Amyotrophic Lateral Sclerosis – A Study in Transgenic Mice

HANNA WOOTZ
Dissertation presented at Uppsala University to be publicly examined in B41, BMC, Husargatan 3, 75123, Uppsala, Friday, December 15, 2006 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with an incidence of 1.5-2.7/100000 people/year. Today there is no cure for the disease and only symptomatic treatments are available. ALS progresses rapidly and only 50% of the patients are alive three years after the symptom debut. In ALS, the upper and lower motor neurons undergo degeneration in a process resembling apoptosis. This leads to muscle atrophy and paralysis. The causes of neuronal death are however unknown. In this thesis we have studied transgenic mice carrying human mutant superoxide dismutase, as a model for familial ALS. These mice develop ALS-like symptoms after four months of age with degeneration of the motor neurons. Our results show an involvement of endoplasmic reticulum stress, caspase-12, -9, -3 and pro-caspase-7 in the ALS mice spinal cord. Overexpression of the anti-apoptotic protein XIAP in spinal cord neurons inhibited the activation of caspase-12 and reduced caspase-3 and calpain activity. Calpastatin, the regulator of calpain activity, was kept intact in the ALS-XIAP mice. These mice showed a 12% increase in the mean survival suggesting a beneficial effect of XIAP in ALS. The reason for the ultimate cell death of motor neurons in the ALS-XIAP mice may be due to the activation of additional cell death pathways. Thus, we observed that lysosomal proteases particularly, cathepsinB, -D, and -L were activated in the ALS mice spinal cord together with a less marked upregulation of the inhibitors, cystatinB and -C. We also found activation of astrocytes and microglial cells in the spinal cord of ALS mice indicating their involvement in the disease. The results show that both caspase-dependent and -independent pathways are activated during neuronal degeneration in the ALS spinal cord. The results obtained may help to identify novel drug targets for future treatments of ALS.

Keywords: ALS, Caspase, Caspase-12, Cathepsin, Cystatin, ER stress, Motor neuron, Neurodegeneration, Sod1, XIAP

Hanna Wootz, Department of Neuroscience, Neurobiology, Box 587, Uppsala University, SE-75123 Uppsala, Sweden

© Hanna Wootz 2006

ISSN 1651-6206
ISBN 91-554-6730-X
urn:nbn:se:uu:diva-7342 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7342)
The best scientist is open to experience and begins with romance - the idea that anything is possible.
Ray Bradbury
List of papers

This thesis is based on the following papers, referred to in the text by their roman numerals:


Reprints were made with permission from the publishers.

All figures appearing in this thesis are made by Hanna Wootz.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AD</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>AR</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>BIP</td>
<td>Binding protein</td>
</tr>
<tr>
<td>CathB</td>
<td>Cathepsin B</td>
</tr>
<tr>
<td>CathD</td>
<td>Cathepsin D</td>
</tr>
<tr>
<td>CathL</td>
<td>Cathepsin L</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>CystB</td>
<td>Cystatin B</td>
</tr>
<tr>
<td>CystC</td>
<td>Cystatin C</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FALS</td>
<td>Familial ALS</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acid protein</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IPC</td>
<td>Insoluble protein complexes</td>
</tr>
<tr>
<td>m</td>
<td>Mouse</td>
</tr>
<tr>
<td>MN</td>
<td>Motor neuron</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>rb</td>
<td>Rabbit</td>
</tr>
<tr>
<td>RING</td>
<td>Really interested new gene (= protein name)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SOD1</td>
<td>Super oxide dismutase (the gene)</td>
</tr>
<tr>
<td>SOD1</td>
<td>Super oxide dismutase (the protein)</td>
</tr>
<tr>
<td>SALS</td>
<td>Sporadic ALS</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
</tbody>
</table>
Introduction

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease without a cure. Although huge leaps forward towards better understanding and treating ALS have been made, we are still searching for the key triggers of the disease to be able to piece together the last important parts of the puzzle.

History

ALS was first described in 1849 by the French neuropathologist Charcot. Originally it was thought to be a muscular disease since the first visual symptoms usually are weakness in the legs or arms. After findings of degeneration of cells in the ventral horn in the spinal cord, which connect and control muscles, Charcot put the two together and described the characteristics of ALS.

Amyotrophic lateral sclerosis means: a = no, myo = muscle, trophic = nourishment, “no muscle nourishment”. When a muscle has no nourishment it “atrophy”es or wastes away. “Lateral” defines the areas in the spinal cord in which portions of neurons are located that signal to the periphery and control muscles. As the neurons in this area degenerate, it leads to scarring “sclerosis” in the spinal cord.

Epidemiology

The incidence is defined as the number of new cases of a disease or a condition in a specific population over a given time. This is usually expressed as a ratio between the number of affected people and the total population. ALS is a relative rare disease with a worldwide incidence of 1.5-2.7/100000 people/year \(^1\). In Sweden, around 200 people are diagnosed every year. The incidence is basically the same around the world except for a few places, the most famous exception being among the Chamorro population on Guam, an island in the western pacific. Here, the incidence peaked between 1950 and 1960 but has now declined to levels more similar to the rest of the world \(^2\). The decrease in incidence on Guam is likely due to altered exposure to risk factors rather than to changes in the gene pool of the Chamorro population.
based on the relatively short time span. Changes in the gene pool would require a longer time period and several generations to affect the incidence of ALS.

The prevalence is defined as the number of people with a disease or a condition in a specific population at a specific time, usually expressed as a ratio between the number of affected people and the total population. The prevalence of ALS ranges from 2.7-7.4/100000 people $^{1}$.

There are only a few known risk factors for ALS where age and gender are the two most obvious. The incidence increases with age and peaks between 50 and 70 years for women and between 55 and 70 years for men. ALS is somewhat more common in men than in women $^{2}$.

**Clinical features**

The human body contains several different kinds of nerve cells (neurons). The neurons affected in ALS are the motor neurons that facilitate voluntary movements and muscle power. Every muscle is connected to the brain via the spinal cord by long motor neuron axons. A single motor neuron can be attached to several muscle fibers and control their activation. If the muscle performs detailed movements each motor neuron is connected to few muscle fibers to increase movement specificity. In large powerful muscles, a motor neuron is connected to several muscle fibers. When a motor neuron repeatedly becomes excited and the muscle contracts due to calcium influx the connection between the muscle and the motor neuron is strengthened. On the contrary, if a muscle doesn’t receive any input the connection is weakened and the muscle will deteriorate and shrink.

ALS is a fatal progressive neurodegenerative disease which affects both upper motor neurons (projecting from the motor cortex in the brain to the brain stem and spinal cord) and lower motor neurons (connecting the upper ones to the muscle). When a motor neuron dies, adjacent neuron will try to take over its function and connect more muscle fibers. To a certain extent, this is a natural process in the body but in ALS this is an uncontrolled continuous process. The loss of motor neurons will decrease muscle power as well as specificity which leads to clumsy and fumbling movement. Muscles will also start to move in an uncontrolled way which looks like twitches under the skin (fasciculation). Typical for ALS is visible twitches in many different muscles. Extensive loss of motor neurons eventually leads to the inability to control and move muscles, which causes muscle atrophy and eventually paralysis. As the disease progresses, additional muscles become affected, their functions are lost and eventually life supporting muscles such as those of the diaphragm loses their motor neurons and the patient is unable to breathe *(figure 1)*. The lung volume decreases together with the ability to clean the airways through coughs and clearing of the throat. In the end the
patient die due to respiratory failure. Three years after the appearance of the first symptom, half of the patients are still alive\textsuperscript{4}.

The initial symptoms for 80\% of the patients are weakness in one of the extremities, which is called the spinal form of the disease. The initial symptoms for the remaining 20\% are speech and/or swallowing difficulties, called the bulbar form.

There is only one neuroprotective drug approved on the market for treatment of ALS, Riluzole. Riluzole is a glutamate antagonist and has been proved to prolong life in ALS patients with two to three months\textsuperscript{5}. The mode of action of Riluzole will be discussed in more detail below. The patients also often receive pharmacological treatments for secondary symptoms and side effects from Riluzole, such as cramps, plasticity and constipation. Feeding tube and respiration support is mainstay in management of this disorder.

Approximately 10\% of all ALS cases are inherited (familial ALS, FALS) and the remainders of the cases are called sporadic (SALS). What causes the sporadic cases of ALS is unknown but among the familial form a few genes have been identified. These genes do not however account for all FALS cases.

Pathological features

Both upper motor neurons descending from the motor cortex to the brain stem and to the ventral horn of the spinal cord as well as the lower motor neurons connecting the upper ones to muscles deteriorate and die in ALS
patients. This can be visualized by a cresyl violet staining of a transverse section of the ventral horn in the spinal cord (figure 2). In the control, healthy motor neurons are distinctly stained whereas in ALS a loss of a significant number of motor neurons in the ventral horn is visible and only holes (scarring) remain in their place. The few remaining motor neurons in the ALS affected spinal cord are smaller than the control ones.

Figure 2. A cresyl violet staining of ventral spinal cord section from control and transgenic ALS mice at disease end stage. Clear differences in the number and size of motor neurons are seen. Also note the “holes” in the spinal cord of the ALS mice.

Interestingly, in ALS urethral and sphincter functions controlled by Onuf’s nucleus, motor neurons in the oculomotor, trochlear and abducens cranial nerve nuclei are spared 6. This might be due to alterations in the composition of the ion channels in the postsynaptic membrane affecting their permeability. This leads to decreased calcium influx in the postsynaptic neuron (reviewed in 7).

Axonal swellings that are thought to contain disarrayed neurofilaments is a pathological feature of ALS 8. Incorrect assembly of neurofilaments has a large impact on motor neurons because of their large axons (up to one meter long). Components must first be synthesized in the cell body and then transported down the extended axon. Retrograde dynein mediated transport returns components back to the cell body. Accumulation of abnormal neurofilaments leading to slower anterograde axonal transport has been showed to be an early event in an ALS mouse model, already prior to disease onset 9.

In ALS various types of inclusions has been observed. Bunina bodies are perhaps the most typical type of inclusions in ALS, although it should be noted that not all ALS patients have them. Bunina bodies are small (1-2 μm) eosinophilic intraneuronal inclusions in the remaining lower motor neurons which stain positive for cystatin C 10. Other types of inclusions seen in ALS are skein-like inclusions which stain positive for ubiquitin 11 and Lewy body like hyaline inclusion 12 (similar in appearance to Lewy bodies in Parkin-
son’s disease). It is not clear however, if the inclusion bodies are damaging for the cell or if it is a way for the cell to neutralize harmful proteins.

In addition, activation and proliferation of nearby astrocytes and microglia is also a common feature of ALS. Their role in the pathogenesis will be discussed in more detail below.

Pathogenesis

The ALS etiology is largely unknown but several cellular and molecular changes have been described and many hypotheses have been formulated. It is not clear however if these changes are the primary cause of the disease or a secondary effect.

Oxidative stress: A free radical is an atom or molecule with an unpaired electron. Free radicals are naturally formed in the cell during chemical reactions and are highly reactive. Free radicals are often referred to as reactive oxygen species (ROS). In a healthy cell, ROS are neutralized by scavenger enzymes, for example Cu/Zn Superoxide dismutase (SOD1). Mutated SOD1 can cause ALS (see below) and oxidative stress has been observed in ALS. Enhanced oxidative stress and peroxide production can lead to apoptosis of motor neurons. However, it is generally accepted that SOD1 causes ALS through an unknown toxic gain of function and not by a loss of function. This is because ALS is dominantly inherited and SOD1 knock out mice do not develop ALS.

Excitotoxicity: The development of Riluzole is based on observations of abnormal glutamate accumulation in the extracellular space. This is thought to be due to defective glutamate clearance by related transporters. This theory is supported by the observation that ALS mice showed downregulation of the glial glutamate transporter GLT1/EAAT2. In addition, it was observed from a drug screen study that certain antibiotics were able to enhance expression of GLT1/EAAT2 and that administration of these drugs delayed the onset of symptoms in an ALS mouse model. Recently, ALS mice heterozygous for GLT1/EAAT2 were shown to have an increase in the rate of motor decline and earlier motor neuron loss. These results indicate that GLT1/EAAT2 and glial cells play an important role in the pathophysiology of these ALS mice. However, GLT1/EAAT2 null mice do not develop motor neuron disease but this might be explained by their short life span (only six weeks) prematurely terminated by seizures. Riluzole inhibits the release of glutamate from the presynaptic membrane and evidence also exist that Riluzole inhibits NMDA and AMPA receptors on the postsynaptic membrane. One should keep in mind though, that Riluzole only has a slight effect on life span which indicates that the death of motor neurons is caused by more than one stimuli. Treatment with the noncompetitive NMDA receptor antagonist Memantine produces a small but significant increase in life span in an ALS mouse model. Overexpression of one of the AMPA recap-
tor subunits with a particular high permeability to calcium resulted in a more rapid decline in motor performance and a shorter lifespan in ALS mice. Treatment with an AMPA antagonist was found to be neuroprotective in the same mouse model.

Inflammation and neighboring cells: Although ALS is not a classic immunological disorder, inflammation is a feature during its progression. The cerebrospinal fluid (CSF) of ALS patients contains inflammatory mediators and activated microglia is seen close to motor neurons in the spinal cord. Both astrocytes and microglia can produce inflammatory signals such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) which can damage nearby motor neurons. Elevated levels of TNF-α and IL-6 have been observed in CSF and sera from hypoxemic ALS patients. Microglia can also produce neurotrophic and protective factors such as brain derived neurotrophic factor. Moreover, microglia can stimulate astrocytes to remove excitotoxic glutamate during an inflammatory response. Cyclooxygenase-2 (COX2) activity has been shown to be increased in post mortem spinal cord sampled from ALS patients. The COX2 products the prostanoids, are potent mediators of inflammation. Treatment with a COX2 inhibitor prolongs the lifespan of ALS mice. Interestingly, the prostaglandins (a prostanoid) stimulate release of glutamate from astrocytes.

The balance between the neurotoxic and neuroprotective properties determines the fate of nearby neurons. The inflammation in ALS mouse and rat models begins at or before onset of disease which indicates that it is just not a response to dying motor neurons but might be part of the cause. The reason for inflammation is not known. Drugs that can inhibit microglial activation, for example minocycline, delays disease onset and prolongs survival in a mouse model for ALS. Minocycline is currently tested in clinical trials.

Data from studies examine excitotoxicity and inflammation in ALS underlines the importance of communication and interactions between different cell types in ALS (figure 3).
Growth factors: During development, neuronal differentiation and death are dependent of growth factors. The survival of neurons in the adult is also controlled by growth factors. About 10-20 years ago, several reports on the protective effect of growth factors on neurons were published 33,34. These reports started extensive investigation of different growth factors role in animal models of ALS. Clinical trials with ALS patients were also rapidly initiated. Examples of growth factors tested in clinical trials are brain-derived neurotrophic factor, ciliary neurotrophic factor and insulin-like growth factor I, (all reviewed in 35). Despite the massive success growth factors displayed in rescuing motor neurons in animal models and in cell cultures, patients with ALS did unfortunately not respond in the same way. These poor results might be due to problems with administration, transportation, uptake, half-life and dose optimization. A perhaps better explanation is that ALS patients do not seem to have decreased levels of growth factors, on the contrary, several studies have reported unaltered or increased levels in brain, spinal cord and muscles 36,37. The most recent growth factor investigated in ALS is vascular endothelial growth factor (VEGF). VEGF received a lot of attention when it was shown to delay disease onset and prolong survival in rat and mice ALS models, presenting the largest effects seen achieved by protein delivery 38,39. However, in humans VEGF face the same problems as the other growth factors investigated with administration etc.
and with studies showing contradictory results regarding the levels of VEGF in ALS patients.

Environment: Quite a few epidemiological studies have tried to identify environmental factors causing ALS. Several factors have been suggested: exposure to heavy metals, exposure to chemicals, welding, electrical trauma, mechanical trauma, physical activity, smoking and several others, (reviewed in 42). Despite this abundance of studies, the only established risk factors for developing ALS are age, gender and genetic factors.

Genetics and ALS: As mentioned above, 10% of all ALS cases are familial and the genes involved in causing ALS will be discussed in more detail in the next section.

Genetics and ALS

ALS is as far as we know today, generally a sporadic disease which affects individuals all over the world with about the same frequency. However, about 10% of the cases are inherited with an increased incidence in certain families. In most families with increased incidence of ALS, the genetic component causing ALS is not known but in some patient mutations in genes important for the development of ALS has been found. The discovery of genes causing ALS has made it possible for researchers to create animal models to further study ALS in detail.

Superoxide dismutase

A major breakthrough in deciphering the mechanisms behind ALS occurred ten years ago when the copper-zinc superoxide dismutase (SOD1) gene, located on human chromosome 21, was identified as the first ALS associated gene (table 1). Of the familial ALS cases, about 20% carry a mutation in SOD1. This mutation does not have to be located at a specific nucleotide to cause ALS, on the contrary there are more than 100 different SOD1 mutations identified. Most mutations in SOD1 are missence mutations but there are also a few cases of deletion and insertion mutations which result in prematurely terminated polypeptides. The expression of the altered polypeptide is required for the development of ALS. This is confirmed by the SOD1 knock out mouse that does not develop ALS. ALS caused by mutated SOD1 is autosomal dominantly inherited and these two pieces of evidence indicates that altered SOD1 is to possess a toxic gain of function rather than a loss of function. This gain of function is still unknown.

The protein SOD1 is an enzyme containing 153 amino acids enclosing one copper and one zinc ion. SOD1 is predominantly located in the cytoplasm as a homodimer but can also be found in the intermembrane space of
The copper ion can scavenge ROS by reducing superoxide to hydrogen peroxide. So what happens when \( SOD1 \) is mutated? We know that the motor neurons die via a process resembling apoptosis but we do not know what triggers it. Adding to the pathogenesis discussed above, altered SOD1 protein can form aggregates that induce cell death. Protein aggregates are frequently associated with neurodegenerative diseases and are also found in ALS. In vitro studies have shown that altered SOD1 has distorted folding properties compared to wild type and that oxidation of the altered SOD1 induced the formation of large aggregates. Oxidation of wild type SOD1 did not induce the formation of aggregates. It is not clear, however, why aggregates are formed only in motor neurons even though mutant SOD1 is expressed in all cell types.

Another theory is that the cell death in ALS could be initiated by abnormal oxidative chemistry catalyzed by the copper atom bound in the altered SOD1. This theory requires copper to be bound to the protein. Contradictory to this theory, it was found that genetic removal of the copper chaperone that facilitates the transfer of copper into the active site did not protect motor neurons from degeneration or alter disease onset or progression in a transgenic mouse model for familial ALS. This theory is also not supported by the observation that elevation or elimination of wild type SOD1 had no effect on disease symptoms or pathology in vivo.

Interestingly, if mutated SOD1 is expressed only in astrocytes or only in motor neurons, the mice do not develop ALS. This indicates that the ultimate degeneration of motor neurons is dependent on both cell types and their interactions.

The finding of \( SOD1 \) and its involvement in ALS has enabled researchers to create animal models for familial ALS. In this thesis I have worked with the Sod1\(^{G93A}\) model which is a transgenic mice expressing ~20 copies of human mutant \( SOD1 \) where glycine at position 93 is changed to an alanine. The first symptom of the Sod1\(^{G93A}\) mice (also referred to as TG-ALS or ALS mouse) is an impairment of the evoked response tested electromiographically (EMG). This usually appears at two months of age. Thereafter they develop tremors, have shorter stride length and impaired ability to stay on the rotarod. At around 150 days the animals are paralyzed and have lost more than half of their motor neurons. The mitochondria become affected early during the disease progression and this is followed by gliosis and formation of intracellular aggregates (figure 4). As a control, a transgenic mouse expressing human \( SOD1 \) without mutation was used.
Figure 4. Illustration of behavioral and pathological features of the Sod1<sup>G93A</sup> mouse during disease progression. MN=motor neuron

The pathology of the Sod1<sup>G93A</sup> mouse is similar to human ALS patients and the transgenic animals also respond to Riluzole as humans do<sup>50</sup>. Working with transgenic animals instead of humans has the advantages of easy access of material from a greater number of individuals and the possibility to acquire material at different time points during disease progression. It is also possible to use genetic approaches and cross the Sod1<sup>G93A</sup> mouse with other mouse strains with different genetic modifications to obtain a double transgenic mouse which might counteract previous condition. To only examine post mortem material from humans might not reveal what initiates ALS.

Silencing of human SOD1 by using RNAi protects against neurodegeneration, delays onset with 100% and extends survival with 80% in the Sod1<sup>G93A</sup> mouse<sup>51</sup>. These data are the first to show substantial increases but only a very small amount of ALS patients could possibly benefit from this type of treatment.

Even though other genes besides SOD1 have been linked to ALS, mutated SOD1 in transgenic animals is the most studied form. This is due to the close resemblance of the phenotype and pathology between sporadic and familial human cases and between the human ALS cases and the mouse model.

Other genes causing ALS

About 20% of the familial cases are caused by a mutation in SOD1 and an intense search for the genetic cause of the remaining 80% is currently undertaken, with a few new findings. A total of five gene defects have been reported in families with ALS (table 1).
Table 1. Genes that predispose to ALS. AD=autosomal dominant, AR=autosomal recessive.

<table>
<thead>
<tr>
<th>Disease Description</th>
<th>Disease</th>
<th>Gene</th>
<th>Inheritance</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical ALS</td>
<td></td>
<td>Superoxide dismutase (Sod1)</td>
<td>AD</td>
<td>Scavenger enzyme, mainly cytosolic, also in mitochondria</td>
</tr>
<tr>
<td>Juvenile onset</td>
<td></td>
<td>Alsin (ALS2)</td>
<td>AR</td>
<td>GEF signalling, endosome trafficking</td>
</tr>
<tr>
<td>Slowly progressive</td>
<td></td>
<td>VAMP-associated protein B (VAPB)</td>
<td>AD</td>
<td>Golgi vesicle-trafficking protein</td>
</tr>
<tr>
<td>ALS</td>
<td></td>
<td>Senataxin (SETX)</td>
<td>AD</td>
<td>DNA/RNA metabolism</td>
</tr>
<tr>
<td>Vocal cord paralysis</td>
<td></td>
<td>Dynactin (DCTN)</td>
<td>AR</td>
<td>Transport motor</td>
</tr>
</tbody>
</table>

Additionally, several loci have been identified for ALS and many genes modifying the disease have been investigated (reviewed in 52 and 53). I will here focus on genes (other than SOD1) that have been found to be mutated in families with ALS.

Mutated Alsin (ALS2) causes a juvenile onset form of slowly progressive ALS 54,55. Most patients are homozygous for the mutation, indicating that the cause of disease is a loss of function. The function of Alsin is not fully understood but it is involved in endosomal trafficking. The effect of Alsin has been tested in mice and loss of Alsin did not trigger motor neuron degeneration but predisposed the mice to oxidative stress 56 and caused neurological defects and altered vesicle trafficking 57.

VAMP-associated protein B (VAPB) has been found to cause typical ALS 58. A missense mutation in the gene was found in all patients from the same Brazilian family and later in six more families with different diagnosis. Although it is not proven, this suggests that the families have a common ancestor. VAPB normally associates with intracellular membranes such as the ER and the golgi apparatus. Mutated VAPB displays a different location pattern that may abort its function 59.

Defects in senataxin causes a form of ALS with juvenile onset 60,61. Onset of disease is usually before 25 years of age but the disease is progressing slowly and the patients have a normal life span. The function of sentaxin is not known but it has homology to genes that are involved in RNA processing.

The neurofilament network is a key part of the axonal transport system in neurons. A mutation in the gene encoding the p150 subunit of the transporter protein dynactin has been identified in a family with a slow progressing form of ALS 62. Dynactin is a component of the dynein complex that comprises the major axonal retrograde motor and defect transport is an early feature in ALS pathology 9. Interestingly, it has been suggested that mutated SOD1 inhibits the dynein/dynactin complex 63 which would connect mutated SOD1 and dynactin to the same toxic mode of action.
Though Charcot described ALS more than 130 years ago and researchers have gained a lot of knowledge about the pathogenesis, genetics, motor neurons and cellular signaling systems since, the molecular mechanisms behind the disease are still largely unknown. The elucidation of the mechanisms will provide us with new drug targets which can possibly help us to design novel and more effective treatments for the disease.

Apoptosis in ALS

Cells can die by different means of which necrosis and apoptosis are the two most studied ones. Necrosis is usually seen after massive tissue injury and is a result of an overwhelming toxic insult, but compared to apoptosis, it is a rare event. Necrosis is uncontrolled, unintentional and characterized by the swelling and rupturing of the injured cell and the cell content escapes and affects neighboring cells in a negative fashion. Apoptosis, on the other hand, is a controlled programmed cell death which requires the active participation of energy and specific genes, characterized by cell shrinkage, collapse of the cytoskeleton, nuclear envelope breakdown, chromatin condensation and degradation of DNA. Apoptosis has minimal impact on neighboring cells and ultimately the apoptotic cell debris is engulfed by the surrounding cells. During development as well as in adults, apoptosis is crucial. For example; in removing the webbing between the digits of hands and feet of the fetus and in recycling the millions of blood cells that die every minute in a healthy human. Every second, around a hundred thousand cells are produced through mitosis and a similar number die via apoptosis.

In the adult, neurons are not readily replaced; therefore apoptosis is a much less common event. Compared with other cell types, motor neurons are very long cells and therefore complicated to replace. Death of motor neurons will thus result in a loss of function.

Apoptosis can be triggered by different factors and events. One is extrinsic via binding of the Fas ligand to the Fas receptor on the target cell. This can occur if a cell is infected with a virus and an immune cell triggers it to initiate apoptosis. Another way to activate apoptosis is intrinsic and can be triggered by damaged DNA, growth factor withdrawal, energy depletion, overload of ROS or imbalance of the calcium homeostasis. Cells that are excessively stressed can also undergo apoptosis, how this occurs is not well understood. Apoptosis consists of a series of basic events. These involve the mitochondria and also sometimes the endoplasmic reticulum (ER) and the activation of caspases.

During ALS progression, motor neurons are thought to die via apoptosis. This is based on several pieces of evidence discussed more in detail below. It should be noted here that some studies have failed to demonstrate “classical” apoptosis in ALS.
Caspases

At the molecular level, the cell executes a number of controlled ordered events that eventually lead to apoptosis. The key players in these events are a family of cysteine proteases, the so called caspases. A typical caspase contains a prodomain, a large subunit and a small subunit. Caspases are synthesized as inactive molecules and become activated by the removal of the prodomain and then they form a tetramer consisting of two small and two large subunits. Caspases can roughly be divided into two classes, the upstream initiator caspases (caspase-2, -8 and -9) and the downstream effector caspases (caspase-3, -6 and -7). The downstream effector caspases can directly degrade multiple substances such as structural and regulatory proteins.

Since caspases can reside inside the cell inactivated, the source of activation is very important. One way to activate caspases is via adaptor molecules that link apoptotic sensors such as death receptors to the initiator procaspases. Alternatively, the caspase cascade can be initiated in a receptor independent manner by a variety of stimuli, for example chemotherapeutic agents or by cellular stress. The effector caspases can not only become activated by initiator caspases but also by other enzymes such as the cathepsins and calpains. The proapoptotic signals can originate from different locations in the cell, for example the nucleus or organelles such as the mitochondria, the ER, lysosomes or the Golgi complex. Some of the caspases have defined locations in the cells and will respond to apoptotic signals in that specific area. For example, caspase-9 is functionally associated with the mitochondria and caspase-12 is localized to the ER. Even though some caspases have defined location, their signaling pathways are interconnected and sometimes redundant due to overlapping substrate specificity. Because of this, the apoptotic signal can be largely amplified. Once activated, caspases can transactivate other caspases to continue the signaling.

Activation of caspases during different types of central nervous system (CNS) disorders featuring apoptosis has previously been reported. During ALS progression, activation of several caspases has also been observed. Activation of caspase-1 and caspase-3 has been reported in mouse models of ALS. Caspase-1 acts upstream, as an initiator, of the effector caspase-3 and these caspases were shown to be sequentially activated during ALS progression indicating a controlled course of the disease. Upregulation of caspase-7 and activation of caspase-9 and caspase-12 are also features of ALS mice (figure 5). The importance of the caspase family in ALS is supported by the results from a mating between the ALS mouse and a dominant negative caspase-1 mouse. These double transgenic mice had an increased life span with about 7.7%, although onset of disease was not affected. ALS mice treated with the broad caspase inhibitor N-benzyloxycarbonyl-Val-Asp-fluoromethylketone (zVAD-fmk) had a delayed disease onset and prolonged life span, again pointing to an active role of caspases in ALS.
Mitochondria

Ultrastructural studies of muscles and motor neuron synapses in spinal cord from ALS patients show aggregates within the mitochondria \(^\text{76,77}\). In some models with mutant \textit{SOD1}, vacuolization of the mitochondria is an early feature \(^\text{78}\). This, together with the alteration observed in the mitochondrial associated proteins discussed below and the caspases discussed above, indicates an important role for the mitochondria in ALS.

The mitochondrion is a two membrane organelle primary involved in oxidative events to extract energy from food and to conserve as much of it as possible in the form of high-energy compound adenosine triphosphate. SOD1 resides primary in the cytosol but also in the intermembrane space of the mitochondria \(^\text{44}\) where it metabolizes ROS.

During apoptosis cytochrome c, which is normally involved in electron transport, is released from the mitochondria into the cytosol \(^\text{79}\). The released cytochrome c recruits the apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9 to form what is called the apoptosome. This permits cleavage and activation of caspase-9, enabling it to process additional caspases downstream (\textit{figure 5}). The extrinsic (Fas induced) and the intrinsic apoptotic pathways converge at caspase-3.

There is a delicate balance in the cell between pro- and antiapoptotic proteins. The Bcl-2 family proteins include both types and are functionally association with the mitochondria. Proapoptotic proteins of the Bcl-2 family include Bax, Bad, Bim and Bid whereas bcl-2 and bcl-xl are antiapoptotic. The bcl-2 family members are the key regulators of cytochrome c release into the cytosol. Under normal conditions Bax is present in the cytosol but following an apoptotic stimuli, it translocates and binds to the mitochondria. Present at the mitochondria membrane, Bax can promote pore formation and the release of cytochrome c. These actions are counteracted by bcl-2 (reviewed in \(^\text{80}\)). The involvement of the mitochondrial dependent apoptotic pathway in ALS has been verified by experiments showing translocation of the proapoptotic protein Bax from the cytosol to the mitochondria. Release of cytochrome c during disease progression has also been shown \(^\text{81}\). It was demonstrated that both wild type and mutant SOD1 bind the antiapoptotic protein bcl-2, indicating a direct link between SOD1 and apoptotic machinery. Bcl-2 was shown to bind mutant SOD1-containing aggregates in mice and human spinal cord, suggesting a depletion of the antiapoptotic bcl-2 protein \(^\text{82}\). Both spinal cord and muscle samples from ALS patients stain positive for bax and bcl-2 \(^\text{83}\). Activation of Bid has been detected in ALS transgenic mice at the beginning of symptoms \(^\text{84}\).

By crossing the ALS mice with mice lacking or overexpressing different bcl-2 family members, more insights into the role of these proteins in ALS has been obtained. ALS mice overexpressing bcl-2 have delayed disease onset and prolonged survival \(^\text{85}\), however it did not alter disease duration.
ALS mice deficient in the proapoptotic protein Bax also enjoy delayed disease onset and prolonged survival. Bax deletion fails to prevent muscular denervation but rescued the motor neurons from SOD1 mediated death. These studies support the view that bcl-2 family members play an important role in ALS.

**Figure 5.** Illustration of pathways leading to apoptosis starting at the mitochondria and at the ER. Cytochrome C is released from the mitochondria by unknown mechanisms and forms the apoptosome together with Apaf-1 and procaspase-9. This activates caspase-9. Caspase-9 might also be activated by caspase-12. Activated caspase-9 cleaves and activates caspase-3 and -7 which in turn propagate the apoptotic signal.

**Endoplasmic reticulum**

The ER is the primary organelle for synthesizing and folding proteins and storage site for calcium but it is also involved in the cellular response to stress. ER stress can be caused by accumulation of misfolded proteins and alterations in the calcium homeostasis within the ER. The ER can respond to stress through translational attenuation, upregulation of the gene expression for ER chaperones and degradation of unfolded proteins. However, if the function of the ER is severely impaired, cell death pathways are activated (reviewed in 87).

Apaf-1 -/- (null) cells, which are resistant to apoptosis via the cytochrome c pathway, still undergo apoptosis if exposed to the ER stress inducing agents such as thapsigargin (an inhibitor of glycosylation) and brefeldin (an inhibitor of the ER specific calcium ATPase). This indicates that apoptosis can be initiated without mitochondria.
The ER has lately been suggested to be involved in neuronal human diseases such as Parkinson’s disease, Alzheimer’s disease, prion disease, neuronal damage by ischemia and ALS. A common pathological feature of neurodegenerative disorders are deposition of aggregated proteins. Aggregates containing SOD1, so called inclusion bodies, have been detected in ALS patients and transgenic mice but how these proteins aggregate and if the inclusion bodies cause neurotoxicity is not clear. SOD1 has also been shown to aggregate into biochemically distinct insoluble protein complexes (IPC) with high molecular weight that are detectable several months before the inclusion bodies are apparent. These IPCs where later reported to induce ER stress in mutant SOD1 transfected COS7 cells. The IPC were co-localized with the ER which strongly suggest association of the aggregates with the ER.

In paper I we have studied the role of ER stress in ALS mice using different markers. These include the chaperone BiP/Grp78 which is upregulated fairly early during ER stress, and the transcription factor CHOP (C/EBP homologous protein) also known as DNA damage-inducible gene 153 (GADD-153). CHOP is ubiquitously expressed at low levels and is present in the cytosol during normal conditions. However, when the cell responds to ER stress, CHOP expression increases and accumulate in the nucleus to induce gene expression. Mouse embryonic fibroblasts derived from CHOP -/- mice exhibit reduced apoptosis in response to ER stress.

Another marker for ER stress is caspase-12. Caspase-12 deficient mice are resistant to inducers of ER stress, suggesting that caspase-12 is essential in ER stress induced apoptosis. Caspase-9 has in some studies been shown to be a substrate for caspase-12.

X-linked inhibitor of apoptosis protein

In order to survive it is important for the cell to regulate and inhibit apoptosis. Mechanisms therefore exist to tightly regulate the balance between pro- and antiapoptotic proteins. Procaspases and activated caspases are tightly regulated in the cell by endogenous caspase inhibitors.

Naturally occurring caspase inhibitors in the cell include the inhibitor of apoptosis protein (IAP) family. The members of the IAP family contain up to three baculovirus inhibitory repeat (BIR) domains in their amino terminal. Some of the IAPs have a zinc finger-like RING (really interesting new gene) domain near the carboxy terminal. X-linked inhibitor of apoptosis (XIAP) is a member of the IAP family and is the most potent inhibitor of caspases.

XIAP regulates apoptotic cell death by direct caspase inhibition, specifically caspase-3, -7 and -9 (figure 5). XIAP has three BIR regions of which the second together with adjacent linker region, between BIR1 and BIR2, is sufficient to bind caspase-3 and -7. The third BIR mediate caspase-9 inhibi-
bition. It has also been shown that XIAP possesses ubiquitin ligase activity which promotes caspase-3 and -9 degradation \(^{100,101}\).

It was reported that the levels of XIAP mRNA decreased in the spinal cord of Sod1\(^{G93A}\) mice \(^{102,103}\) and that caspase-9 was activated \(^8\) indicating an important role for XIAP in ALS.

**In paper II**, we took advantage of XIAPs proapoptotic properties and studied its effects on ALS by crossing the Sod1\(^{G93A}\) mouse with a mouse that overexpresses XIAP in neurons to create a double transgenic mouse, ALS-XIAP. As a control, we used the Sod1\(^{G93A}\) mouse with normal XIAP expression. In the double transgenic mice we analyzed life span as well as the caspase cascade and calpain and its inhibitor, calpastatin.

**Calpain**

Calpain belongs to a family of cysteine proteases, just like caspases do, and plays a role in cell death. The calpain family has at least six members where the two most studied once are m-calpain and \(\mu\)-calpain. Their names reflect the concentration of calcium required for their activation in vitro, millimolar and micromolar respectively \(^{104}\). Imbalance of the intracellular calcium concentration is a feature during ER stress and can activate the calpains. Activation of calpain has been proposed to contribute to neuronal cell death in different neurodegenerative disorders such as ischemic brain injury \(^{105}\) and Alzheimer’s disease \(^{106}\). Caspase-12 can also be activated during ER stress and it has been shown that calpain can activate caspase-12 \(^{107}\).

Calpain and caspase-3 share a series of different substrates \(^{108}\) and the activity of these two proteases can be investigated by observing the breakdown products of their substrates. Caspase-3 and calpain use different cleavage sites and thus produce products of different sizes. Calpastatin is an endogenous inhibitor of calpains, reviewed in \(^{109}\) and there are reports demonstrating degradation of calpastatin by caspase-3 \(^{110}\). This is interesting because it connects calpastatin, calpain, caspase-12, caspase-3 and XIAP to the same pathway. **In Paper II** we used \(\alpha\)-spectrin which is a substrate for calpain and caspase-3 and analyzed the effects of XIAP on this pathway during ALS.

**Cathepsins and Cystatins**

Cathepsins are lysosomal proteases. They are important in proper proteins turnover and function of the cell \(^{111}\). The activity of cathepsins can be regulated in different ways. Most cathepsins are synthesized as inactive precursor proteins that require cleavage for activation. The pH is also a factor controlling the cathepsin; they are optimally active in the slightly acidic milieu found in lysosomes. Once activated, cathepsins are controlled by cystatins, the endogenous protein inhibitors of lysosomal cysteine proteases.
When first discovered cathepsins were mainly thought of as scavenger enzymes but several knockout animal models have linked them to different genetic disorders. Deficiency of cathepsin B and -L in mice is lethal during the second to forth week of life. Theses mice suffer from massive apoptosis and brain atrophy\textsuperscript{112}. Cathepsin D deficiency has been linked to a human neurodegenerative disease with mental retardation\textsuperscript{113}. There is also evidence for cathepsin B working as a β-secretase in Alzheimer’s disease\textsuperscript{114}. Problems can also be caused by mutations in enzymes inhibiting cathepsins, for example, the cystatins. Cystatin B and -C are endogenous inhibitors of the cysteine cathepsins, such as -B and -L. A mutation in the gene encoding cystatin B has been linked to the hereditary form of myoclonal epilepsy\textsuperscript{115}.

One link between the cathepsins and the apoptotic machinery is the pro-apoptotic protein Bid. Bid was shown to be cleaved by lysosomal extracts and the cleaved Bid could in turn release cytochrome c from the mitochondria\textsuperscript{116}. Thus, cleavage of Bid may represent a mechanism by which cathepsins that have leaked from the lysosomes can facilitate cytochrome c release and subsequent caspase activation. In human fibroblasts, cathepsin D has been shown to act upstream of cytochrome c release and caspase activation when cells were exposed to staurosporine induced apoptosis. Pretreatment with a cathepsin D inhibitor prevented this chain of events\textsuperscript{117}.

In paper I and II we identified molecular mechanisms of apoptosis including ER stress, oxidative damage, and caspase activation. We also noted that inhibition of caspase -3, -7 and -9 only slightly increased the life span of the ALS mice. Based on this, we decided to investigate a different apoptotic pathway including the cathepsins and cystatins and their roles in ALS.

In paper III, we therefore investigated whether the mRNA- and protein levels as well as protein localization of cathepsin B, -D, -L, and cystatin B and -C were changed in ALS mice. We also used the double transgenic ALS mice which overexpress XIAP in neurons, to investigate whether inhibition of caspase-3, -7 and -9 affected the upregulation of cathepsins and cystatins.
Aims of the present study

The overall aim of this thesis was to study ALS in a mouse model to gain more insight into its neuropathological features. More specifically:

**Paper I**
To investigate whether endoplasmic reticulum stress is a feature during ALS progression, visualized by caspase-12 activation and specific mechanisms in the transgenic Sod1<sup>G93A</sup> mice.

**Paper II**
To characterize and study the influence of overexpression of XIAP in spinal neurons on ALS pathology, by generating a double transgenic mouse.

**Paper III**
To investigate whether lysosomal cysteine proteases, the cathepsins, and their inhibitors, the cystatins, are involved in ALS.
Materials and Methods

Animals

Strains and breeding

Transgenic mice expressing human mutant \textit{SOD1} with a point mutation at position 93, glycine changed to alanine (Sod1\textsuperscript{G93A}) (referred to as “TG-ALS” or “ALS” mice in the articles) were used as a model for familial ALS \textsuperscript{118}. As a control, transgenic mice expressing human \textit{SOD1} without point mutation (Sod2Gur) were used (referred to as “control” in the articles). The mice were obtained from Jackson lab, Bar Harbor, Maine, USA.

Transgenic mice overexpressing XIAP in neurons under the \textit{Thy1} promoter \textsuperscript{119} (generated as described in \textsuperscript{120}) were crossed with Sod1\textsuperscript{G93A} and Sod2Gur mice respectively to obtain double transgenic mice (referred to as “ALS-XIAP” and “Control-XIAP” in paper II).

To confirm the genotype of the Sod\textsuperscript{G93A}, Sod2Gur, Sod\textsuperscript{G93A}-XIAP and Sod2Gur-XIAP animals, genomic DNA was extracted from tail biopsies at three weeks of age and analyzed with PCR. For primer sequences and PCR programs, see table 3 and 4.

Approval for animal experiments was obtained from the Local Ethical Committee.

Symptom development and life span

Animals were checked for signs of disease twice a week by holding the animal by the tail and looking for fine tremors and changes in the hind limbs. End point for disease progression is represented by total paralysis of the hind limbs.

The Sod1\textsuperscript{G93A} mice were sacrificed either presymptomatic or at disease end stage and the Sod2Gur mice were sacrificed at the same age. The Sod1\textsuperscript{G93A} animals developed symptoms at five months of age. The Sod1\textsuperscript{G93A} - XIAP mice were sacrificed at disease end point and the Sod2Gur-XIAP mice were sacrificed at the same age.

Spinal cords were frozen immediately upon dissection, and kept at -80°C for protein and DNA analyses.
Protein based assays

Western blotting

Mouse spinal cords were homogenized in ice cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris–HCl, and 0.1% SDS, pH 8.0) supplemented with the protease inhibitor cocktail (Roche, Germany). Protein concentrations were determined by protein assay DC (Bio-Rad, Hercules, CA) and equal amounts of protein (20 μg) were separated on a 12% SDS–PAGE gel by electrophoresis. The proteins were transferred to a nitrocellulose membrane (Serva, Heidelberg, Germany), blocked for 1 h in blocking solution (Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween, 20, and 5% skim milk), and incubated overnight with the appropriate primary antibody (see table 2).

Primary antibodies for cathepsins were a kind gift from E. Weber. The antibodies were raised against affinity-purified enzymes. Characterization of the antibodies was done using ELISA and Western blots and they were all found to be free of crossreactivity with other members of the papain family of cysteine proteinases.

After washing, the membrane was incubated with horseradish peroxidase conjugated secondary antibody (1:2000, Jackson ImmunoResearch Laboratories) followed by detection with the enhanced chemiluminescent (ECL) method. Filters were stripped for 30 min at 40 °C (62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 0.68% v/v β-mercaptoethanol) and reprobed.

Immunohistochemistry

Mice were anesthetized with 0.5ml 2.5% avertin and perfused with 4% paraformaldehyde in phosphatase buffered saline (PBS). Spinal cords were dissected, dehydrated and embedded in paraffin. Five micrometer thick sections were deparaffinized and rehydrated in a series of descending ethanol concentrations. Epitope retrieval was performed by boiling the samples in 0.01M citrate buffer (pH 6) for ten minutes in the microwave. After cooling for 30 minutes the samples were blocked in 4% goat serum for 30 minutes and incubated with primary antibodies over night. After washing three times in PBS, the sections were incubated with appropriate fluorescent Cy2 or Cy3 conjugated secondary antibody (1:200, Jackson Laboratories) and counterstained using 4 μg/ml Hoechst 33342 (Sigma) and finally mounted in Kaiser Glyceringelatine (Merck, Darmstadt, Germany). Sections were analyzed using a Leica Axiovert fluorescent microscope or a Zeiss confocal microscope.

The primary antibody against XIAP was produced in rabbits by immunization as described before 121.
Table 2. Primary antibodies. WB=western blot, IHC=immunohistochemistry, rb=rabbit, m=mouse

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Dilution WB</th>
<th>Dilution IHC</th>
<th>Company</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfa-spectrin</td>
<td>m</td>
<td>1:1000</td>
<td></td>
<td>Chemicon</td>
<td>I, II</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>rb</td>
<td>1:2000</td>
<td></td>
<td>Sigma-Aldrich</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Bip/GRP78</td>
<td>m</td>
<td>1:250 1:300</td>
<td></td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>rb</td>
<td>1:200</td>
<td></td>
<td>Santa Cruz</td>
<td>II</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>rb</td>
<td>1:200</td>
<td></td>
<td>Sigma-Aldrich</td>
<td>II</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>rb</td>
<td>1:1000</td>
<td></td>
<td>Cell Signaling</td>
<td>I</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>m</td>
<td>1:1000</td>
<td></td>
<td>Cell Signaling</td>
<td>I</td>
</tr>
<tr>
<td>Caspase-12</td>
<td>rb</td>
<td>1:1000</td>
<td></td>
<td>Oncogene</td>
<td>I</td>
</tr>
<tr>
<td>Caspase-12</td>
<td>rb</td>
<td>1:1000</td>
<td></td>
<td>Calbiochem</td>
<td>II</td>
</tr>
<tr>
<td>CathepsinD</td>
<td>rb</td>
<td>1:1000 1:200</td>
<td></td>
<td>Kind gift E. Weber</td>
<td>III</td>
</tr>
<tr>
<td>CathepsinL</td>
<td>rb</td>
<td>1:6000 1:200</td>
<td></td>
<td>Kind gift E. Weber</td>
<td>III</td>
</tr>
<tr>
<td>CystatinB</td>
<td>rb</td>
<td>1:200</td>
<td></td>
<td>Biogenes</td>
<td>III</td>
</tr>
<tr>
<td>CystatinC</td>
<td>rb</td>
<td>1:1000 1:200</td>
<td></td>
<td>Upstate Biotechnology</td>
<td>III</td>
</tr>
<tr>
<td>Chop/GADD153</td>
<td>m</td>
<td>1:1000</td>
<td></td>
<td>Santa Cruz Biotech.</td>
<td>I</td>
</tr>
<tr>
<td>cIAP/MIAP</td>
<td>rb</td>
<td>1:1000 1:200</td>
<td></td>
<td>Cell Signaling</td>
<td>II</td>
</tr>
<tr>
<td>GFAP</td>
<td>m</td>
<td>1:200</td>
<td></td>
<td>Sigma</td>
<td>III</td>
</tr>
<tr>
<td>Mac-2</td>
<td>rat</td>
<td>1:100</td>
<td></td>
<td>Cederlane laboratories</td>
<td>III</td>
</tr>
<tr>
<td>Myosin</td>
<td>m</td>
<td>1:1000</td>
<td></td>
<td>Sigma</td>
<td>I,II</td>
</tr>
<tr>
<td>NeuN</td>
<td>m</td>
<td>1:200</td>
<td></td>
<td>Chemicon</td>
<td>II,III</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>rb</td>
<td>1:200</td>
<td></td>
<td>Molecular Probes</td>
<td>I</td>
</tr>
<tr>
<td>XIAP</td>
<td>m</td>
<td>1:2000</td>
<td></td>
<td>BD Biosciences</td>
<td>II</td>
</tr>
<tr>
<td>XIAP</td>
<td>rb</td>
<td>1:1000</td>
<td></td>
<td>Own production</td>
<td>II</td>
</tr>
</tbody>
</table>

Histology

Tissues were embedded and sectioned as described above. Five micrometer thick sections were deparaffinized and rehydrated in a series of descending ethanol concentrations. The sections were pretreated for 15-20 min in potassium disulphite solution before stained for 20 min in staining solution (1.5g cresyl fast violet (sigma) in 98ml water, 1.0ml 1M sodium acetate solution and 1.0ml glacial acetic acid). After washing in acetate buffer and 70% ethanol, sections were dehydrated with a series of ascending ethanol concentrations finishing with xylene. Sections were mounted in Entellan (Merck).
PCR
Genotyping
To confirm the genotype of the Sod1<sup>G93A</sup> mice, Sod2Gur and the cross breedings with the XIAP overexpressing mice, a tail biopsy was removed at three weeks of age. Genomic DNA was extracted by dissolving the biopsy in 0.5ml lysis buffer (50mM Tris-HCl, 10mM EDTA, 100mM NaCl and 1% SDS) supplemented with proteinase K (Roche) for three-four hours in 55°C. An equal volume of phenol (DNA phenol Sigma, pH 7.9) was added and the aqueous phase was transferred to a new tube where the DNA was precipitated with 0.5ml 99.5% ethanol and 3µl 3M sodium acetate. The DNA was pelleted for 5 min, dissolved in 100µl H<sub>2</sub>O and incubated at 37°C for 15 min before the PCR reaction.

Reversed transcriptase real time qPCR
To analyze the presence of transcripts for genes of interest total RNA from frozen spinal cord was prepared from Sod1<sup>G93A</sup> and Sod2Gur mice. In paper I and paper II, the two step PCR method was employed where 5µg RNA were used in the cDNA synthesis with dTT primers according to the manufacturer’s manual (StrataScript First-Strand synthesis system, Stratagene). Real time PCR was performed using the SYBR Green PCR master mix (Applied biosystems, Warrington, UK). Duplicate cDNA samples were used. The result from each sample was normalized against its corresponding β-actin reaction.

In paper III, the one step qPCR method was employed where only the mRNAs of interest are converted into cDNA. One step real time PCR was performed using iScript one-step RT-PCR SYBR green (Bio-Rad). 10ng RNA and 300nM primers were used according to the manufacturer’s manual. Duplicate samples were used.
### Primers and PCR programs

**Table 3. Primers used for PCR and real time qPCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Size (bp)</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ǻ-Actin fwd</td>
<td>5' CTCAACACCCAGCCCATG 3'</td>
<td>68</td>
<td>I</td>
</tr>
<tr>
<td>Ǻ-Actin rev</td>
<td>5' GTGTTAGCAGGAGGCATAC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bip fwd</td>
<td>5' AAGGTAACCCTACAAACAA 3'</td>
<td>135</td>
<td>I</td>
</tr>
<tr>
<td>Bip rev</td>
<td>5' GTCACCTGGAGAATACCATATCTCT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-12 fwd</td>
<td>5' CTCTAAGTGCTGGAGTCTGAGAAACA 3'</td>
<td>113</td>
<td>I</td>
</tr>
<tr>
<td>Caspase-12 rev</td>
<td>5' TCAGCAGTGATCCCTTCCTGG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CathepsinB fwd</td>
<td>5' TGGGTCCTGGTGAGGAGGATA 3'</td>
<td>67</td>
<td>III</td>
</tr>
<tr>
<td>CathepsinB rev</td>
<td>5' CGGGCAGTGGAGCCATTG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CathepsinD fwd</td>
<td>5' TGGGTCCTGGTGAGGAGGATA 3'</td>
<td>68</td>
<td>III</td>
</tr>
<tr>
<td>CathepsinD rev</td>
<td>5' CGGGCAGTGGAGCCATTG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CathepsinL fwd</td>
<td>5' TGGGTCCTGGTGAGGAGGATA 3'</td>
<td>70</td>
<td>III</td>
</tr>
<tr>
<td>CathepsinL rev</td>
<td>5' CGGGCAGTGGAGCCATTG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CystatinB fwd</td>
<td>5' AAGGCAATCTCTTTCAAGAGAGAGAGA 3'</td>
<td>79</td>
<td>III</td>
</tr>
<tr>
<td>CystatinB rev</td>
<td>5' AAGGCAATCTCTTTCAAGAGAGAGAGA 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CystatinC fwd</td>
<td>5' AGCTCAGTGCTGGAGTGAAC 3'</td>
<td>74</td>
<td>III</td>
</tr>
<tr>
<td>CystatinC rev</td>
<td>5' ATGGCTCTGGGAGGAGTGAAC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chop fwd</td>
<td>5' GTCCCTAGCTGCTGGAGCAGAGAGA 3'</td>
<td>55</td>
<td>I</td>
</tr>
<tr>
<td>Chop rev</td>
<td>5' ATGGCTCTGGGAGGAGTGAAC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sod human fwd</td>
<td>5' TGGGTCCTGGTGAGGAGGATA 3'</td>
<td>235</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Sod human rev</td>
<td>5' TGGGTCCTGGTGAGGAGGATA 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thy1 (XIAP) fwd</td>
<td>5' GTATCTCTCTCCACCCAGTA 3'</td>
<td>381</td>
<td>II</td>
</tr>
<tr>
<td>XIAP rev</td>
<td>5' GTATCTCTCTCCACCCAGTA 3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4. PCR programs**

<table>
<thead>
<tr>
<th></th>
<th>Stage1</th>
<th>Stage2</th>
<th>Stage3</th>
<th>Stage4</th>
<th>Stage5</th>
<th>Stage6</th>
<th>Stage7</th>
<th>Stage8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotyping</td>
<td>94°C</td>
<td>94°C</td>
<td>58°C</td>
<td>72°C</td>
<td>33 rep from stage2</td>
<td>72°C</td>
<td>10min</td>
<td>4°C</td>
</tr>
<tr>
<td>PCR</td>
<td>8min</td>
<td>30s</td>
<td>60s</td>
<td>60s</td>
<td></td>
<td>stage2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Real time</td>
<td>95°C</td>
<td>95°C</td>
<td>60°C</td>
<td>40 rep from stage2</td>
<td>95°C</td>
<td>60°C</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>10min</td>
<td>15s</td>
<td>60s</td>
<td>stage2</td>
<td>15s</td>
<td>20s</td>
<td>15s</td>
<td>4°C</td>
</tr>
</tbody>
</table>

During a real time PCR, data was collected once in stage1 and 40 times in stage2 and stage3. Stage6 and stage7 are for the dissociation curve and the ramping time was set to 19min 59s, during which time data was collected. Dissociation curves were analyzed with Dissociation 1.0 (applied biosys-
tems) and only samples with one peak at the correct melting temperature were kept for further analysis.

Statistical analysis

PCR data was analyzed using two tailed Student’s t-test (Excel software). Quantification of western blots was done using the ImageJ program (available on the net http://rsb.info.nih.gov/ij/) and analyzed using Student’s t-test.

When examining life span and survival of the mice in Paper II, The GraphPad Prism 3.0 (GraphPad software Inc., San Diego, USA) was used and data was analyzed with a Kaplan-Meier test.

Error bars indicate standard error of the mean.

*P<0.05, **P<0.01, ***P<0.001
Results and Discussion

Paper I

Spinal cord motor neurons in ALS show characteristics of undergoing apoptosis with the activation of the cell death machinery. Caspases and other proteins involved in apoptosis has been shown to be sequentially activated during disease progression. The mitochondrion plays an important role during apoptosis and apoptotic proteins associated with this organelle has been extensively studied. A decrease in the antiapoptotic protein Bcl-2 and an increase in the proapoptotic protein Bax has been shown in spinal cord from ALS patients. Overexpression of Bcl-2 extends the life of ALS mice. Compared with the mitochondrion much less is known about other organelles and their roles in ALS. In this first paper, we have studied the involvement of the ER in ALS during disease progression using a transgenic mouse model for ALS.

To study factors involved in ALS progression and the degeneration of motor neurons we analyzed the levels of expression and activation of different caspases as well as proteins involved in ER stress and oxidative stress in control, presymptomatic and symptomatic TG-ALS mice.

We observed that caspase-12 was cleaved early during the course of the disease prior to hind limb paralysis. The cleavage of caspase-12 was accompanied by a reduced level of procaspase-12. Analysis of spinal cord mRNA with quantitative PCR showed an upregulation of caspase-12 levels. This indicated a rapid turnover and processing of caspase-12 in TG-ALS animals.

Caspase-12 is normally localized to the ER and its activation is linked to ER stress. We therefore analyzed the induction of the ER specific proteins BiP and CHOP. BiP is an ER specific chaperone which also has been shown to be antiapoptotic and CHOP is a transcription factor involved in ER stress induced gene expression mediating cell death. In contrast to caspase-12, we observed no changes of BiP and CHOP at the protein or mRNA level. This may be due to the transient change of these ER proteins or that the changes are too small to detect using these methods.

To study if caspases other than caspase-12 were activated in TG-ALS mice we analyzed the expression of caspase-3, -7 and -9. Caspase-9 was activated prior to observed symptoms. Recent data support the view that caspase-12 can activate downstream caspases such as caspase-9 independently of apaf-1 and the apoptosome. Caspase-9 can cleave and activate...
caspase-3. The activation of caspase-3 was detected by analyzing the cleavage pattern of alpha-spectrin which is a substrate for caspase-3 \(^{108}\). We observed an increase in caspase-3 activity in the TG-ALS animals. Caspase-3 can cleave the inhibitor of the caspase-activated DNase (ICAD) which leads to DNA and chromatin breakdown \(^{124}\). There are reports on increased DNA breakage in ALS shown by TUNEL labeling in TG-ALS mice \(^{125}\) and in humans \(^{122}\). The cleavage pattern of alpha-spectrin also revealed that the activity of calpain increased in the spinal cord of symptomatic TG-ALS mice. Calpains are activated mainly by elevated intracellular calcium levels \(^{108}\). The calcium levels might change during disease progression due to ER-stress. Caspase-7 was also upregulated but we failed to detect the cleaved form. The role of caspase-7 in TG-ALS mice remains to be investigated.

To study factors leading to apoptosis we explored if oxidative stress was present in the TG-ALS animals. We performed a staining with an antibody against nitrotyrosine, a marker for oxidative stress, and observed a marked increase mainly in large cells characteristic of motor neurons. Elevated levels of nitrotyrosine have previously been reported in TG-ALS mice \(^{126}\) and in humans \(^{14}\). Though studied mainly in cell cultures, it is important to note that enhanced oxidative stress and peroxide production can lead to apoptosis of motor neurons \(^{15}\).

The human homologue to the mouse gene caspase-12 encodes a truncated version of the protein \(^{527}\). Caspase activities similar to that of caspase-12 in mouse have been reported in humans indicating a related course of events \(^{91}\). Our findings of activated caspase-12 and ER stress are important for an increased understanding and might lead to the discovery of novel targets for treating the disease.

**Paper II**

In this work we generated and studied a double transgenic mouse that expressed mutated SOD1 (Sod1\(^{G93A}\)) in conjunction with overexpression of XIAP in spinal neurons (referred to as “ALS-XIAP” in the article). The ALS-XIAP mouse was compared with ALS, control and control-XIAP mice. The expression of XIAP is driven by the neuron specific promoter \(\text{Thy1}\). The \(\text{Thy1}\) promoter is turned on during early postnatal life and no developmental abnormalities were found in these mice \(^{120}\).

We analyzed the expression of XIAP and cIAP (MIAP), a protein belonging to the same protein family, and observed a decrease within the spinal cord of ALS mice compared with control. The decrease was visibly in large motor neurons indicating that the neurons are more vulnerable to insults caused by caspase activation. A decrease in XIAP has previously been noted in ALS mice \(^{103}\). In the ALS-XIAP mouse XIAP was expressed in large mo-
tor neurons and in smaller interneurons. This is important because other cells than motor neurons might also be involved in disease progression.

In paper I, we showed cleavage and activation of caspase-12 in the spinal cord of ALS mice. We found that the cleaved form of caspase-12 present in ALS mice decreased in ALS-XIAP mice. Immunohistochemistry showed colocalization of caspase-12 and XIAP in large neurons in the ventral spinal cord.

Caspase-12 has been suggested to act upstream of caspase-9 possibly independent of the mitochondria ⁹⁷. The mode of activation of caspase-12 in ALS has not been clarified. It has previously been shown that caspase-12 is activated in glial cells after oxygen and glucose deprivation and in cortical neurons treated with amyloid beta peptide and that this activation was blocked by calpain inhibitors ⁹⁶.

To analyze the activity of calpain and caspase-3 we investigated the cleavage pattern of alpha-spectrin which is a substrate for both proteins. We observed marked differences where ALS mice had a clear upregulation of calpain and caspase-3 activity compared to ALS-XIAP which more resembled that of the control mice. This data suggests that the upregulation of calpain and caspase-3 can be counteracted by overexpression of XIAP in the ALS-XIAP animals. How XIAP influences calpain in the spinal cord is however not clear. Calpain can be activated by elevated intracellular calcium levels which may arise during ER-stress or by sustained stimulation of excitatory glutamate receptors ¹²⁸. The inhibition of calpain activity by XIAP might in turn decrease caspase-12 activation. This is supported by the observation that we detected no caspase-12 cleavage in the ALS-XIAP mice. XIAP can directly block caspase-3 in the nervous system shown in XIAP transgenic mice after ischemia ¹²⁰.

Besides calcium, calpain is also regulated by the calpastatin protein. In our model, we found that calpastatin is cleaved in the spinal cord of ALS mice which leads to an inability to control calpain activity. In contrast, in the spinal cord of ALS-XIAP mice calpastatin was not degraded and thus able to inhibit calpain activity. Immunohistochemistry revealed calpastatin in large neurons in the ventral spinal cord. Caspases have been showed to be able to cleave calpastatin ¹¹⁰. A possible mode of action for XIAP to influence calpastatin cleavage would be via caspase-3, -7 and -9. However, it can not be excluded that XIAP influences calpastatin via other mechanisms.

Overexpression of XIAP in spinal neurons increased mean survival by 12%. However it did not completely inhibit the disease progress even though it altered the activation of caspase-12, caspase-3, calpain and calpastatin. This can be due to a too low general overexpression of XIAP or that XIAP only was overexpressed in neurons and not in glial cells. A mouse model that expresses mutated SOD1 only in motor neurons do not develop ALS ⁴⁹. This shows the importance of the interactions between neighboring cells in disease development. Another reason might be that caspases became acti-
vated by a mitochondrial independent pathway not controlled by XIAP. Previous studies on ALS mice with overexpression of XIAP in motor neurons using the ChAT promoter showed an increase of 6% in mean survival \(^\text{74}\). One explanation to why we observed a greater increase in life span might be because XIAP in our study was expressed under the Thy1 promoter that is active in all neuronal subtypes. It is possible that our results are due to a more widespread expression of XIAP. Taken together our results demonstrate the importance of XIAP in ALS shown by the inhibition of caspase-3, caspase-12 and calpain activity as well as breakdown of calpastatin. There was also a significant increase in life span of mice overexpressing XIAP. One could speculate that overexpression of XIAP in other cell types as well as in neurons would have an even greater positive effect on ALS mice.

The pathology of ALS-XIAP mice did not differ significantly from ALS mice at disease end stage. This might be because at disease end stage equal numbers of motor neurons have been lost. Note that in ALS-XIAP mice disease end stage came on average 17.7 days later than in ALS mice. The possible difference in the number of motor neurons might have been reduced during this time.

The present results indicate that blocking of caspase-12, calpain and calpastatin breakdown is not sufficient to hinder disease progression in ALS. This suggests that therapies interfering with cellular caspases could be best considered in combination with other approaches.

**Paper III**

In this study we investigated changes in mRNA-, protein levels and protein localization of cathepsinB, -L, -D, cystatinB and -C in ALS mice. We also studied whether inhibition of caspase-3, -7 and -9 by neuronal overexpression of XIAP affects the activation of the cathepsins and cystatins.

We found that expression of cathepsinB and -L mRNA levels increased about twofold and cathepsinD mRNA levels increased about fivefold in the ALS mice compared with controls. In contrast, cystatinB and cystatinC mRNA levels only increased about 40% and 20% respectively. We then investigated if the observed changes in mRNA levels were accompanied by corresponding changes in protein levels. Western blotting showed increase in all cathepsins and cystatinC compared to controls. We were unable to detect cystatinB with the available antibodies. CathepsinB protein levels started to increase already at the presymptomatic stage. In keeping with the results from the mRNA experiments, cathepsinD protein levels increased the most. The cleaved, or active, form appeared for all cathepsins on the western blots.

Distribution of the cathepsins and cystatins in the ALS mice were studied using immunohistochemistry. All cathepsins appeared within dot like struc-
tures, representing lysosomes in large neurons with morphology of spinal motor neurons. NeuN was used as a neuronal marker. CathepsinB and -L displayed dot like staining in motor neurons in both control and ALS mice. Double staining with GFAP, a marker for astrocytes showed that astrocytes in ALS mice also expressed cathepsinB in the spinal cord. These stainings reveal that the increases seen in mRNA and protein levels are caused by the surviving motor neurons and, in the case of cathepsinB also by astrocytes. For cathepsinD we observed a shift in immunoreactivity with a loss in motor neurons and an increase in astrocytes. These results show that cathepsinB and -D in particular have altered cellular distribution patterns in ALS compared with control.

The cathepsins play an essential role in the cell regulating protein turnover in the lysosomes. Disturbances in this system can cause several different phenotypes. In our experiments, CathepsinB and -D displayed the most prevalent changes. CathepsinB has previously been linked to the regulation of tumor cell death and invasiveness as well as apoptosis. CathepsinB immunoreactivity is reported to be upregulated early after stroke and to colocalize with caspase-1 and -11 which indicates a possible link to caspase activation during apoptosis. Cathepsin activation is also connected to other neurological diseases, such as Alzheimer’s disease in which cathepsinB and -D were found to be upregulated. During oxidative stress, cathepsinD has been shown to be released from the lysosome and translocated to the cytosol. Taken together, the alterations in cathepsin level and distribution might play an essential role in ALS progression.

Immunostaining with specific antibodies for cystatinsB and -C, revealed that cystatinB was located in neurons in control spinal cord and downregulated in neurons in ALS spinal cord. However, small surrounding cells also expressed cystatinB in ALS mice. These cells did not overlap with GFAP but with a marker for activated microglia, Mac-2. These data show that there is an upregulation in cystatinB expression in activated microglia and a downregulation in neurons in ALS spinal cord. CystatinC showed expression in neurons in control mice. In the ALS mice cystatinC accumulated in larger protein aggregates known as Bunina bodies. We also observed expression of cystatinC in GFAP positive cells in ALS spinal cord. These results show that cystatinC has altered distribution patterns in ALS mice compared with controls.

The levels and distribution of cathepsins are regulated by the cystatins, and their altered levels and distribution might therefore have a widespread impact on the cell survival. CystatinC has been shown to be involved in other neurodegenerative diseases. For example, cystatinC colocalizes with amyloid β protein in brains of Alzheimer’s patients. CystatinC gene deletion aggravates brain damage following focal ischemia. We observed an increase in cystatinC protein in ALS samples but some of the cystatinC was found as aggregates and it is therefore not clear if the increase is represented
by active or inactive protein. A cystatinB gene deletion study has revealed cystatinB may have a role in preventing neuronal apoptosis. We speculate that cystatinB may have a protective function in ALS and the decrease in the protein may render the motor neurons more vulnerable to apoptosis. Interestingly, we found cystatinB to be expressed by activated microglia in ALS spinal cord suggesting a role in brain inflammation. However, the exact roles for cystatinB and -C in ALS are so far unknown and deserve further investigation.

To investigate whether activation of caspases affected the cleavage of cathepsinD or the upregulation of cystatinC we analyzed ALS transgenic mice overexpressing XIAP in neurons (ALS-XIAP). XIAP inhibits caspase-3, -7 and -9. We found that inhibition of these caspases by overexpression of XIAP had no effect on the changes seen in protein levels of cathepsinD and cystatinC. This suggests that the alterations seen in cathepsinD and cystatinC occurs independently of caspase-3, -7 and -9, and is dependant of other cellular pathways.

To summarize

In this thesis I have found pronounced changes in the ALS mice compared with control mice. For example; ER stress represented by caspase-12 cleavage and oxidative stress shown with nitrotyrosine staining occur during disease progression. I have also investigated the effects of reducing caspase activation in the ALS mice by overexpression of XIAP and observed positive effects. I have also described changes in mRNA and protein levels as well as alterations in localization among proteins involved in apoptosis which normally reside in the lysosome. These findings all contribute to a better understanding of ALS which can stimulate further research and lead to the development of novel drugs to treat this severe disease.
Future perspectives

After the introduction of the first drug against ALS progression on the market more than ten years ago, no other drug has proved effective against this devastating disease in humans. The significance of Riluzole treatment has also been wildly debated and its effects variable. We still know too little about ALS to arrive at a rational treatment. Several drug screening studies are currently underway however, studies of the molecular mechanisms modulating the disease are crucially important to reveal the underlying causes of ALS.

ALS animal models have been developed and are excellent tools to study the disease. The bcl-2 family proteins have been proved to play a role in ALS and genetic manipulation of some of the family members has lead to an increased lifespan. Detailed information about the actions of the bcl-2 family members during disease progressing might turn out to be crucial for the understanding of ALS. It would therefore be interesting to further characterize the role of the bcl-2 family members in the ALS mice by using both a genetic approach and a pharmacological.

In paper I we observed ER stress in the form of cleaved caspase-12. Cellular pathways leading to cleavage of caspase-12 and its downstream substrates are currently unknown. Paper II shows that overexpression of XIAP inhibits caspase-12 activation. Caspase-12 may escape the ER membrane during cell death and the ER may become the target of some proapoptotic molecule as shown for Bax. The impact of activated caspases and ER stress on disease progression need further investigation.

Since inhibition of caspases (paper II) and overexpression or knock out of certain bcl-2 family molecules had only a slight impact on survival, these therapies might be best considered in combination with each other or with other treatments. In paper III we observed changes in the lysosomal proteins the cathepsins and the cystatins, which became activated during ALS. Upregulation of certain cathepsins and cystatins were not affected by caspase inhibition. Their significance in ALS has not been thoroughly explored and it would be interesting to test how inhibition of one or more of them would affect ALS development and progression. To continue this study, a combination of several drugs inhibiting the apoptotic machinery at different places could be considered. Crucial for the continuation of cathepsin and cystatin inhibition in ALS mice is to examine human ALS material and explore if the same changes occur in humans compared to mice.
In paper III, we also found activated microglia that express cystatinB, which leads us to cell communication and to the microenvironment of the spinal cord. Questions remaining to be answered include the role of astrocytes and microglia in ALS and their impact on the death of motor neurons. Glial cells are involved in metabolizing glutamate and can modulate the excitotoxicity of motor neurons. Increased levels of glutamate in the extracellular space have been reported in ALS patients. The role of glutamate transporters during reuptake, calcium channels that responds to glutamate and the amount of glutamate released from the presynaptic membrane might prove important for the death of the motor neurons.

The ALS mouse model is excellent to use for a genetic approach to better understand this disease. Several mice strains with different parts of the apoptotic pathway knocked out are currently available. Mating between one of these and the ALS mice can reveal mechanisms involved in ALS.

A future perspective is to use the information gathered from the above experiments to facilitate drug discovery. Finally, use the drugs in clinical trails with patients carrying the mutant \textit{SOD1} gene and then investigate if the drug has the same effect on patients with the sporadic form of ALS.
Svensk sammanfattning

Amyotrofisk lateralskleros (ALS) är en neurodegenerativ sjukdom som drabbar motoriska nervceller (motorneuron) i hjärnbarken, hjärnstammen och ryggmärgen. Alla motorneuron är inte drabbade utan endast de som kontrollerar viljestyrda muskler. När dessa motorneuron dör förlorar patienten förmågan att röra musklerna och dessa förvintar med tiden. För ca 80% av patienter startar förlamningen i ben eller armar (spinal form) och för de övriga 20% är de första symptomen tal- och sväljningssvårigheter (bulbär form). Under sjukdomsförloppet drabbar succesivt fler muskelgrupper och tillslut även motorneuron som styr andningsmuskulaturen. Tre år efter symptomdebut har hälften av patienterna avlidit på grund av andningsinsufficiens. Debutålder för ALS är i de flesta fall mellan 50-70 år för kvinnor och 55-70 år för män. Män drabbar ofta än kvinnor. I Sverige drabbar ca 200 personer per år.

Orsaken till ALS är oftast okänd, sk sporadisk ALS (SALS) men ca 10% av fallen har en ärftlig form, sk familjär ALS (FALS). Utav dessa bär ca 20% på en mutation i genen Cu/Zn Superoxid dismutas (SOD1). Hur mutationen i SOD1 orsakar motorneurondöd är inte känd.

Fyndet att muterat SOD1 orsakar ALS har möjliggjort utvecklandet av djurmodeller i vilka sjukdomen kan studeras. I mina arbeten har jag använt mig av en transgen mus som bär på muterat humant SOD1 där glycine på position 93 är utbytt mot alanin, sk Sod1G93A. Denna mus utvecklar ALS-liknande symptom efter ca fyra månader.


I det andra arbetet undersökte vi om inhibering av kaspas-3, -7 och -9 via överuttryck av det anti-apoptotiska proteinet XIAP skyddar mot ALS i Sod1G93A musen. Det har tidigare visats att XIAP är nedreglerat i ALS, detta skulle kunna vara en orsak till kaspasaktiviteten. Vi fann att överuttryck av XIAP i neuron i Sod1G93A musen ledde till minskad aktivering av kaspas-12. Vi såg också minskad aktivitet av kaspas-3 och calpain. Calpain kan aktivera kaspas-12. Överuttryck av XIAP ledde också till minskat nedbrytning av calpastatin, som inhiberar calpain. Sod1G93A mössen som överuttryckte XIAP i neuron levde i snitt 12% längre än de vanliga Sod1G93A mössen. Att vi inte såg en större förändring i livslängd indikerar att andra signalvägar som inte inhiberas av XIAP också är aktiverade i ALS. Det kan också bero på att överuttryck av XIAP endast skedde i neuron och att vi hade uppnått en större effekt om även tex. glialceller överuttryckt XIAP.

I det tredje arbetet ställde vi oss frågan huruvida lysosomala proteiner såsom cystatiner och katepsiner är förändrade i ALS. Katepsiner är enzymer som degraderar andra proteiner i lysosomen. Cystatiner inhiberar vissa katepsiner. Vi undersökte också om överuttryck av XIAP i neuron påverkade dessa lysosomala enzymen. Vi fann att både mRNA och protein nivåer av katepsinB, -D, -L, cystatinB och -C var uppreglerade i Sod1G93A musen. KatepsinB var uppreglerat redan före symptomdebut och katepsinD visade den största förändringen. Western blot visade nära av den aktiva klyvnings-
produkten av alla katepsiner hos Sod1\textsuperscript{G93A} proverna. KatepsinB, -D och -L fanns hos kontrollmössen lokaliseraade i prickformade strukturer i neuron, antagligen lysosomer. Hos Sod1\textsuperscript{G93A} mössen fanns katepsin-B och -L fortfarande i neuron men katepsinB var även närvarande i gliaceller. Uppregleringen hos Sod1\textsuperscript{G93A} mössen vi tidigare observerade på mRNA- och protein-nivå kommer således från fortsatt uttryck hos överlevande neuron och när det gäller katepsinB, även från gliaceller. När det gäller katepsinD såg vi ett förändrat uttrycksmönster med minskade nivåer hos neuron och en ökning extracellulärt.

CystatinB visade sig vara närvarande i neuron i ryggmärgen hos kontrollmöss. Hos Sod1\textsuperscript{G93A} mössen observerade vi minskade mängder i neuron men här fanns cystatinB även i aktiverade mikroglia. CystatinC fanns i neuron hos både kontroll och Sod1\textsuperscript{G93A} möss och även i gliaceller hos Sod1\textsuperscript{G93A} mössen. CystatinC accumulerar i proteinaggregat när sjukdomen fortskridit men det är inte känt om dessa aggregat är skadliga för cellen eller om de är ett sätt att oskadliggöra toxiiska proteiner.

För att studera om de ovan beskrivna förändringarna är kopplade till kaspas-3, -7 och -9 studerade vi Sod1\textsuperscript{G93A} möss som överuttrycker XIAP i neuron. XIAP inhiberar kaspas-3, -7 och -9. Vi fann att XIAP inte påverkar förändringarna av katepsinD eller cystatinC. Detta tyder på att uppregleringen och aktivering av dessa proteiner sker oavsett kaspasaktivitet i cellen och är beroende av andra signalvägar.

I denna avhandling har jag hittat tydliga förändringar av apoptosmekanismer hos ALS musen, Sod1\textsuperscript{G93A}, jämfört med kontrollmöss. Exempel på detta är ER stress, oxidativ stress, aktivering av olika kaspaser och aktivering och distributionsförändringar av lysosomala proteiner. Jag har även genetiskt manipulerat apoptotiska signalvägar genom överuttryck av kaspasinhibitorn XIAP och studerat dess effekter på ALS musen. Dessa nya fynd medverkar till en ökad förståelse av ALS vilket tillsammans med mera forskning kan hjälpa till att utveckla nya terapier för denna svåra sjukdom.
Acknowledgement

This work was carried out at the Department of Neuroscience, unit of Neurobiology at Uppsala University and was supported by Uppsala Universitet, Björklunds foundation, Vetenskapsrådet and Mary, Åke och Hans Ländells stiftelse.

I would like to acknowledge my supervisor and professor Dan Lindholm, for introducing me to the field of neurodegeneration, constant flow of ideas and for the cheerful “Let’s publish!” as soon as I got results!

The neurobiology group members, past and present:
Inga Hansson, for fantastic help in the lab and priceless hands-on tips when working with animals, running encouragement and friskvård!
Håkan Steen, for sharing an office, western blot discussions and for exposing me to real skånska and perhaps improving my ability to understand!? Karin Brännvall, for Friday burgers, drinks on your balcony, laughs and good stories!
Laura Korhonen, for help with various laboratory problems.
Former post docs in our lab, Beat and Rodrigo for helping me to settle in, in the beginning.
All students who have done their projects in our group, especially Maria and Olof.

Fellow PhD students and fika friends:
Ulrika Lönnegren, our own Sverker Olofsson ;-) for PCR discussions, sport events and kantarelljakt!
Lotta Israelsson, for taking time to talk about small and big matters, lively debate at Snerikes and great Valborg!
Per-Henrik Edqvist, easy going always close to jokes and laughs.
Sojeong Ka, for chocolate chip cookies and friendly atmosphere.
Robert Olinski, for adding a polish touch to the lab and for teaming with me in “BMC-stafetten”.
Annika Kyllberg, for enjoyable lunch and fika conversations as well as highlights from bondepraktikan!
Mikael Corell, for a very funny Hajk imitation!
Madeleine Lek and Henrik Boje for spicing up the lunch conversations!
Surrounding group leaders

Håkan Aldskogius my co-supervisor, Klas Kullander for beer sessions and future projects! Ted Ebendal, Finn Hallböök, Elena Kozlova and Åsa Fex Svenningsen for inspiring discussions and for creating our neuro-lab feeling.

Lena Karlsson, Marianne Jonsson and Birgitta Hägerbaum for help with various practical things.

All my super friends!

My wonderful family Pappa, Mamma, Ylva, Tuva, Calle, Magali and Mira for believing in me!

And especially my sweetheart Daniel for everything every day!
References


94. Johnston, J.A., Dalton, M.J., Gurney, M.E. & Kopito, R.R. Formation of high molecular weight complexes of mutant Cu, Zn-


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 205

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-7342

ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA
2006