2 FALS patients (aged 33 and 49 years) and 13 SALS patients (mean age 70 ± 10 [55–88] years), from whom material was subjected to histological study. Statistical comparison was made between prospectively and retrospectively collected patients with the same type of ALS. Since no differences were found within the groups, the materials were combined in the final analysis, giving one control group of 8 FALS patients (mean age 58 ± 12 [33–68]; years) and one control group of 29 SALS patients (mean age 70 ± 9 [49–88] years).

3:1:2 MICE

3:1:2:1 Making of a transgenic mouse

The cDNA of the desired human gene is fusioned with a promoter sequence. If instead a knockout mouse is generated, a cDNA with a deletion that inhibits the expression of the targeted gene is inserted.

Adolescent female mice are stimulated with hormones PMS - Pregnant Mare Serumgonadotropin and hCG – human Choriogonadotropin to superovulate. They are mated and the embryos are dissected out. The transgene is injected into the pronucleus of the fertilised egg and is then implanted into the oviduct of a pseudopregnant female mouse.

If the transgene is incorporated into the genome before the first cell division the embryo will have the transgene in all its cells, including the germ cells. If the transgene is incorporated at a later stage the embryo will be a mosaic, where some cells contain the transgene while others don’t.
The multiple copies of the transgene will normally incorporate in tandem into one chromosome, making the embryo hemizygous. However the exact chromosomal localisation and the number of copies inserted can not be predicted. This will vary between the different founders.

The success rate of delivered transgenic mice may vary, as well as the numbers of incorporated copies of the transgene into the genome. A mouse that has the transgene incorporated is called a founder and can be used for further breeding.

3:1:2:2 C57BL/6J inbred mouse strain

The C57BL/6J inbred strain was developed by Clarence C. Little in 1921 after crossing female N57 with male N52 from Abbie CC Lathrop, Granby, Ma ,USA. Stocks were then received by Hall. Jackson Laboratory received stock from Hall in 1948, when the strain was F22. Zentralinstitut für Versuchsteirzucht, Hannover Germany (Han) received stock in 1971. M&B A/S (now Taconic Europe) received stock at F141 in 1988. The mice were derived by embryo transfer in January 2000. The Taconic US foundation colony was at F156 + 35 in 2005 [70-72].

C57BL/6J mice have a high preference for ethanol and morphine, and are susceptible to streptozotocin-induced diabetes. They are relatively resistant to mycobacterium leprae murium infection [70]. They have good hearing, good learning capacity and are usually not aggressive [73].

It is known that pups occasionally have to be terminated due to feeding problems because of overgrowth of teeth. Exceptionally pups are born with small eyebulbs uni- or bilaterally. Hydrocephalus is seen in rare occasions. Well kept the C57BL/6JBomTac mouse can become very old, up to three years of age.

One of the great advantages of C57BL/6J is their good capacity as breeders. They tend to have frequent litters with reasonable sized pups which can be weaned at 21 days in general. They take good care of their offspring, even if the first litter sometimes is lost. They can also be kept as breeders for a prolonged period of time. Even if the fertility in female mice is considered to be reduced by half at six to eight months of age [74, 75] they can be used for breeding in higher ages if necessary. However females are somewhat difficult to superovulate, and hybrid females e.g. C57BL/6JxCBA are therefore preferred when constructing transgenic mice.

3:1:2:3 Termination

The mice were euthanized at preselected intervals or when showing established paralysis. This stage will if continued lead to terminal illness when they could no longer reach the food in their cages and thus not feed themselves.

Mice were anesthetised with an intraperitoneal injection of midazolam, fentanyl and fluanizone. They were euthanized by an overdose of sodium pentobarbitone [76]. The mice used for histopathology were perfusion-fixed with 4% paraformaldehyde in 0.1 M Na phosphate, pH 7.4 and post-fixed in the same medium for at least overnight before dissected.
Experiments and animal care were accomplished in accordance with the European Communities Council Directive (86/609/EEC) [76] and the experimental protocol was approved by the Ethics Committee for Animal Research at Umeå University.

3:1:2:4 Mice histologically investigated in paper IV

<table>
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<td>-</td>
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<td>-</td>
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</tbody>
</table>

3:1:2:5 Genotyping of mice

For all of the mouse strains except G127X genotyping were done on erythrocytes using an enzyme linked immunosorbent assay (ELISA) for human SOD1 [77] or by measuring the SOD activity.

G127X mice were genotyped using PCR to detect DNA. Southern blot technique was used to detect DNA and to quantify number of copies of the transgene inserted into the mice.

3:1:2:6 D90A

A 0.6 kb PCR fragment containing HindIII-NsiI exon 4, amplified from genomic DNA of a D90A ALS patient (primers 5’-CAC TAG CAA AAT CTA CTA-3’ and 5’-TCT TAG AAT TCG CGA CTA ACA ATC-3’) was ligated into a HindIII-NsiI cleaved PvuII-PstI subclone of the human SOD1 gene [78]. This subclone includes exons 2 - 4. Exon 5 with flanking sequences was added by ligating a PstI-BamHI fragment to it. Finally a complete SOD1 fragment with a D90X mutation in exon 4 was completed by ligating the PvuII-BamHI to an exon 1 containing EcoRI-PvuII subclone. The 11.6 kb EcoRI-BamHI fragment was then excised from an agarose gel, electroeluted and used for microinjection into fertilised ova from C57BL6/CBA mice.

Transgenic mice were then generated using standard methods [72].

Twelve lines of transgenic mice carrying the mutant human D90A SOD1 gene were generated. Transgenic mice were identified by Southern blot.

3:1:2:7 G127X

A 0.6 kb fragment containing Ncol-BclI exon 5, amplified from genomic DNA of a G127X patient (primers 5’-AAA GTA AGA GTG ACT GCG GAA CTA-3’ and 5’-CTG GCA AAA TAC AGG TCA TTG A-3’), was ligated into a Ncol-BclI-cleaved PstI-BamHI subclone of
the human *SOD1* gene. To restore a complete 11.6 kb *EcoRI*-*BamHI* genomic fragment the *PstI*-*BamHI* fragment with mutant exon 5 was isolated and ligated to a *PvuII*-*PstI* subclone of the *SOD1* gene. In a final step the *PvuII*-*BamHI* fragment containing exons 2-5 was isolated and combined with an *EcoRI*-*PvuII* fragment adding exon 1 and upstream sequences to the construct. This 11.6 kb SOD1 fragment was then excised with *EcoRI* and *BamHI*, electroeluted and used for microinjections into fertilised ova from C57BL6/CBA mice.

Nineteen lines of transgenic mice carrying the mutant human G127X *SOD1* gene were generated. Transgenic mice were identified by Southern blot [79].

The founders initially used for breeding purposes had incorporated a high number of copies of the transgene as determined by southern blot. Continuous brother-sister mating combined with backcrossing into C57BL/6JBomTac mice was not sufficient to maintain the line, and thus a second set of new founders had to be generated.

### 3:1:2:8 OTHER MICE STRAINS

#### 3:1:2:8:1 G93A mice and G93Adl mice

G93AGur² [47, 80] and a line with loss of copies of the transgene, G93AGurdl [81] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Both these lines are hemizygous for the transgene.

The strain of origin is (C57BL/6 x SJL)F1 and they are mated F1 x hemizygote, female x male while at the Jackson Laboratory.

The mice were then backcrossed with C57BL/6JBomTac mice. The backcrossing into C57BL/6JBomTac mice using congenic breeding were up to six generations when sacrificed for experimental purposes.

#### 3:1:2:8:2 Human wtSOD1 mice

Male human wtSOD1 mice B6SJL-Tg(SOD1)2Gur/J² [47] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). This transgenic strain carries the normal allele of the human *SOD1* gene. Originally published as N1029, it has been reported that the SOD1 protein level is the same as in the transgenic strain carrying the SOD1-G93A transgene (002726), even though the copy number in the SOD1-G93A transgenic mice is higher [82].

The strain of origin is (C57BL/6 x SJL)F1 and they are mated F1 x hemizygote, female x male while at the Jackson Laboratory.

The human wtSOD1 mice are hemizygous for the transgene and upon arrival to our facilities they were mated with C57BL/6JBomTac female mice. The backcrossing into C57BL/6JBomTac mice using congenic breeding were up to 14 generations when sacrificed for experimental purposes.

#### 3:1:2:8:3 G85R mice

G85R mice, both males and females, were obtained from D.W. Cleveland [51]. The mice were F20 into C57/BL6NcrI BR (Charles River) upon arrival at our facilities and were then...
backcrossed into C57BL/6J BomTac mice. The backcrossing into C57BL/6J BomTac mice using congenic breeding were up to three generations when sacrificed for experimental purposes.

3:1:2:8:4 C57BL/6J BomTac mice
C57BL/6J BomTac mice were continuously obtained from Taconic Europe (Bomholt, Denmark), and were used for backcrossing the transgenic mouse strains as well as non-transgenic controls in some biochemical experiments.

3:1:2:8:5 Control mice
Non-transgenic litter mates of the transgenic mice were regularly used as controls. These mice were bred, kept and fed exactly as their transgenic litter mates and are thus ideal as controls.

3:1:3 ANTIBODIES

3:1:3:1 SOD1 antibodies
Primary polyclonal rabbit antibodies was raised against key hole limpet haemocyanin coupled peptides corresponding to amino acids 4-20, 24-39 (human specific), 43-57, 58-72, 80-96, 100-115, and 131-153 in the human SOD1 sequence. The later sequence is the same in both murine and human SOD1, but is lacking in the G127X mutation truncated protein.

Mouse SOD1 specific antibodies were raised against a peptide corresponding to amino acids 24-36 in the murine sequence.

In order to detect the G127X mutation truncated protein two specific antibodies were created against the carboxy terminal of this protein. One antibody has the sequence CIIGRTLVVHEKADDLGGQWRK (Agaz antibody) and the other has the sequence ADDLGQQRWK (Inger antibody). The sequence GQRWK is the novel epitope [83].

The rabbit antisera were first purified on Protein-A Sepharose (Amersham Biosciences, Uppsala, Sweden) and subsequent purified on a Sulfolink coupling gel (Pierce, Rockford, IL, USA) with the corresponding peptides coupled.

3:1:3:2 Commercial available antibodies
Commercially available antibodies against proteasomes; intermediary filaments, microtubuli, intermediary filament related structures, chaperones, nuclear structures and microglia/macrophages were used.

Ubiquitin carboxyl-terminal hydrolase isoenzyme 1 (UCLH1) also known as Protein gene product 9.5 (PGP 9.5) [84, 85] is a major protein component of the neuronal cytoplasm. It is found in cell bodies/processes in the neocortex. It is also expressed in neurons and other cells of the neuroendocrine system and in tumours derived from those cells. Small amounts can be found in ovary tissue.

The protein is a thiol hydrolase that recognises and hydrolyses a peptide bond at the C-terminal glycine of ubiquitin. Another function is to bind free monodimers of ubiquitin and
thus prevent degradation in lysosomes. It’s stipulated that the homodimer might have an ubiquitin ligase activity that is ATP independent.

Ubiquitin-protein hydrolase is involved both in the processing of ubiquitin precursors and of ubiquitinated proteins. The antibody is used as a marker of protein degradation.

Table 4 Commercial available antibodies used in this study.

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<td></td>
</tr>
<tr>
<td>Neuronal nuclei</td>
<td>monoclonal</td>
<td>Chemicon, Chndlers Ford, UK</td>
<td>[91]</td>
</tr>
<tr>
<td>TAR DNA binding protein 43</td>
<td>monoclonal</td>
<td>Abnova Taipei, Taiwan</td>
<td>[92]</td>
</tr>
<tr>
<td>CD 68</td>
<td>monoclonal</td>
<td>DAKO Glostrup, Denmark</td>
<td></td>
</tr>
</tbody>
</table>

Ubiquitin [93] is a protein responsible for degradation of most proteins in the mammalian cells. In humans the protein is found in both the nucleus and in the cytoplasm.
It is covalently bound to target lysines in other proteins, and as a polymer enhances degradation in proteasomes. However if the attachment is monomeric or as a linked polymer the functions are different i.e. stress response, DNA repair and regulation of gene expression. Ubiquitin is also vital for the formation of ribosomes. The antibody is used as a marker of protein degradation.

*Glial fibrillary acidic protein* is a class-III intermediate filament and a cell-specific marker that during the development of the central nervous system distinguishes astrocytes from other glial cells. The protein exists in three different isoforms and is found in the cytoplasm of the astrocyte. The antibody is used as a marker of gliosis.

*Neurofilaments* are intermediate filaments that are part of the cytoskeleton of neurons, which also includes neurotubuli and microtubuli. They are divided in to three classes according to molecular weight – light (68-70 kDa), medium (160 kDa) and heavy (200 kDa). Neurofilaments play a crucial part in intracellular transport.

*τ-proteins* are microtubuli associated proteins found in neuronal cells. They are highly soluble proteins that maintain the stability of the microtubule in the axons. There are six different splicing isoforms of τ-proteins. All of these can be found in a hyperphosphorylated state in Alzheimer’s disease, where they form paired helical filaments. Aggregates of τ-proteins have also been described in other neurodegenerative diseases as Parkinson’s disease and progressive supranuclear palsy as well as in dementia [88].

*Alzheimer precursor protein* is integral membrane protein which is found in many tissues in the body. It is concentrated in synapses of neuronal cells and thought to be part of formation of the synapses and important for neuronal plasticity. The human gene is found on chromosome 21 and alterations in its splicing give a defunct Aβ peptide. This peptide is the major component in β-amyloid found in Alzheimer’s disease.

*aβ-crystallin* is a major protein in the lens that acts as a chaperone. It is normally is absent in neurons, but can be found in oligodendrocytes. The protein is found in ubiquitinated intermediate filament inclusion bodies such as Lewy bodies [90].

*aβ-crystallin* has been shown to be upregulated in astrocytes in the spinal cords of mice with motor neuron disease [54].

*a-synuclein* is a neuronal protein mostly found in presynaptic terminals, but it can also be found in glial cells. It is located in various parts of the brain as neocortex, thalamus, hippocampus, striatum and cerebellum. The function is unknown, but it might be part of the transport mediating SNARE complex.

The protein is mostly in a soluble unstructured state. However during pathological conditions it aggregates and turns into insoluble fibrils. a-synuclein is a major component of Lewy bodies, which are found in Parkinson’s disease, Multiple System Atrophy, Lewy Body Dementia and Alzheimer’s disease.

*Cystatin C* is an abundant small 13 kDa polypeptide which is found both intra cellular and extra cellular in most organs and body fluids. The polypeptide is excreted at a constant rate from all cells with a nucleus. Clinically cystatin C is mostly used when evaluating the glomerular filtration rate of the kidneys. In the brain cystatin C in most abundant in
astrocytes, but can also be detected in neurons. It is thought to inhibit the activity of cystein proteases which occur in lysosomes and other locations.

*TAR DNA binding protein* also known as *TDP-43* is a nuclear factor with a weight of 43 kDa. It regulates transcription and alternative splicing. The protein is described in frontotemporal dementia [92], but has also been described as a component in skein-like inclusions and intra neuronal inclusions as well as in glial inclusions of spinal cords in ALS patients.

*CD68* is a cytoplasmic antigen expressed throughout monocyte differentiation. It is more intensely staining macrophages and microglial cells than monocytes. The location is considered to be closely associated to lysosomes.

3:1:4 STAININGS USED TO VISUALISE CELLS AND TISSUE

*Hematoxylin eosin* is the standard staining used to visualize histological structures. Mayer’s haematoxylin stains the cell nuclei and the chromatin well, and the eosin component which colours surrounding cytoplasm and tissue fibres thus making validation of tissue components possible [94].

The glasses are rehydrated and deparaffined, after which they are rinsed in distilled water. Following 10 minutes in Mayer’s hematoxylin solution, the glasses are rinsed in lithium carbonate water for 1 minute. After 15 minutes rinsing in running tap water, the glasses are stained in eosin solution for 4 minutes. The glasses are then dehydrated and mounted.

*Van Gieson* staining is used to visualize connective tissue, and as counter stain to visualize cell nuclei from the cytoplasm. Fresh collagen, though, is less well stained [94]. The glasses are rehydrated and deparaffined, thereafter they are placed in Weigert’s hematoxylin solution for ten minutes. After seven minutes rinse in running tap water, the glasses are placed in van Gieson’s solution for five minutes, after which they are dehydrated and mounted.

*Periodic acid-Schiff* staining is used to visualize inclusion bodies [95, 96]. The glasses are rehydrated and deparaffined, whereafter they are placed in 1% periodate solution for five minutes. After rinsing in distilled water and 70% ethanol the glasses are put in Schiff’s reagent for twenty minutes. This is followed by rinsing in running tap water and staining in Mayer’s haematoxylin for ten minutes. After rinsing in running tap water, the glasses are dehydrated and mounted.

Periodic acid-Schiff staining can also be combined with diastase (*α*-amylase), an enzyme which is found in saliva. *α*-amylase cleaves the 1,4 linkages of starch or glycogen and thus yields water soluble sugars, and thus make them easy washable [94].

*Klüver-Barrera* staining is used to visualize changes in myelinated nerve fibres in both the central and the peripheral nervous system as well as changes in glial cells and in nerve cells. Myelin is stained bluish green and the cell nuclei are stained purple against a weak blue background [97].

The glasses are rehydrated and deparaffined, whereafter they are placed in Luxol solution and kept in 56°C over night. After rinse 70% ethanol the glasses are then differentiated in 0.05% lithium carbonate. After rinsing in 70% ethanol and distillate water, the stained glasses are
dyed in cresyl violet and rinsed in water. After a final dehydration and mounting the stained glasses are ready for inspection.

*Congo red* is used for detection of amyloid. The dye binds to amyloid with nonpolar hydrogen bonds, and can be seen as red to green birefringence in polarised light.

The glasses are rehydrated and deparaffined, whereafter they are stained with hematoxylin for five minutes. After rinsing in distilled water they are placed in *congo red* solution for 20 minutes. Then the glasses are rinsed in 95% ethanol and in xylene after which they are mounted [94].

*Bielschowsky’s technique* is used to visualise nerve fibres. It is a silver staining starting with a “sensitization” in silver nitrate after which the section is treated with ammoniacal silver. The section is then developed by using formalin added to silver. Gold and sodium thiosulphate is added to remove unbound silver.

The glasses are rehydrated and deparaffined, and put into hydrochloric acid. The glasses are stepwise treated with the different silver solutions and rinsed in distilled water in between. They are then stained with gold and rinsed in ammonium water and sodium thiosulphate, dehydrated and mounted with Canada balsam [94].

*Laidlaw’s technique* is used to visualise collagen type III fibres, also known as reticulin fibres. These thin fibres will be shown in black. Laidlaw’s technique is a modification of Bielschowsky’s technique.

The glasses are rehydrated and deparaffined, whereafter they are treated with potassium permanganate for three minutes. They are then washed in water and bleached with oxalic acid solution. After washing and rinsing several times in distilled water the glasses are treated with ammoniacal lithium-silver solution for five minutes at 55º C. They are then rinsed in distilled water and reduced with 1% formalin for three minutes. After rinsing in distilled water the glasses are treated with gold chloride for ten minutes, after which they are rinsed again in distilled water. They are then treated with with oxalic acid solution and sodium thiosulphate with rinsing in distilled water in between. After a final rinse in distilled water the glasses are dehydrated and mounted with Canada balsam [94].

*Perl’s technique* is used to visualise ferric iron, and can also be used to demonstrate ferritin. The tissue is treated with hydrochloric acid to separate the iron from the ferritin. This makes it possible for the potassium ferrocyanide to bind to the ferric iron and form ferric ferrocyanide or Prussian Blue.

The glasses are rehydrated and deparaffined, and put into a mixture of hydrochloric acid and potassium ferrocyanide for 30 minutes. After washing the glasses are counterstained in neutral red for five minutes and rinsed. They are then dehydrated and mounted.

*Fouchet technique* is used to visualise bile pigments. Aqueous ferric acid is used in the presence of trichloracetic acid to oxidise bile pigments. Bilirubin will be stained green and cholecyanin will be blue.

The glasses are rehydrated and deparaffined, where after they are stained with Fouchet’s reagent for five minutes. After rinsing in distilled water the glasses are counterstained with
van Gieson’s solution for two to three minutes. After rinsing in distilled water, the glasses are dehydrated and mounted [94].

3:2 METHODS

3:2:1 HISTOLOGY

3:2:1:1 Preparations of tissues
The dissected fixed central nervous tissue pieces from humans as well as from mice were initially collected and fixed in 4% paraformaldehyde in 0.1 M Na phosphate, pH 7.4 as described above. After being thoroughly fixed they were dehydrated and embedded in paraffine.

For the prospectively collected human material, tissue samples were at autopsy taken from the cervical, thoracic and lumbar regions of the spinal cord. In addition, half the brain, brainstem and cerebellum were collected for histopathological studies. After fixation for at least six weeks, the brain, brainstem and cerebellum were serially cut and blocks from defined areas dissected for further histological processing. For the retrospective material the spinal tissues available mostly consisted only of blocks from the cervical and/or thoracic parts of the spinal cord.

The tissue from the human control patients corresponded to the defined areas mentioned above, and for the neurological control patients mainly of spinal tissue from the cervical level while for the non-neurological control patients tissue from all three levels of the spinal cord was available.

Murine tissue was obtained from perfusion fixed mice as described above. The brain, brainstem, cerebellum and spinal cord were dissected after additional fixation of the mice in 4% paraformaldehyde in 0.1 M Na phosphate, pH 7.4 for at least 24 hours.

From selected mice in each of the examined cohorts sections from lung, heart, liver, kidney and quadriceps femoris muscle were examined using standard stainings as described above as well as for presence of SOD1. These sections were treated according to the same protocols as used for the central nervous tissue.

3:2:1:2 Microscopy slides
Paraffine embedded 5 μm thick sections of central nervous tissue, brain and spinal cord at three levels (cervical, thoracic and lumbar) were slided onto positive charged glass slides and used for staining procedures. Sections used for Klüver-Barrera staining were 10μm thick.

3:2:1:3 Immunohistochemistry
Paraffine embedded 4μm thick sections of central nervous tissue were immunostained using the Ventana ES automated slide stainer and ES reagents (Ventana Medical Systems Inc. Illkirch, France) according to the manufacturer’s recommendations.
As pre-treatment to enhance antigen–antibody coupling the sections after being placed onto positively charged glass slides, were preincubated for 30 minutes in 3% H$_2$O$_2$ in methanol and then heated in 0.5 M citrate buffer pH 6.0 for 20 minutes in a microwave oven. The primary antibodies were located with biotin conjugated secondary antibodies coupled to an avidin-horseradish peroxidise conjugate. To visualise the complex the chromogen AEC (3-Amino, 9-Ethyl Carbazole) was used.

The sections were counterstained with hematoxylin, washed and mounted with glycerine-gelatine.

These procedures were done using the above mentioned commercial available antibodies as well as the anti-SOD-1 antibodies.

**3:2:1:3:1 Grading of immunhistochemistry**

A four graded semiquantitative scale was used to estimate the number of neurons in each section showing inclusions. A two tiered scale was used evaluating non-cellular staining for white matter tracts and cerebellum and cortical areas outside subiculum and hippocampal formation.

<table>
<thead>
<tr>
<th>LEVEL</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No neurons with inclusions</td>
</tr>
<tr>
<td>1</td>
<td>&lt; 25% of the neurons show inclusions</td>
</tr>
<tr>
<td>2</td>
<td>25 – 75 % of the neurons show inclusions</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 75 % of the neurons show inclusions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LEVEL</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>negative</td>
</tr>
<tr>
<td>1</td>
<td>positive</td>
</tr>
</tbody>
</table>

**3:2:1:4 Stereology of mice spinal cords**

From the upper end of each of the six paraffine blocks containing the thoracic spinal cord of the terminally ill mice or the approximately 600 day old mice with corresponding controls, 50 μm thick sections were cut on a sliding microtome, mounted on glass slides and stained with cresyl violet [98].

The CastGrid System (Olympus, Solna, Sweden) connected to a BX61 motorised microscope was used to calculate the number of neurons in the ventral horn of the thoracic spinal cord. The ventral horn was defined as the area enclosed by the grey-white matter border of the spinal cord ventral to a plane at right angles to the long axis of the spinal cord and passing through the midpoint of the central canal. For each of the blocks this area was calculated using a 10x objective. Using a 60x oil objective the numeric density of neurons in the ventral horn was calculated using the optical dissector technique [99]. Neurons were defined as cells possessing a nucleus with an identifiable nucleolus [98].

The number of neurons in the thoracic ventral horn in each block was calculated as the density of neurons x the area of the ventral horn x one sixth of the length of the entire spinal
cord. The number of neurons in the thoracic ventral horn was found by adding the number in all six blocks.

3:2:2 CHEMISTRY

3:2:2:1 Preparation of tissues
The collected human central nervous tissues were snap frozen in liquid nitrogen and stored at -80º C until analysed. To obtain select areas of the tissue pieces for analysis, the central nervous tissue was dissected under a microscope at a frozen aluminium plate at -20º C.

3:2:2:2 Southern blotting
Southern blot technique was used to detect DNA and to quantify the number of copies of the inserted transgene in the mice.

DNA was prepared from mouse tails using standard methods and separated on 0.7% agarose gels and blotted onto Hybond XL nylon membranes (Amersham Biosciences, Uppsala, Sweden). The EcoRI-BamHI fragment of the SOD1-gene was used as a probe. Band labelling was done with MegaPrime random labelling kit (Amersham Biosciences) and then visualised with a Bio-Rad GS-525 Molecular Imager and quantified using Molecular Analyst software (Bio-Rad, Hercules, Ca, USA).

DNA copy numbers were quantified using known amounts of the EcoRI-BamHI fragment.

3:2:2:3 Northern blotting
RNA from mouse brain was prepared using the Trizol reagent in agreement with the manual provided by the manufacturer (Invitrogen, Carlsbad, Ca, USA). Electrophoresis was carried out using the NorthernMax-Gly kit (Ambion, Austin, Tx, USA) and RNA was blotted onto Hybond XL nylon membranes (Amersham Biosciences). Blots were probed using ULTRAhyb hybridisation buffer (Ambion) and MegaPrime random labelling kit (Amersham Biosciences) and then visualised with a Bio-Rad GS-525 Molecular Imager and quantified using Molecular Analyst software (Bio-Rad). SOD1 mRNA was detected using a purified PCR fragment as probe. Primers were 5'-GCGTGGCCTAGCGAGTTATG-3' AND 5'-ATCCTTTGGCCCACCGTGTTTTCTG-3' producing a 248 bp amplicon of axons 1-3. The PCR amplicon was purified using High Pure PCR Product Purification kit (Roche Diagnostics, Mannheim, Germany).

Samples were normalised against β-actin (Clontech, Palo Alto, Ca, USA).

3:2:2:4 Immunoblotting [Western blotting]
The dissected human central nervous tissue pieces were weighed and generally homogenised in 25 volumes of a buffer made of 50mM K phosphate, ph 7.4; 3mM DTPA; 0,3M KBr and Complete with EDTA using an Ultraturrax (IKA, Staufen, Germany) for two minutes followed by sonication using a Sonifer Cell Disruptor (Branson, Danbury, Ct, USA) for one minute.

The pretreated tissue samples were then solubilised 1:1 in 2 x SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) sample buffer (100 mM Tris-HCl, pH 6.8; 10%
β-mercaptoethanol; 20% glycerol; 4% sodium dodecyl sulphate and bromophenolblue) and heated at 95º C for five minutes after which they were separated on 12% SDS-polyacrylamide gels.

The gels were electroblotted onto polyvinylidene difluoride membranes (Amersham Biosciences). Blots were probed with antibodies and horseradish peroxidase labelled anti-rabbit IgG antibody or biotinylated anti-rabbit IgG antibody and horseradish peroxidase labelled streptavidin (Amersham Biosciences) were used as secondary antibodies. Chemiluminescence was generated using Supersignal West Dura substrate (Pierce). Bands were visualised on film or by using a Fluor-S Multiimager and Quantity One software (Bio-Rad).

### 3:2:2:5 2-D gel electrophoresis

Spinal cords from mice (100 day old and terminal G127X) were homogenised in 8M urea; 4% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS) and 2% Bio-Lyte, swollen into 7-cm pH immobilised pH gradient strips (Bio-Rad) and electrofocused following the manual from the manufacturer. The strips were then top-loaded onto 12.5% SDS-PAGE gels, electrophoresed and electroblotted to polyvinylidine difluoride membranes and immunoblotted with the 24-39 anti-SOD1 antibody and an anti-ubiquitin antibody (Sigma, St Louis, Mo, USA).

### 3:2:2:6 Quantification of SOD1

Immunoblotting was also used for quantification of SOD1. Wild type human SOD1 with the concentration determined by quantitative amino acid analysis was used as standard [100]. Samples were blotted in triplicates.

<table>
<thead>
<tr>
<th>SOD1 TYPE</th>
<th>ANTIBODY (AGAINST AMINO ACIDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse SOD1</td>
<td>24-36</td>
</tr>
<tr>
<td>Human G127X in murine tissue</td>
<td>24-39</td>
</tr>
<tr>
<td>Human G93A in murine tissue</td>
<td>24-39</td>
</tr>
<tr>
<td>Human wild type SOD1 in murine tissue</td>
<td>24-39</td>
</tr>
<tr>
<td>G127X in human tissue</td>
<td>ADDLGGQWRK (Inger antibody)</td>
</tr>
</tbody>
</table>

### 3:2:2:7 Centrifugations

The dissected human central nervous tissue pieces were weighed and homogenised in 25 volumes of a buffer made of phosphate buffered saline [PBS; 10Mm K phosphate, pH7.0 in 0.15M NaCl with complete without EDTA (Roche Diagnostics) antiproteolytic cocktail added] using an Ultraturrax followed by sonication.

### 3:2:2:8 Analysis of detergent-resistant aggregates

Human and murine central nervous tissues were homogenised in the pH 7.0 PBS with 0.1% of the detergent Nonidet 40 (NP40) (Roche Diagnostics) added were centrifuged at 20 000 g for 30 minutes at 4º C, after which supernatants were carefully removed. The pellets were resuspended and sonicated in double the initial amount of homogenising solution, after which they were centrifuged at 20 000 g for 30 minutes at 4º C. This procedure was repeated five
times. After the final washing the pellets were resuspended and sonicated in 1 x SDS-PAGE sample buffer. The resuspended pellets were analysed by immunoblotting using the 24-39 anti-SOD antibody which is human specific.

To quantify the relative amounts of mutated SOD1 in the pellets they were compared against dilutions of the original homogenates.

3:2:2:9 Analysis of detergent-soluble aggregates

Fresh murine central nervous tissue from G127X mice homogenised in the pH 7.0 PBS were centrifuged at 20,000 g for 30 minutes at 4°C, after which supernatants were carefully removed. The resulting pellets were resuspended in the pH 7.0 PBS with 0.1% NP40. The resuspended pellets were analysed by immunoblotting using the 24-39 anti-SOD1 antibody which is human specific.

To quantify the relative amounts of mutated G127X SOD in the pellets they were compared against dilutions of the original homogenate in duplicates. The amounts of material contained in the “processomes” in the pellets were estimated by probing the blots with the 131-153 anti-SOD1 antibody, which in the G127X mice only detects murine SOD1.

The percentage of mouse SOD1 pelleted in processomes were then subtracted from the percentage of G127X mutated pelleted by centrifugation, to give the amount of detergent soluble aggregates.

3:2:2:10 SOD activity analysis

The dissected human central nervous tissue pieces were treated as before immunoblotting. [weighed and homogenised in 25 volumes of a buffer made of 50mM K phosphate, pH 7.4; 3mM DTPA; 0.3M KBr and Complete with EDTA using an Ultraturrax followed by sonication]. The samples were analysed spectrophotometrically using the KO2 assay [101]. One unit is defined as the SOD activity that brings about a decay of O2·− at the rate of 0.1 s\(^{-1}\) in 3 ml buffer. One unit in the assay equates to 4.2 ng human wild type SOD1 and mutant D90A SOD1 [100].

3:2:2:11 Stabilities of human and murine SOD1s

In order to estimate the stability of SOD1s pools of packed erythrocytes from three homozygous D90A humans, three human controls and three control C57Black/6J mice were mixed with 1.6 volumes of 37.5/62.5 vol/vol chloroform/ethanol at -20°C to precipitate and remove the haemoglobin. The samples were vortexed and centrifuged at 2,500 g for ten minutes. 200 μl of the supernatant, which contains SOD1, was added to 400 μl 3.75 M guanidiniumchloride in 0.1 M HEPES, pH 7.4 with 3 mM DTPA and incubated at 37°C. Denaturation was stopped by adding 50 μl aliquots of the mixture after different time intervals to 400 μl 50 mM Na HEPES, pH 7.4 with 0.25% BSA followed by analysis of the SOD activity.

Thermal stability were analysed by adding 100 μl of the chloroform/ethanol supernatant to 200 μl 0.1 M HEPES, pH 7.4 with 3 mM DTPA which then was placed in 70°C water bath. Denaturation was stopped by adding 50 μl aliquots of the mixture to 400 μl 50 mM Na
HEPES, pH 7.4 with 0.25% BSA at room temperature followed by analysis of the SOD activity. The denaturation was done at preset time intervals.

3:2:2:11 Isoelectric focusing and SOD staining
Brains from mice 100 days of age were homogenised in ten volumes of pH 7.0 PBS and centrifuged at 20 000 g for 30 minutes. According to instructions from the manufacturer (Amersham Biosciences) 20; 40 and 60 μl of the supernatant were separated by isoelectric focusing on Immobuline pH 3.5 – 9 gels. The gels were stained for SOD activity by immersion in 0.25 mM nitroblue tetrazolium, 30 μM riboflavin in 200 mM Tris cacodylate, pH 7.8 for 30 min followed by irradiation, as adapted from Beauchamp and Fridovich [102].

3:2:2:12 Effects of buffer composition, pH and chelators on SOD activity
In our laboratory tissues are routinely extracted in a buffer containing 3 mM DTPA. DTPA reduces the activity of some metal dependent proteases. The chaotropic salt KBr are added at a concentration of 0.3 M in order to facilitate extraction of proteins. The SOD activity is analysed with a direct assay at pH 9.5, in medium with a chelator.

The truncated G127X mutant protein lacks several parts of the protein which are important for conformation and subunit interaction. Therefore it is possible that SOD activity might be lost because of metal ion extraction by the chelators, and by denaturation by the high pH and by the chaotropic salt.

To analyse eventual loss of SOD activity brain homogenates from a G127X mouse and from a wild type mouse were made in 50 mM sodium phosphate, pH 7.4; these were then split into aliquots to which were added nothing; 0.3 M KBr; 1 mM DTPA or both. After further homogenisation the extracts were analyse by the direct KO₂ assay at pH 9.5 and the pyrogallol autooxidation assay at pH 7.8 [103] in either presence or absence of chelators in the medium [83].

3:2:2:13 Effects of post-mortem time
Time between death and post-mortem examination may vary, as well as the conditions under which the remains of the deceased person have been stored in the mean time. Family members must be given sufficient time for farewells and the availability of cold storages depends on the location for death.

In order to evaluate the effects of post-mortem times on the biochemical analyses a time mimicking set-up as carried out using brain tissue from G127X mice.

Small pieces of mouse brain tissue were put using aseptic technique into sealed tubes. They were kept at 37° C for 6h followed by storage in room temperature for another 6h. The tubes were then placed in 4° C. Immediately after this, and after preselected time intervals they were snap frozen to -80° C for subsequent thawing and analysis.

3:2:3 STATISTICAL ANALYSIS
Statistical analysis was done using the STATISTICA data analysis software system (version 7.1; StatSoft Inc., Tulsa, OK, USA) or SPSS (version 15; SPSS Inc., Chicago, IL, USA). The
nonparametric Kruskall-Wallis statistics (when comparing three groups) and Mann-Whitney statistics (when comparing two groups) were used for the morphological comparisons. In estimation of staining, the numbers given are median and range. When comparing SOD activities, analysis of variance (ANOVA) was used.
4 RESULTS

4:1 PAPER I

Motor neuron death, inclusions and gliosis are predominant in the spinal cords of both the patient and the transgenic mice with the corresponding mutation

4:1:1 The G127X patient

4:1:1:1 Histopathology

Three levels of the spinal cord – cervical, thoracic and lumbar – as well as three areas of the cortical brain – frontal, temporal and precentral – were examined.

Astrogliosis were found in all areas, especially in the corticospinal tract. No apparent spongiosis or major cell death were seen in the investigated cortical areas. However, in the spinal cord there was a major loss of motor neurons at all levels. Bunina bodies were present in many of the remaining motor neurons.

Small inclusions were present in both neurons and astrocytes in the cortical regions. These inclusions reacted with the anti SOD1- antibody ADDLGGQRWK (Inger antibody), which is specific for the G127X mutation.

Motor neurons contained ubiquitin immunopositive inclusions, of which some also stained positive for the Inger SOD1 antibody. Lewy body like hyaline inclusions were seen in both motor neurons and in astrocytes in the spinal cord. These inclusions often reacted with the Inger SOD1 antibody and some of the inclusions also showed weak staining with the 131-153 antibody. This might be due to presence of small amounts of wild type SOD1 in accordance with findings with detergent washed aggregates. Small round inclusions reacting with the Inger SOD1 antibody were seen in the motor neuron somata and in the neuropil, as were skeins and Lewy body like hyaline inclusions. No aggregates of SOD1 were seen in motor axons.

The findings are in concordance with findings in other studies [51, 104-107], however the antibodies used in these studies did not distinguish between wild type SOD1 and mutant SOD1.

Skein like ubiquitin immunopositive inclusions were seen in some motor neurons. Astrocytes were commonly positive for αβ crystalline and UCHL1. The latter antibody was also often positive in motor neurons.

4:1:1:2 Biochemistry

Immunoblotting of CNS extracts using an antibody raised against the middle part of the SOD protein showed a lower content of the wild type enzyme, and only a faint band representing the mutant enzyme, despite of prolonged exposure time. However, this band was clearly showed using the mutation specific antibody Inger. The highest levels were found in the ventral horn of the cervical and the lumbar spinal cord (2-4 % of the SOD1 content in control brain), followed distantly by the precentral gyrus and other parts of the CNS. In the ventral horns two bands were seen, one at 17 kDa representing the native size protein and one at 36
kDa. The latter band appears to be almost specific for areas afflicted by disease. Long exposure revealed additional high molecular weight bands as well as high molecular weight smearing in the ventral horn samples.

Precentral gyrus from the patient was homogenised with buffer without detergent and centrifuged for 30 minutes in 20,000 g. All of the G127X protein was found in the pellet suggesting that all mutant enzyme present was present in aggregates. The detergent resistance of the aggregates was examined by sonication in room temperature in 1% solutions of NP40; Tergitol NP40 and CHAPS in 10 mM K phosphate pH 7.4, 0.15 M NaCl resulted in less than 10% solubilisation. However, all mutant G127X was solubilised by 1% SDS. All these treatments were gentle and thus neither SOD1 nor SOD2 from the precentral gyrus homogenate of the G127X patient were inactivated.

The composition of the aggregated material was then analysed with five fold washing of material pelleted at 20,000 g in pH 7.0 buffer containing 0.1% NP40. No mutant enzyme was solubilised in the first extraction step, but there was some wt SOD1 together with the mutant G127X enzyme in the final washed pellet.

The SOD1 activity in the brain of the patient was half of the levels of the controls. This is in concordance with other SOD1 mutations [108]. The activity of SOD2 (Mn-SOD) and SOD3 (extra cellular SOD) were comparable to controls. The SOD1 activity in the erythrocyte of G127X carriers are about 40% of that of controls which may suggest a dominant negative effect of the mutant protein [109]. The findings in this study do not confirm any such mechanism in the CNS.

4:1:1:3 Fibroblast cultures
Immunoblotting of skin fibroblast cultures from the G127X patient using the mutation specific antibody Agaz could not detect any mutant G127X protein. Culture of the fibroblasts in the presence of 20 μM lactacystin, a proteosomal inhibitor, for 2 days resulted in a barely visible band whereas culturing the fibroblasts with the proteosomal inhibitor 3-nitro-4-hydroxy-5-iodophenylacetyl-LLL- vinylsulfone (NLVS) made the mutant SOD1 easily detectable. Previous studies on mutant SOD1s using proteasomes inhibitors showed similar results [110].

Neither the 33 kDa nor the 36 kDa bands found in brain and spinal cord of the patient or in mice of all ages were found in the cultured fibroblasts. There was a single 17 kDa band which probably represented the full length mutant subunit. When centrifuged at 20,000 g about 60% of the G127X mutant protein in the homogenate was pelleted. There was no sedimentation of the wild type human SOD1.

4:1:2 Mice
Nineteen founders with the G127X mutation were generated and breeding was established using C57BL/6J Bombay mice. The two lines with the highest numbers of inserted copies showed signs of motor neuron disease and was used for further breeding.

The 832 line had 28 copies and the mean survival time for homozygous mice was 126 days and for hemizygous mice was 213 days. The hemizygous mice were bred using congenic breeding and the homozygous mice were obtained by continuous brother – sister mating. This
approach was not entirely successful since a sudden decline in litter size, six pups in the last available litter, caused the end of this line.

The 716 line had 19 copies inserted by the transgene and the mean survival time was 250 days for homozygous mice and 477 days for hemizygous mice. The breeding strategy was the same with congenic breeding for the hemizygous mice and continuous brother – sister mating for the homozygous mice. This strategy has been proven successful with close to 30 generations at present. This study focused on the homozygous mice of line 716 and these mice are henceforth referred to as G127X mice. While most transgenic mice show paralysis of hind leg onset, approximately one third of the G127X showed paralysis of front leg as onset sign. The course of disease is rapid; in general it takes 7-10 day from first sign of disease until the mice reached final stage.

The expression of the SOD1 transgene in the brain of the G127X mice was about 60% of the amount found in G93A mice [47], a strain whose mean survival time in our settings was 126 days.

4:1:2:1 Histopathology
Sections from the brain and from the cervical, thoracic and lumbar spinal cord were examined in mice at the ages of 2, 50, 100 and 200 days as well as from terminally ill mice and corresponding non-transgenic control mice.

4:1:2:1:1 SOD stainings and SOD1 positive inclusions
Diffuse immunoreactivity with the mutation specific CIIGRTLVVHEKADDLGGQRWK (Agaz) antibody was seen from 2 days of age and onwards. In mice 100 days of age small immunoreactive inclusions were seen in the motor neuron somata, and these inclusions increased in size at 200 days of age and in terminally ill mice. Agaz positive inclusions were also seen in the neuropil and, in the end-stage mice, also in a few astrocytes.

SOD1 positive inclusions were also seen in terminal G93A mice. In the terminal G127X mice there were no inclusions or swelling in the ventral roots or in the ventral funiculi. The unstable G127X mutant SOD1 protein possibly only enters the axonal transport system on rare occasions. This suggests that axonal injury does not possess a major role in the degenerative process of the motor neurons, at least in the G127X model. In hematoxylin/eosin staining the ventral root fibres of the G127X mice looked unremarkable.

4:1:2:1:2 aβ-crystallin, UCHL1 and ubiquitin stainings
When examining the 2 day old mice nothing remarkable was noticed. However, from the age of 50 days and onwards aβ-crystallin immunoreactive astrocytes were seen throughout the spinal cord of the mice, including the white matter and the dorsal horn. In mice 200 days of age and in terminally ill mice this immunoreactivity was also seen in motor neurons.

Intense staining for an antibody directed against UCHL1 was seen in the spinal motor neuron somata from 50 days of age and onwards. The staining in the neuropil was of somewhat less intensity in the same age groups of mice.

Ubiquitin staining increased with age in the mice, starting in the mice 50 days of age and culminating in the terminally ill mice. This increase was more pronounced than for the age
matched human wtSOD1 mice. Moreover some of the ubiquitin staining appeared to co-localise with inclusions staining positively for human SOD1.

4:1:2:1:3 Gliosis
In the spinal cord of 50 day old mice weak GFAP immunopositivity was seen, a finding that became more tangible in mice 100 and 200 days of age as well as in terminally ill mice. In cortical regions of the brain weak immunopositivity was seen in mice 200 days of age and in terminally ill mice. No apparent differences from human wtSOD1 mice were seen in younger mice.

4:1:2:1:4 Similarities between mice and patient
Comparing sections from the patient and terminally ill mice many similarities were noted. Skein like and Lewy body like hyaline inclusions were seen in motor neurons and in some astrocytes in both the patient and in the mice. Some of these inclusions reacted with the mutant specific antibodies Inger and Agaz. Astrocytes immunopositive for antibodies against αβ-crystallin were also seen in both species as well as astrogliosis.

4:1:2:2 Biochemistry
4:1:2:2:1 Immunoblots
Whole homogenates of brain and of spinal cord of G127X mice were immunoblotted. Two heterogenous bands showed, one band at 17 kDa, accounting for ~ 60% of the mutated G127X mutated enzyme and the other band at 33 kDa accounting for ~ 40 % of the enzyme. The pattern was the same in all age groups of the mice, starting already at 2 days of age. The 17 kDa band probably represents a full length subunit of the mutated G127X SOD1 protein.

Spinal cord from G127X mice at 100 days of age were analysed by 2D-gel electrophoresis subjected to immunoblotting. Both the bands, 17 kDa and 33 kDa, were composed of multiple species.

Total amount of G127X mutant SOD1 was estimated from the sum of the two major bands in the immunoblots. The levels in brain and spinal cord of mice 100 days of age were 97% respectively 45% of those in wild type mice. In brain the level of the mutant G127X protein rose by 30% between 2 days of age and 50 days of age, but thereafter a plateau was reached. Contrary in the spinal cord the rise in the level of the mutant protein only appeared in the last stage of the disease. Relative amounts of the mutated G127X SOD protein in CNS were much higher than seen in kidney and liver. The amount of the mutated G127X SOD protein in erythrocytes was below the detection limit (0.2 % of wild type murine SOD1) in spite of the fact that total amount of SOD1 is about the same as in kidney and liver.
Fig 3 Survival and mutant protein in G127X mice

4:1:2:2:2 Ubiquitin
Replicate 2D electrophoresis gels were blotted, and gels were probed with anti-ubiquitin antibodies. A spot with low molecular weight, probably representing monomers, as well as higher molecular weight spots were found. No co-staining between SOD1 and ubiquitin were found when analysing mice 100 days of age. In the terminal mice a train of diagonal spots co-stained and suggested presence of some ubiquinated SOD1.

4:1:2:2:3 SOD activity
SOD activity were comparable between G127X mice and non transgenic mice of the same age. G127X mutated SOD1 is a very labile protein and might thus be inactivated of the high
concentration of KBr and chelator in the extraction medium usually utilised. Therefore
analysis also was done using medium without chelators having physiological ionic strength
and pH.

All the different extractions were analysed with the two different KO₂ and pyrogallol assays
with and without chelators as described above in the methods’ section. Omission of the
chaotropic salt and the chelators did not increase the SOD1 activity of the tissue extracts from
the G127X mice.

SOD activity was also measured by isoelectric focusing gels stained for SOD activity.
Extracts and staining solution were prepared without chelators. In order to increase the
possibility of detecting a minor activity band line 716 G127X mice hemizygous for the
transgene in SOD1 knockout background were analysed. No activity band was detected that
could be contributed to the G127X mutant SOD1 protein. This assay has high sensitivity and
able to detect activity levels as low as 0.5% of level of the murine SOD1.

4:1:2:2:4 Comparison with the G93A mice
The G93A and the G93A\textsuperscript{dl} mice \cite{47} contained 35 fold and 20 fold respectively more mutant
protein in spinal cord than homozygous G127X mice at 100 days of age. In the G93A\textsuperscript{dl} mice
the SOD1 activities also were markedly increased.

4:1:2:2:5 Detergent soluble aggregates
CNS tissues are frequently extracted with buffers containing weak detergents e.g. \( \sim 1\% \) NP40
and deoxycholate in order to solubilise membranes and myelin \cite{111-113}. In order to avoid
potential artefacts on the mutant SOD1 murine CNS were extracted with PBS pH 7.0 in the
absence of detergents. Samples were disrupted with the Ultrarax followed by extensive
sonification and yet \( \sim 15\% \) of wild type SOD1 of murine and human spinal cord and brain
was sedimented by centrifugation at 20 000 g for 30 min. Further sedimentation was not
achieved by higher speeds.

When liver and kidney extracts from wild type mice were analysed 5-10 \% of the wild-type
murine SOD1 were pelleted, a phenomenon further explored.

Brain from a SOD1 knockout mouse was homogenised and sonicated in the presence of a
haemolysate to yield the same SOD1 activity as in a homogenate of a wild type mouse. Only
2\% of the enzyme was present in the sediment. Thus the pelleting is not caused by affinity of
SOD1 for sedimenting components.

Sediments from centrifugation at 20 000 g from control mouse CNS were dissolved by
sonification in buffer containing 0.1\% NP40 and the SOD1 enzymic activity released to the
supernatant corresponded to \( \sim 95\% \) of the protein that had been sedimented. Thus the
sedimented trapped SOD1 enzyme is enzymatically active. Similarly \( \sim 85\% \) of the SOD2
activity was present in the first supernatant and 15 \% was released from the NP40 solubilised
pellet. SOD2 is localised to the mitochondrial matrix, and this is good evidence that
mitochondria were efficiently disrupted by the primary homogenisation. SOD1 localised to
the intramembranal space of the mitochondrion should have been released. These findings
suggest that sedimented SOD1 mainly derives from cytosol trapped in structures formed by
disruption and closure of abundant neuronal and glial processes existing in the CNS. This
sedimentable material is referred to as “processomes”.

43
To determine the amount of detergent sensitive aggregates spinal cords from mice of different ages (2 days of age until moribund stage of disease) were homogenised in pH 7.0 PBS. Large proportions of the mutant G127X protein were sedimented in mice of all ages by centrifuging at 20 000 g. The amount of processomes were determined in all experiments by analysis of the wild type murine SOD1 in the pellet and the original homogenate. The processomes corresponded to ~4 % at 2 days of age and 15 % of the wild type SOD of the homogenate at 50 days of age and later. These figures were subtracted and it was found that at all ages examined an additional 20% of the mutant G127X SOD1 protein was sedimented. This additional material might represent aggregated mutant G127X SOD1 protein. The real amount of aggregated G127X SOD1 protein is probably even higher since the processomes most likely trapped some aggregated enzyme. The SOD1 immunoreactive complexes forming the 33kDa band were twice as prone to form aggregates and accounted on average for ~60 % of the sedimented material.

In the brain the circumstances were similar. In brains from mice 100 days of age on average ~20 % of the wild type murine SOD1 was pelleted at 20 000 g and additionally ~17% of the mutant G127X SOD1 protein. Similar processed tissue from kidney and liver showed a minimal amount and 20% respectively more of the mutant G127X SOD1 protein was sedimented compared with murine SOD1.

4:1:2:2:6 Detergent resistant aggregates
Brains and spinal cords from mice of different ages were homogenised, centrifuged and analysed in buffer containing 0.1% NP40. This was the lowest concentration found to dissolve or prevent the formation of processomes. The sediments were washed five times in this buffer and for each washing step some aggregated mutant SOD1 was dissolved. The final pelleted material thus showed relatively high detergent resistance.

The content of the final pellet varied over time. For mice up to 150 days of age small but successively increasing amounts of mutant G127X SOD1 was sedimented, and the 33 kDa band were more abundant than the 17 kDa band. The closer terminal illness the mice were the more marked was the accumulation of detergent resistant aggregates. There was a marked increase from 200 days of age and onwards. Similar to the findings in the patients the accumulation was more pronounced in spinal cord than in brain.

Aggregates in the terminal stages of disease were composed of multiple molecular forms and there was extensive high molecular weight smearing. No immunoreactive material were found at the application point or in the stacking gel. No murine SOD1 could be demonstrated by blotting with the mouse specific 131-153 antibody.

Spinal cords from G93A mice were analysed in the same way as G127X mice for comparison. There was a marked accumulation of detergent resistant aggregates in the spinal cords of terminal mice, but not in the brain. The total amount of aggregates in both strains of mice when moribund was similar, ~ 15 μg/g wet weight.
**4:1:2:2:7 Stability**

Since the G127X mutant protein seems to be very labile, there was a possibility that handling of specimens might cause aggregation. In order to evaluate this series of control experiments were performed.

Brains and spinal cords from G127X mice at 100 days of age were cut into pieces, homogenised and analysed by centrifugation, either immediately after the animals had been euthanized or after being kept frozen at -80°C. No differences in amounts of sedimented SOD1 or patterns on immunoblots could be discerned. Neither was any differences found between homogenates immediately or kept at 4°C for 2, 6, 12 and 24 hrs before centrifugation. Freezing of a whole homogenate overnight at -80°C did not alter the distribution of the mutated G127X SOD1 protein. Thus, following disruption of the tissue and dilution in homogenisation buffer, the physical and structural properties of the mutant protein appeared to be relatively stable.

**4:1:2:2:8 Post mortem time effects**

In order to evaluate the effects of post mortem change and time effects pieces of brain from G127X mice were taken care of and handled under conditions mimicking the temperature changes of a dead body; see above under “methods”.

The pieces of brain were homogenised in pH 7.0 PBS without detergent and centrifuged. Both pellet and supernatants were immunoblotted. Half of the mutant enzyme was sedimented from the homogenate of the snap frozen piece; nearly all of the mutant enzyme was sedimented by centrifugation after 6, 12 and 24 hours of storage. No evidence of degradation or other structural alterations were showed in immunoblots of the whole homogenates. Similarly stored and processed pieces of brains from human wtSOD1 mice did not show any alterations.

**4:2 PAPER II**

**4:2:1 Mice**

Two of the founders A134 and A154, who had the highest numbers of inserted copies of the transgene, were used for establishing a colony of D90A transgenic mice. Line 134 is used for experimental purposes. The lines are bred using congenic breeding.

Considering line 134 occasionally hemizygous offspring from hemizygous × hemizygous mating are used for breeding. Homozygous brother-sister matings have not generated any offspring. Neither have attempts of mating a homozygous mouse with a hemizygous or a non-transgenic mouse. It is therefore likely that homozygous D90A line 134 mice have reduced fertility or are unfertile.

Occasionally pups have to be terminated due to consequences of inbreeding, see above. Considering that the D90A line 134 substrain is F31 these issues have been rarely occurring and only a minor discomfort.

None of the F1 generation from A134 or A154 developed any signs of motor neuron disease, even though been kept until high age, more than 800 days. This finding has been confirmed by letting hemizygous mice from subsequent generations live until dying of old age.
Homozygous mice of the 134 line start to show signs of muscle weakness at an age of approximately 350 days. Their capability to extend their hind limbs decrease. When lifted in the tail one of the hind limbs is not stretched out properly, and the weakness progress to the other hind limb within one to two weeks. The progression of paralysis continues and the wasting of muscles is apparent. Another sign of the declining condition is that the mice are no longer able to groom themselves properly and their coats become rough. Just as the patients with the D90A mutation the mice have micturition problems when in terminal stage, and when pressure is applied urine leakage is seen. A distended urinary bladder is commonly found at dissection.

From the first sign of muscular weakness until the stage where the mice no longer could feed themselves the duration was approximately 50 days. The mice were euthanized when they could no longer reach the food in their cages; mean age 404 days ± 53 days.

Both homozygous 154 and mixed 134/154 mice develop similar phenotype but at a later age.

4:2:1:1 Histopathology

4:2:1:1:1 Stereology

Spinal cord ventral horn neurons were counted using the CastGrid system. The rationale behind choosing the thoracic part of the spinal cord is the relative simpleness of delineation. In order to circumvent bias due to phenotypic changes or cell size all neurons were counted.

Regardless of strain all transgenic mice showed substantial loss of neurons at terminal stage/600 days of age. Using 700 day old non transgenic mice as controls the terminal D90A mice lost 40% of their ventral horn neurons at terminal stage and terminal G93A mice 45% of their ventral horn neurons. The 600 day old human wtSOD1 mice had lost 31% of their ventral horn neurons while 600 day old hemizygous D90A mice lost 38% of theirs. Terminal G93A mice had lost most ventral horn neurons of all the examined strains. On the other hand the D90A mice showed the greatest amount of pyknotic nuclei. Pyknotic nuclei were seen already at 100 days of age and were not correlated to neuron loss.

4:2:1:1:2 Vacuolisation

In the D90A homozygous mice vacuoles were seen throughout the ventral neuropil from 200 days of age, and they increased in size and numbers with age.

Vacuolisation and rim SOD1 positivity in the ventral funiculi and roots were seen in 100 day old D90A homozygous mice. These findings indicate that at least some of the ventral neuropil vacuoles derive from swollen motor axons.

D90A hemizygous mice showed vacuoles in the ventral neuropil at 400 days of age, which increased in size and numbers at 600 days of age. However, they always fell short of the occurrence in the homozygous mice. Progressive vacuolisation and rim SOD1 immunoreactivity were seen from 200 days of age and onwards in the ventral roots and ventral funiculi, but not in the dorsal roots.

Human wtSOD1 mice showed vacuolisation of the ventral neuropil at 100 days of age, and it was more pronounced than what was seen in the D90A hemizygous mice from 400 days of
age and onwards. The changes in the ventral funiculi and in the ventral roots were somewhat more pronounced than in the D90A hemizygous mice.

Extensive vacuolisation as well as severe cell loss was seen in the sacral part of the spinal cord of the terminal homozygous D90A mice. The cell loss was seen in the ventral neuropil, and was more pronounced than at corresponding parts of the cervical, thoracic or lumbar parts of the spinal cord. However these findings do not seem to relate to the bladder dysfunctions in these mice, since similar changes also were found in terminal G93A mice. Likewise the sacral spinal cord in 600 day old hemizygous mice and 600 day old human wtSOD1 mice showed SOD1 positive inclusions widespread throughout the ventral neuropil as well as a lesser degree of vacuolisation of ventral roots and ventral neuropil.

4:2:1:3 SOD1 stainings
Diffuse SOD1 staining was seen in motor neuron somata and in the cells of Clarke’s nucleus in homozygous D90A mice, hemizygous mice as well as in human wtSOD1 mice from 50 days of age and onwards. The intensity increased with age. The other cells in the spinal cord did not show any SOD1 staining. These findings were confirmed by using a more diluted solution of the 4-20 antibody. No staining at all was seen in the non-transgenic control mice.

In human wtSOD1 mice the SOD1 specific staining was very scarce at 100 days of age, and thereafter it steadily increased in size and intensity. When the mice were 600 days of age the neuropil staining exceeded the occurrence in the D90A hemizygous mice. The background intensity of the SOD1 specific motor neuron somal staining was similar to what was seen in the D90A hemizygous mice, but more localised staining could be seen.

4:2:1:1:4 SOD1 positive inclusions
In the D90A homozygous mice large SOD1 positive inclusions were seen in the ventral neuropil at 100 days of age, and the number and size increased in frequency with age. At 200 days of age large SOD1 positive inclusions were seen in the motor neurons of these mice, and the inclusions were pronounced at terminal stage.

D90A hemizygous mice showed some defined and inspissated somal SOD1 in motor neurons was seen at 100 days of age and increased with advancing age.

SOD1 positive inclusions were seen in the dorsal roots of the terminal D90A homozygous mice, but not in the 400 day old D90A homozygous mice. This indicates that swollen axons also occur in peripheral neurons.

4:2:1:1:5 Gliosis
Progressive gliosis was seen in the homozygous D90A mice starting already at 50 days of age. Interestingly the hemizygous D90A mice did not show any noteworthy gliosis until 400 days of age.

Human wtSOD1 mice in general showed alterations intermediate to the ones seen in the both zygotic variants of D90A mice. The astrogliosis was seen in the ventral neuropil and in the ventral roots where it was more pronounced that it was in the hemizygous D90A mice.
4:2:1:2 Biochemistry

All ALS associated mutant SOD1s that have been tested have shown to be less stable than the wild type enzyme [114, 115] which indicates that the noxious effects might be linked to the structural instability of the mutants. The stabilities of D90A, human wtSOD1 and murine SOD1 were compared by incubation of guanidinium chloride or at high temperature in presence of DTPA, a chelator. As previously found for the purified human enzyme [100] D90A mutant SOD1 was nearly as stable as human wtSOD1 and both were substantially less stable than murine SOD1.

SOD1 activity was measured in the central nervous system (brain, cerebellum and spinal cord), liver, kidney, heart and skeletal muscles from homozygous D90A mice and compared to non transgenic controls, human wtSOD1 mice and to G93A mice.

The levels in the CNS of the homozygous D90A mice were 6 - 8 times higher than the levels seen in the controls, and somewhat lower than those seen in G93A and human wtSOD1 mice. The levels in the D90A mice were rather constant throughout the entire life span, whereas the human wtSOD1 mice showed a slight increase with advancing age. The SOD1 activity levels in the innards were higher than in the CNS in all the examined transgenic strains, whereas the activity levels of the skeletal muscles were roughly the same.

Using the human specific 24-39 SOD1 antibody the amount of total SOD1 protein was analysed. The amount of active SOD1 was calculated using the measured SOD1 activity and calculating the amount of SOD1 protein from that [100, 116]. Only 20 to 40 % of the human SOD1 protein expressed in murine CNS is enzymatically active, which is less than in the innards and in skeletal muscle.

See table 1 in paper II for a more detailed description of SOD1 activity and protein levels in the mice.

Western blots of homogenates of spinal cords from homozygous D90A mice showed the expected band at the place for the molecular weight of human SOD1. Prolonged exposure revealed a second band with higher molecular weight. The pattern was the same using all the anti human-SOD1 antibodies and consistent with previous findings in other murine models [111]. The high molecular weight SOD1 species was predominantly seen in spinal cord homogenates and increased with age. Similar bands were also found in G93A and human wtSOD1 mice. See figure 2 in paper II.

Mouse pups have relatively seen a higher amount of erythrocytes than older mice since the rate of producing is higher and there is also a remaining pool of erythrocytes with foetal haemoglobin. The mean surviving time for an erythrocyte in mice is 60 days [117]. Proteins are synthesised only during erythropoiesis, but the ability to degrade misfolded proteins remains high in the mature erythrocyte.

Enzyme linked immunosorbent assay used for detecting human SOD1 in murine erythrocytes shows an age dependent decline in the amount of human SOD1, reflecting the stability of protein. The stability is high for the human wtSOD1, nearly similar for D90A but G93A is less stable.

Large amounts of detergent resistant aggregates in terminal mutant SOD1 transgenic mice models have been previously shown [53, 112, 118]. In the spinal cords of D90A mice there is
a marked accumulation of detergent resistant aggregates containing human SOD1 in the monomeric form as well as high molecular weight form. Totally the aggregates account for approximately 3% of the total amount of human SOD1 in the spinal cord of the D90A mice. No similar accumulations were found in the mouse brains. In the hemizygous D90A mice only a minor accumulation of detergent resistant aggregates was seen in the 700 day old mice. The amount of similar aggregates in human wtSOD1 mice were in between the both varieties of D90A mice.

![Fig 4 Detergent resistant aggregates in spinal cords of D90A and human wtSOD1 mice](image)

Murine SOD1 is a structurally more stable protein. When staining the detergent resistant aggregates for murine SOD1 much less age related accumulation was detected in the spinal cords. When the hemizygous D90A mice were 600 days of age murine SOD accounted for 0.06% of the total amount of murine SOD1 in the spinal cord homogenate. The corresponding amount for human wtSOD1 mice at 600 days of age was 0.3%.

**4:3 PAPER III**

Five patients with clinically manifest ALS carrying the D90A SOD1 mutation in homozygotic form underwent full post mortem examination. Central nervous tissue from cervical, thoracic and lumbar spinal cord as well as from predefined areas of brain, brainstem and cerebellum was examined. Tissues from heart, kidney, liver and skeletal muscles were also examined.
4:3:1 Histopathology

Tissues were processed as described in the method’s section and examined by light microscopy.

4:3:1:1 SOD1 positive inclusions

By utilising the previously described antibodies raised against selected peptides in the SOD1 sequence small round inclusions were detected in the spinal cord motor neurons of all the five D90A patients examined. The inclusions measured 0.5 – 3 μm, are homogenous and they were present in the soma of the motor neuron, and in many cells especially prevalent in the axon hillock. Even if only a small proportion of the motor neurons revealed such inclusions, they were seen in all sections from all levels of the spinal cord and in all D90A patients. Compared to SALS and FALS patients without SOD1 mutation, the number of motor neurons with SOD1 immunopositive inclusions was lower. However the number of inclusions in each positive motor neuron was comparable to those seen in the non-SOD1 ALS groups. In Clarke’s neurons small granular SOD1-immunoreactive inclusions were also seen.

Examination of the control patients with other degenerative diseases revealed a few granular SOD1-immunoreactive inclusions in some motor neurons in three of the 27 patients in this group. These three patients had Huntington’s disease; Parkinson’s disease and Alzheimer’s disease respectively. The density of inclusions was lower than in any of the ALS patients in this study.

ALS patients homozygous for the D90A mutation have many small round inclusions immunoreactive for SOD1 in some of their motor neurons, the same type of inclusion which are seen in sporadic ALS patients as well as in ALS patients carrying the G127X SOD1 mutation [68]. Only rarely skein-like TDP-43 immunopositive inclusions were seen in motor neurons of the D90A patients.

No SOD1 inclusions could be seen in cortical neurons of the brain in any of the patients or in any of the peripheral organs examined.

4:3:1:2 Spinal cord

Sections from the spinal cords of all the patients showed an extensive loss of motor neurons, some sections were even without recognisable large neurons in the ventral horn. Likewise the neuropil of the ventral horns was sparse and thin. All patients had Bunina bodies [119] in at least some of the motor neurons.

All the five examined patients showed degeneration of the corticospinal tract and of the dorsal columns.

Staining with TDP-43 immunohistochemistry showed a few motor neurons with Skein-like inclusions, as well as thread-like immunopositivity in the dorsal horn neuropil of three patients. This immunoreactivity was mainly located in the medial border of laminas I-IV and seen at all levels.
4:3:1:3 Brain
Gliosis were present cortically as well as subcortically in all five patients. The areas affected were mainly the ventral cingulated lobe and the insula, where the gliosis was present both in the cortical mantle and in the subcortical white matter. In three of the patients a superficial and mild gliosis was seen in anterior temporal and frontal areas.

We also found gliosis in cortical areas of the frontal and temporal lobes and in these areas as well as in adjacent white matter there were frequent ubiquitin neuropil threads.

A low amount of senile plaques and sparse neurofibrillary tangles was seen in three of the patients. These changes did not qualify for a diagnosis of Alzheimer’s disease [120] in any of the affected patients. Some of these senile plaques were immunopositive for SOD1.

No SOD1 immunopositive inclusions were seen in cortical neurons in any of the patients.

4:3:1:4 Brainstem
Cranial nerve motor neurons in all D90A patients showed the same kind of granular type SOD1 immunopositive inclusions as found in spinal motor neurons. Two of the patients also showed larger SOD1 immunopositive conglomerate inclusions in the pigmented neurons of the caudal dorsal parts of substantia nigra, oculomotor nucleus and in the nucleus ambiguous. The inclusions in the oculomotor motor neurons were intranuclear as well as intracytoplasmatic.

In the inferior olivary nuclei and in the pontine nuclei as well as in a subset of reticular neurons there was a noticeable increase in cystatin C immunoreactivity.

4:3:1:5 Peripheral organs
Sections from liver revealed steatosis in two of the patients, and one of these patients also showed a periportal infiltration of lymphocytes. The patient with steatosis without periportal lymphocytosis and two other patients had accumulation of iron in liver and Kupfer cells. No mutations in the hemochromatosis gene HFE were found when tested.

Muscle tissue was examined by histochemistry, and there was neurogenic atrophy in trunk and body muscles as well as in upper and lower muscles of all the patients. The degeneration was most pronounced in the lower limb muscles.

Sections from hearts and kidneys from all the patients did not reveal anything remarkable. Neither was any SOD1 immunopositive inclusions seen in the investigated peripheral organs.

4:3:2 Biochemistry
4:3:2:1 Western blots
Using the aa4–20, aa57–72, and aa100–115 anti-SOD1 antibodies homogenates of central nervous tissue from brain (temporal lobe and the precentral gyrus) and from the spinal cord (cervical and lumbar ventral horns) of 5 SALS patients, 4 FALS patients and 5 control patients were examined.
The denatured SOD1 monomer was seen as a single band of the appropriate molecular weight in all samples. There was no evidence of smearing or accumulation of SOD1 protein in the loading wells, or were there any other alterations detected with the three antibodies used that could correlate with disease.

### 4:3:2:2 SOD activity

Table 6 SOD1 activities in different tissues from the D90A patients and controls, U/g ww (Means ± SD)

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>D90A PATIENTS</th>
<th>CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal lobe</td>
<td>10,900 ± 540</td>
<td>16,000 ± 2,300</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>11,000 ± 1,000</td>
<td>15,100 ± 2,900</td>
</tr>
<tr>
<td>Precentral lobe</td>
<td>11,500 ± 1,100</td>
<td>14,600 ± 1,300</td>
</tr>
<tr>
<td>Cervical ventral horn</td>
<td>13,600 ± 1,300</td>
<td>11,900 ± 1,500</td>
</tr>
<tr>
<td>Cervical dorsal horn</td>
<td>12,100 ± 1,500</td>
<td>13,900 ± 3,900</td>
</tr>
<tr>
<td>Cervical dorsal funicle</td>
<td>11,900 ± 2,100</td>
<td>10,200 ± 800</td>
</tr>
<tr>
<td>Cervical cortico-spinal tract</td>
<td>12,800 ± 1,900</td>
<td>9,200 ± 900</td>
</tr>
<tr>
<td>Lumbar ventral horn</td>
<td>12,400 ± 600</td>
<td>13,800 ± 1,800</td>
</tr>
<tr>
<td>Lumbar dorsal horn</td>
<td>9,800 ± 2,600</td>
<td>13,600 ± 1,700</td>
</tr>
<tr>
<td>Lumbar dorsal funicle</td>
<td>8,700 ± 1,300</td>
<td>11,700 ± 1,100</td>
</tr>
<tr>
<td>Lumbar cortico-spinal tract</td>
<td>9,500 ± 2,400</td>
<td>12,900 ± 1,100</td>
</tr>
<tr>
<td>Liver</td>
<td>65,100 ± 18,400</td>
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</tbody>
</table>

¹n = 5, except for cervical dorsal horn where n = 3 and lumbar dorsal horn where n = 4.
²n = 5, except for frontal lobe where n = 4 and cervical dorsal horn where n = 3. In control kidney, liver and heart muscle n = 13, 7 and 6 respectively.
SOD activity was measured in eleven different parts of the central nervous system of the five D90A patients and five control patients. There was a slight, but not significant difference in SOD1 activity in almost all parts of the CNS and in the peripheral organs.

No activity difference specific for motor areas could be seen for any of the three SOD isoenzymes. Liver tissue has five times higher SOD1-activity than the CNS. The SOD1 activity is also higher in kidney tissue.

4:4 PAPER IV
SOD1 positivity in spinal cord, brainstem and motor areas of the brain are visible in early ages and increases throughout the life span, as well as the number of SOD1 positive inclusions in motor neurons. Degenerative features as vacuolisation and gliosis precedes phenotypic alterations.

4:4:1 Clinical phenotype of the different strains
The clinical phenotype of the parent strains have been extensively described earlier [48, 51, 83, 121, 122]. Notably is that the low-expressing G93A<sup>di</sup> mice’s mean survival is directly comparable to that of the G127X mice’s.

<table>
<thead>
<tr>
<th>MICE STRAIN</th>
<th>MEAN LIFE SPAN&lt;sup&gt;a&lt;/sup&gt; (DAYS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6JBom non-transgenic&lt;sup&gt;b&lt;/sup&gt;</td>
<td>688 (S.D. ± 57; 589-785; N = 27)</td>
</tr>
<tr>
<td>Hemizygous human wt transgenic&lt;sup&gt;c&lt;/sup&gt;</td>
<td>No ALS symptoms at 672 days</td>
</tr>
<tr>
<td>Hemizygous G85R transgenic</td>
<td>345 (S.D: ± 57; 129-472; N = 66)</td>
</tr>
<tr>
<td>Hemizygous D90A transgenic&lt;sup&gt;b&lt;/sup&gt;</td>
<td>613 (S.D. ± 40; 546-672; N = 41)</td>
</tr>
<tr>
<td>Homozygous D90A transgenic</td>
<td>404 (S.D. ± 53; 294-655; N= 73)</td>
</tr>
<tr>
<td>Hemizygous G93A transgenic</td>
<td>126 (S.D. ± 8.5; 105-140; N = 46)</td>
</tr>
<tr>
<td>Hemizygous G93A&lt;sup&gt;di&lt;/sup&gt; transgenic</td>
<td>255 (S.D. ± 31; 185-396; N = 112)</td>
</tr>
<tr>
<td>Homozygous G127X transgenic</td>
<td>221 (S.D.± 34; 151-333; N = 632)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values refer to mean (standard deviation; minimum-maximum; number of observations).

<sup>b</sup> Values refer to death unrelated to ALS symptoms.

<sup>c</sup> Human wildtype transgenic animals have been harvested at predefined intervals, but have not shown signs of ALS at end the above reported end stage. No survival data is hence reported.

Regardless of mouse strain the initial symptom of motor neuron disease in the mice is paresis of one of the hind limbs. The only exception from that is about a third of the G127X where the initial symptom is paresis of a front limb. Rather soon after the initial paresis of one limb the contra lateral limb is affected, and thus the animal gets a paraparesis.
The usual first observed sign in an affected mouse is failure of extending the hind limbs when it is lifted by the tail. In the G127X mice, the symptomatic phase is between 10 to 30 days, as in the G93A and G85R mice. In the D90A homozygous mice and the G93A<sup>d</sup> the symptomatic phase was approximately double that of the other mice strains. The hemizygous D90A and the human wtSOD1 transgenic strains did not show any signs of paresis. The weakness in the front limbs is harder to detect, and the mice with initial front limb paresis might be detected at a later stage of disease. However, since the front limbs are more crucial for the mice for feeding, it might well represent a shorter duration of disease. Weight loss precedes loss of motor abilities.

### 4:4:2 SOD1 inclusions in spinal motor neurons

Previous studies have shown accumulation of SOD1 inclusions in the D90A, G127X, G93A and G85R strains [51, 83, 111, 121]. In the present study both G93A mice and the homozygous D90A mice already at 100 days of age show vacuolisation in the ventral funiculus, and often these vacuoles are lined by material immunopositive for SOD1. This is accompanied by vacuoles being seen in the ventral horn neuropil in the same mice, indicating axonal damage.

All transgenic strains show accumulation of SOD1 reactive product in spinal motor neurons at this age. The proportion of neurons in the human wtSOD1 mice being less than in the other transgenic strains in which the majority of neurons are labelled. At this stage, this is not accompanied by any increased staining for ubiquitin and UCHL-1 either in motor neurons or in adjacent neuropil of any of the strains. Only in the G93A mice there is an increase in GFAP staining of the corticospinal tract at 100 days of age, while the same is staining is seen at 200 days of age in the G93A<sup>d</sup> and G85R strains, at which age there is also a conspicuous glial limitans staining in these strains.

In most terminal mice, except G127X, SOD1 is also present in axons and in peripheral nerves.

### 4:4:3 Brainstem

Vacuoles are seen in all strains terminally, but only sparse in controls. Accumulation of SOD1 positive inclusions are seen in motor nuclei in all transgenic strains terminally, except in G85R. SOD1 positivity is seen in the white matter in all strains except in G85R and only sparse in terminal D90A hemizygous mice.

Vacuolization are present in terminal D90A homozygous mice, G93A, G85R and human wt SOD mice. Purkinje cells in terminal G93A mice show hyperchromasia. SOD1 positivity is present in purkinjecells of D90A mice from young age, as well as in terminal human wt SOD mice and in G127X mice. In G93A and in G85R mice sparse positivity is seen. Sparse SOD1 positivity is seen in the oldest D90A mice.

### 4:4:4 Cerebellum

Degenerative changes in cerebellum are most pronounced in D90A mice, but hyperchromasia are also found in other strains.
Vacuolization are present in terminal D90A homozygous mice, G93A, G85R and human wt SOD mice. Purkinje cells in terminal G93A mice show hyperchromasia. SOD1 positivity is present in purkinjecells of D90A mice from young age, as well as in terminal human wt SOD mice and in G127X mice. In G93A and in G85R mice sparse positivity is seen. Sparse SOD1 positivity is seen in the oldest D90A mice.

**4:4:5 Cortical changes**

Hyperchromasia is a persistent feature within all transgenic strains, which is more marked with increasing age. Vacuolization in the hippocampal area is found in D90A hemizygous mice at 600 days and in the D90A homozygous mice from 200 days. Vacuolization in the subiculum are seen increasingly in D90A homozygous mice from 200 days, in D90A hemizygous mice from 200 days and in human wtSOD mice at 600 days. Other strains are not affected.

Marked degenerative changes are seen in subiculum and hippocampus in D90A and human wtSOD1 mice, but less in other strains.

**4:4:6 Gliosis**

Gliosis increase with age, and is most marked in the G85R mice.

D90A homozygous mice show gliosis from 50 days of age, hemizygous mice from 400 days of age. Human wtSOD1 mice show gliosis from 100 days of age. G127X show increasing gliosis from 50 days of age and onwards. G93A mice show intermediate amount of gliosis. G85R show the most prominent gliosis.
5: DISCUSSION

5:1 MICE

5:1:1 Importance of genetic background in mice

Mouse breeding in a more planned mode started in the turn of the 19th century. The founding mother of mouse genetics, Abbie CC Lathrop, Granby, Ma ,USA, established a small mouse farm around 1900 where she aimed to breed mice as pets. The mice were sought for as experimental animals by nearby laboratories, and Ms Lathrop expanded her breeding programme. She also conducted studies to evaluate effects of genetic background, inbreeding and pregnancy on incidence of spontaneous tumours.

The mice used as first breeders included North American wild mice caught in Michigan and Vermont; fancy mice from European and American suppliers as well as Japanese “waltzing mice”. The resulting mice breed became the source of many of the classical inbred laboratory mouse strains as C57BL and its substrains; C58; 129; DBA; CBA and BALB and its substrains [72, 123].

An inbred mouse strain is defined as a strain that has been maintained at least 20 generations by mating siblings and thus is homozygous at all genetic loci. Spontaneous mutations might have arisen though. [72, 124] It’s also of great importance to stay with the same provider, since there always is a certain genomic drift within a colony, and thus a variation between different colonies of the same strain of inbred mice.

When changing genetic background to another inbred mouse strain it takes at least ten generations before the strain of origin can be neglected [125].

There are however pitfalls working with mouse models.

Every single inbred mouse strain has traits that seriously must be evaluated before choosing which strain to use both for making the transgene and for backcrossing. Studies comparing common inbred mouse strains have shown differences in for instance learning ability, motor performances and contextual fear-conditioning [123, 126].

Depending on which inbred mouse strain that is chosen for backcrossing the phenotype can vary significantly. It’s well known that the G93A mouse survival is highly influenced on the background. Backcrossing into C57BL/6J background increased survival with almost thirty days (50% survival at 157.1+/-9.3 days) compared to B6SJL background (50% survival at 128.9+/-9.1 days) [80].

5:1:2 Breeding strategies

Continuous inbreeding of littermates (+/+; +/-; -/-) in order to maintain the line might lead to creation of unusual alleles that might interfere with the preferred behavioural phenotype. There is also an increased risk of reproductive failure which might increase over time.

Congenic breeding where transgenic mice are successively backcrossed into an existing inbred mouse strain is a much safer approach. The genetic background will remain stable and the phenotype of the transgene will be consistent.
The founder mouse is mated with for instance a C57BL/6J BomTac mouse and the resulting offspring are genotyped. Offspring carrying the transgene are selected for breeding and mated with C57BL/6J BomTac mice. This principle is used subsequently, and this will give a good supply of hemizygous mice for breeding as well as for experiments. If homozygous mice are wanted hemizygous siblings are mated and they will generate 25% homozygous offspring. After ten generations “the new strain” is genetically uniform and the influence from the strain used for construction of the founder is eliminated.

This backcrossing strategy can be shortened by applying the so called speed congenic breeding. Using this strategy optimal breeder mice are selected by using for instance micro satellite markers to follow modes of inheritance of the desired transgene or chromosomal region of interest [123]. If used consequently five generations of backcrossing can be sufficient for creating a “new strain”.

5:1:3 Survival data
The survival data presented here differs from earlier studies [48, 127]. In these studies both the G93A and the G93A<sup>Δ4</sup> mice lived up to 187 days and 332-354 days respectively. The G85R mice show the same survival times as previously described [51], but the reported front limb onset has not shown in this study.

One possible explanation may be differences in the definition of end stage between different animal facilities and countries. Another important factor is which inbred strain that is use for backcrossing since the phenotype for a transgenic strain may vary due to their genetical background [80].

5:2 COMPARISONS WITH OTHER NEURODEGENERATIVE DISORDERS
Proteins that are involved in familial cases are of importance in sporadic cases of the same disorder. In Alzheimer’s disease, Parkinson’s disease and Creutzfeldt-Jakob disease proteins mutated in familial forms are involved in the pathogenesis of sporadic forms of the diseases.

Newly synthesised proteins are assisted by other proteins such as chaperones to fold properly and arrive to their target receptor. If something is malfunctioning during this way, the protein might get misfolded and aggregate. Inadequate folding may expose hydrophobic amino acids to the surface of the protein and thus make it more sticky and prone to aggregation [128-130].

Misfolded proteins are commonly degraded by the ubiquitin-proteasome system or by lysosome-autophagosome system. Mutations either in the gene encoding for the protein itself or for constituents of the degrading system might be the cause of the earlier onset in familial cases of neurodegenerative disorders [130].

5:2:1 Alzheimer's disease
In the brains of patients with Alzheimer’s disease placques containing β-amyloid as well as tangles containing τ-protein are prevailing findings.

β-amyloid is a cleavage product of amyloid precursor protein, APP. The enzymes β- and γ-secretases can cleave APP and the resulting β-amyloid can vary in size and structure. Both
mutations in the gene coding for APP as well as in the genes coding for the secretases and the accessory protein presenilin 1 and 2 affects the onset and severity of disease.

Similarly mutations in the gene coding for the microtubule associated protein τ affects the onset and outcome of Alzheimer’s disease as well as other forms of taupathies like progressive supranuclear palsy [130, 131].

5:2:2 Parkinson's disease
Lewy bodies are eosinophilic inclusions containing, among many other proteins, α-synuclein are found in Parkinson’s disease. Lewy neurites are similar but filamentous structures. Lewy bodies and Lewy neurites are also found in dementia with Lewy bodies. In multiple system atrophy other inclusions containing α-synuclein as filamentous inclusions in the cytoplasm of the oligodendrocytes and nuclear inclusions are found [130, 131].

5:2:3 Creutzfeld-Jacob’s disease and other prion diseases
Protease resistant subspecies of prion protein, PrP, cause a variety of conditions when accumulated in the brain. These protease resistant species are seen both in sporadic cases of Creutzfeld-Jacob’s disease as well as on familial conditions such as familial Creutzfeld-Jacob’s disease, fatal familial insomnia and Gerstmann-Straussler-Schienker disease. The familial diseases are the result of mutations in the PRNP gene, which codes for PrP.

It is suggested that conformation changes of the prion protein makes it more prone to aggregate and also resistant to proteases normally involved in the metabolism of the prion protein [130, 132, 133].

5:2:3 Polyglutamine disorders
A rather large group of neurodegenerative diseases like Huntington’s disease, Kennedy’s disease and the various forms of spinocerebellar ataxia, SCA, share the occurrence of polyglutamine repeats. Long polyglutamine tracts were shown to easily make protein fibrils in vitro.[134] The longer the polyglutamine repeat, the earlier is the onset of the condition and the more severe it is. Inability of proteolysis and the concomitant accumulation of toxic products are suggested to play a major role in pathology of the polyglutamine disorders [131, 135, 136].

5:3 INCLUSIONS
ALS patients homozygous for the D90A mutation have many small round inclusions immunoreactive for SOD1 in some of their motor neurons, the same type of inclusion which is seen in sporadic ALS patients as well as in ALS patients carrying the G127X SOD1 mutation [68].

Using the specific set of antibodies cytoplasmic inclusions of the mutated enzyme were detected in spinal cord motor neurons [68, 83]. These inclusions were either large, mimicking lewy body-like hyaline inclusions, or small granular inclusions. SOD1 containing inclusions are considered to be hallmarks of ALS caused by mutations in the enzyme [51, 79, 83, 105, 137-139].
Only rarely skein-like TDP-43 immunopositive inclusions were seen in motor neurons of the D90A patients.

In transgenic mice over expressing mutant SOD1 similar SOD1-containing inclusions are found [53, 79, 83, 113, 140, 141] and there is evidence of an early occurring toxicity in the spinal cord [142-145], whereas the main accumulation of inclusions and aggregates is in the terminal symptomatic phase of the disease [53, 79, 113, 140]. Maybe the inclusions are only terminal markers in injured cells.

The lack of detectable SOD1 inclusions in cortical neurons in the D90A patients might be explained by the fact that the inclusions themselves are not toxic, but rather represent a secondary event which is limited to a subset of those cells injured by cytotoxic SOD1 species. The accumulation of granular SOD1 immunopositive inclusions may represent an end-stage process of rather short duration and that in the long survival D90A patients; a smaller proportion of the motor neurons are at this end-stage.

Bunina bodies [119] are eosinophilic intraneuronal inclusions of 2-5 μm size and are considered to be histological hallmarks of ALS. They are considered to be of lysosomal [64, 146] or endoplasmic reticulum [147] derivation. Some authors [61] claim that cystatin C can be found in Bunina bodies.

The small granular inclusions that are labeled by cystatin C which coincides with earlier findings that the small granular inclusions in the SALS and FALS patients without mutations in the SOD1 gene also are positive for cathepsin D, a marker for lysosomes, in double labeling confocal microscopy [68].

Studying SALS and FALS cases without mutations in the SOD1 gene [68] using the same antibodies found small granular inclusions in all patients. Only a few of the control cases showed such inclusions and in a lesser magnitude. SOD1 containing neuronal inclusions may represent a hallmark of ALS in general.

It is generally believed that all SOD1 mutations cause ALS by essentially the same mechanism and thus the type of mutation should not result in different types of nerve cells being affected. This is also supported by the fact that when comparing different SOD1 mutations with respect to SOD1 neuronal inclusions [83, 139], the motor neurons seems to be almost the only cell type affected.

5:4 SOD1 ACTIVITY

The level of SOD1 activity seems to be unrelated to the severity and outcome of the disease. Mice deficient for SOD1 do not develop motor neuron disease [34]. Patients with the D90A mutation show activity levels that are close are to those seen in normal controls. At the same time the mutant SOD1s G85R and G127X appear to lack SOD1 activity in patients and mice.

5:5 SOD1 PROTEIN PROPERTIES

Levels of mutant human SODs in spinal cords vary more than 200-fold in ALS patients carrying different mutations. Thus it is likely that common cytotoxic conformational species
of SOD1 exist in very low concentrations and due to that hard to detect by immunohistochemistry.

In transgenic mice the noxious effect of the mutant human protein shows an evident gene dosage effect. In general hemizygous mice live twice as long as homozygous mice do. The gene expression in the strain correlates with the lifespan, even if the human wtSOD1 and D90A seems to be less noxious than the other mutated SOD1 variants [79].

Table 8 Expression of human SOD1 mRNA and lifespan in transgenic mice.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>percentage of G93A ± SD</th>
<th>Lifespan (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G93A</td>
<td>100%</td>
<td>126 days</td>
</tr>
<tr>
<td>human wtSOD11</td>
<td>60 ± 4%</td>
<td>&gt; 600 days</td>
</tr>
<tr>
<td>D90A</td>
<td>51 ± 8%</td>
<td>404 days</td>
</tr>
<tr>
<td>G93A&lt;sub&gt;dl&lt;/sub&gt;</td>
<td>50 ± 10%</td>
<td>255 days</td>
</tr>
<tr>
<td>G85R</td>
<td>43 ± 6%</td>
<td>345 days</td>
</tr>
<tr>
<td>G127X</td>
<td>63 ± 8%</td>
<td>221 days</td>
</tr>
</tbody>
</table>

Both mutant and human wtSOD1 accumulate in later life stages in the transgenic mice. These accumulations are most pronounced in brain and spinal cord, and only seen in lesser extent in peripheral organs. Mutants lacking the disulphide bond and liganded metal ions seem to be more prone to aggregation [129] which might be due to an increased exposure of hydrophobic amino acids making the protein more “sticky”.

### 5:6 SOD1 SPECIFIC ANTIBODIES

A set of seven primary antibodies were generated against specific amino acid sequences of the SOD1 protein covering more than 80% of the enzyme [68]. Another two which were directed against the truncated G127X mutation were also generated.

These antibodies recognised denatured SOD1 specifically and thus detected the misfolded SOD1 present in the affected cells. Previous studies [105, 137, 138, 148-152] have commonly used antibodies against the whole SOD1 protein which might explain the absence or lower detection rate of SOD1 inclusions.
6. CONCLUDING REMARKS

- All SOD1 mutations probably cause ALS by essentially the same mechanism and thus the type of mutation should not result in different types of nerve cells being affected.

- SOD1 containing neuronal inclusions may represent a hallmark of ALS in general.

- SOD1 may play an important role in all forms of ALS, not only in the familial cases.

- The SOD1 antibodies generated for this study recognised denatured SOD1 specifically and thus detected the misfolded SOD1 present in the affected cells.

- The level of SOD1 activity seems to be unrelated to the severity and outcome of the disease in both humans and mice.

- Selecting the optimal inbred mouse strain is essential for the outcome of transgenic mice studies.

- Both mutant and human wtSOD1 accumulate in later life stages in the transgenic mice. These accumulations are most pronounced in brain and spinal cord.
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8 REFERENCES


