Transmethylation, Polyamines and Apoptosis in Amyotrophic Lateral Sclerosis

BY

TITTI EKEGREN
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Abstract


Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive disorder characterized by degeneration of motor neurons in the cortex, brainstem and spinal cord. The patients usually die within 3-5 years after onset. The full etiology of ALS is unknown and many hypotheses have been proposed to explain the neurodegeneration. However, basic mechanisms of cellular function such as transmethylation and polyamine metabolism have not been extensively studied in ALS. Transmethylation reactions are very important in the synthesis of substrates such as proteins, neurotransmitters, DNA and RNA. The polyamines, putrescine, spermidine and spermine, are involved in essential functions such as cellular growth, proliferation and differentiation.

An initial study in this thesis concerned the process of neuronal death (apoptosis) in ALS spinal cord. The results showed increased levels of an apoptosis-stimulating protein and increased levels of DNA fragmentation indicative of an apoptotic process in the tissue. A comparative study of MAT-enzyme activity in spinal cord from different mammalian species was undertaken to provide a background for future studies on transmethylation and neurodegeneration. Transmethylation reactions were found altered in erythrocytes from males with ALS but not in spinal cord from ALS patients as compared to controls. An adaptation of previously described polyamine assays was made for the study of polyamines in ALS spinal cord. The method was validated and applied for polyamine analysis in human materials of different characteristics. Determination of polyamines in control and ALS spinal cords showed no major differences. However, in female ALS patients, significantly increased spermidine and spermine levels were observed in ventral horn regions. These gender-related alterations in transmethylation and polyamine metabolism are of interest since there is a male preponderance for the disease.

The lack of major differences in polyamine levels between ALS and control spinal cord suggests a maintained regulation of polyamines at the end stage of this neurodegenerative disease.

Keywords: amyotrophic lateral sclerosis, neurodegeneration, transmethylation, polyamine, apoptosis

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The key to every biological problem must finally be sought in the cell.

_E.B. Wilson_
List of Papers

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

I  

II  

III  

IV  
Ekegren T, Gomes-Trolin C. Determination of polyamines in samples of human brain, spinal cord, CSF, muscle and muscle microdialysate by precolumn derivatization with 9-fluorenylmethyl chloroformate and high performance liquid chromatography. Submitted for publication.

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### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>AdoHcy</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>AdoMet</td>
<td>S-Adenosyl-L-methionine</td>
</tr>
<tr>
<td>AdoMetDC</td>
<td>Decarboxylated AdoMet</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma/leukemia 2</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>FALS</td>
<td>Familial amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>FMOC</td>
<td>9-fluorenylmethyl chloroformate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ICH-1L</td>
<td>Ice and ced-3 homolog, the long protein</td>
</tr>
<tr>
<td>Kir-channels</td>
<td>Inward rectifier K$^+$-channels</td>
</tr>
<tr>
<td>MAT</td>
<td>Methionine adenosyltransferase</td>
</tr>
<tr>
<td>MND</td>
<td>Motor neuron disease</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>ODC</td>
<td>L-ornithine decarboxylase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP nick end labeling</td>
</tr>
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</table>
Introduction

Amyotrophic Lateral Sclerosis

History
In 1853 a patient with progressive motor weakness associated with degeneration of the spinal cord was described by Charles Bell (Swash 2000); similar cases were also presented in lectures by Cruveilhier (Cruveilhier 1852-1853). Previously, progressive muscular atrophy (PMA) had been defined by Aran (Aran 1850) and Duchenne (Duchenne de Boulogne 1849). Duchenne also described the syndrome of progressive bulbar palsy (PBP) in 1860 (Duchenne de Boulogne 1860). The same year reports were made on degeneration of anterior cells in the grey matter region of the spinal cord (Lüys 1860). In 1869 the French neurologist Charcot and his colleague Joffroy brought together these observations and introduced the term "la Sclérose Amyotrophique Latérale"; thus, the first classical description of amyotrophic lateral sclerosis (ALS) was made (Charcot et al. 1869).

The conditions of PMA, ALS and PBP, all characterized by the spectrum of upper and lower motor neuron degeneration, were termed motor neuron diseases (MND) in 1962 (Brain 1962). Only the lower motor neurons are affected in PMA whereas when only upper motor neurons are involved the condition is called primary lateral sclerosis (PLS). In PBP and ALS dysfunction of both upper and lower motor neurons are found. Today the syndromes of PMA and PLS are considered as variants of ALS (Kato et al. 2003; Rowland et al. 2001; Swash 2000).

Clinical features and epidemiology
ALS is a devastating neurodegenerative disorder characterized by degeneration of motor neurons in the cortex, the brain stem and the spinal cord resulting in muscle weakness and muscle atrophy. The disorder is relentlessly progressive and death usually results from respiratory failure within 3-5 years after onset. In about 80% of the ALS cases symptoms start with limb or axial involvement; in about 20% a bulbar onset is found,
leading to difficulties in speaking and swallowing. Patients with bulbar onset show a more rapid progress of the disease (Swash 2000).

The incidence in ALS is estimated to 1 - 3 individuals per 100 000 per year and the prevalence is 4 - 6 per 100 000. The incidence is highest between the ages of 65 to 74 years and quite similar worldwide although there are reports of areas with much higher incidence in the western pacific and Japan (Kato et al. 2003). Most of the ALS cases are believed to be of sporadic origin but in 5 to 10% a familial form (FALS) has been found (Rowland et al. 2001). The clinical and pathological characteristics of both forms of ALS are almost identical. A male preponderance for the disease is reported with a male to female ratio of 3:2 but after ages of 65 - 70 years the ratio becomes 1:1 due to an over-representation of older women with bulbar onset (Kato et al. 2003; Worms 2001).

The diagnosis of ALS is based on clinical and neurophysiological findings. Diagnostic criteria were defined at consensus conferences held in El Escorial, Spain in 1994 (Brooks 1994) and Airlie House, USA in 1998 (Miller et al. 1999). Updated versions can be found at: http://www.wfnals.org/guidelines/1998elescorial/elescorial1998.htm

Pathogenesis

The full etiology of ALS is still unknown and many hypotheses have been proposed to explain the cause and consequence of the selective, progressive and irreversible loss in motor neurons. Since the clinical and pathological characteristics of sporadic and familial ALS are almost identical, extensive research is focused on the mechanisms of copper/zinc superoxide dismutase (SOD1) mutations found mainly in patients with FALS. The use of transgenic mouse models carrying SOD1 mutations has also facilitated the search for pathogenic mechanisms in the neurodegenerative process.

Genetic defects

In the group of patients with FALS, 15-20% presents mutations in the gene in chromosome 21 that encodes the free radical scavenging enzyme SOD1 (EC 1.15.1.1) (Rosen et al. 1993). SOD1 mutations are also observed in 2-3% of patients with apparently sporadic ALS (Cohen et al. 1996; Kato et al. 2003). Today more than 100 mutations have been reported in the SOD1 gene. An updated list of these mutations can be found in the ALS online database (http://www.alsod.org). The main function of the SOD1 enzyme in the cell is to convert superoxide free radicals into hydrogen peroxide. A free radical is any molecule that has one or more unpaired electrons and, because of this, becomes unstable. Under this condition free radicals can attract electrons from neighboring molecules and induce oxidative damage. The mechanism of SOD1 mutations in ALS pathology is not fully understood and researchers were slightly puzzled by findings of normal or near normal
levels of SOD1 enzyme activity in ALS patients carrying the mutation. Furthermore, although transgenic mice with mutant human SOD1 show normal or near normal levels of enzyme activity, they still develop motor neuron degeneration whereas SOD1-knockout mice develop normally (Kato et al. 2003). Mutations in the SOD1 gene do not reduce the enzymatic activity but instead they produce a mutant form of the enzyme with toxic functions in the cell. It has been suggested that mutant SOD1 converts hydrogen peroxide to toxic hydroxyl radicals and that a reaction of the radical peroxynitrite with mutant SOD1 can lead to abnormal tyrosine nitration that could damage proteins, see Figure 1 (Beckman et al. 1993; Cleveland et al. 2001; Yim et al. 1996).

![Figure 1](image.png)

**Figure 1.** Normal (A) and neurotoxic (B and C) functions of the SOD1 enzyme. (A) transformation of free superoxide radicals (O$_2^•$) to hydrogen peroxide (H$_2$O$_2$), (B) conversion of hydrogen peroxide to hydroxyl radicals (OH$^•$) by mutant SOD1, (C) formation of nitronium ions (NO$_2^+$) and nitrotyrosine (NO$_2^{-}$Tyr) from peroxynitrite (ONOO$^-$) catalyzed by mutant SOD1.

Increased levels of free nitrotyrosine have been reported in both sporadic and familial ALS (Beal et al. 1997). The inability of mutant SOD1 to bind zinc may also cause oxidative damage and toxicity when copper and/or zinc ions are released into the cytosol (Kato et al. 2003; Rowland et al. 2001).

Other genetic defects have also been observed in ALS, such as mutations in mitochondrial DNA (Wiedemann et al. 2002) and isolated point mutations in genes coding for the astroglial glutamate transporter (EAAT2) (Honig et al. 2000; Jackson et al. 1999) and the DNA repair enzyme APEX nuclease (Hayward et al. 1999). Mutations in the neurofilament heavy gene seem to be the most reported (Al-Chalabi et al. 1999; Figlewicz et al. 1994), occurring in about 1% of patients with apparently sporadic ALS (Kato et al. 2003). Abnormal neurofilament function will be further discussed below.
Oxidative stress

Oxidative stress refers to an imbalance between the production of free radicals, often referred to as reactive oxygen species (ROS), and the ability of the cell to defend against them. This imbalance results in increased levels of oxidatively modified molecules that can cause cellular dysfunction and cell death of neurons (Simonian et al. 1996).

Markers of oxidative damage to proteins and DNA have been identified in postmortem central nervous system (CNS) tissue from patients with ALS. Biochemical changes such as increased expression and activity of free radical-scavenging enzymes that might implicate a compensatory response to oxidative stress have also been reported (Kato et al. 2003).

Excitotoxicity

Glutamate is the major excitatory neurotransmitter in human CNS. It binds to two classes of receptors in the cell membrane of the motor neuron: the G-protein coupled receptor that controls the intracellular calcium ($\text{Ca}^{2+}$) stores and the glutamate-gated ion channels (N-methyl-D-aspartate, NMDA and $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, AMPA). The NMDA receptor is also permeable for $\text{Ca}^{2+}$.

During normal glutamate neurotransmission, released glutamate travels in the synaptic cleft, binds to glutamate ion receptors on the motor neuron and mediates the stimuli to the cell. The excitatory signal is terminated by reuptake of glutamate by excitatory amino acid transporters (EAATs) (Rowland et al. 2001). An excessive or prolonged activation of the glutamate receptors can promote an influx of sodium and chloride ions that will disrupt the cell potential and cause destabilisation of intracellular $\text{Ca}^{2+}$ homeostasis leading to increased $\text{Ca}^{2+}$ concentrations (Shaw et al. 1997; Simonian et al. 1996). This increased intracellular $\text{Ca}^{2+}$ concentration may trigger a cascade of harmful events in the cell, leading to lipid peroxidation and membrane rupture, nuclear and mitochondrial damage and finally cell death (Louvel et al. 1997).

Glutamate-mediated excitotoxicity in ALS was suggested after reports of increased levels of glutamate in cerebrospinal fluid (CSF) and defects in the glutamate transport system in patients with the sporadic form of the disease (Rothstein et al. 1990, 1992; Shaw et al. 1995). However, normal glutamate levels in CSF from patients with ALS have also been reported (Perry et al. 1990). Investigations in postmortem ALS spinal cord show significantly decreased levels of glutamate as compared to controls (Ono et al. 1999). In the group of patients with FALS the mutant SOD1 is thought to inactivate the glutamate transporter GTL1 and mediate excitotoxic neuronal death (Trotti et al. 1999).

Treatment with riluzole, which inhibits glutamate neurotransmission (Louvel et al. 1997; Shaw et al. 1997; Xu et al. 2001), has been shown to...
improve survival in ALS patients (Bensimon et al. 1994; Lacomblez et al. 1996).

Protein aggregations
Inclusion bodies are often found in postmortem ALS tissue and some characteristic inclusions in sporadic ALS will be mentioned here. Ubiquinated inclusions (Skein-like inclusions) are mainly found in lower motor neurones and consist of filamentous proteins. Bunina bodies, first described in 1962 (Bunina 1962) are small eosinophilic bodies in anterior horn cells. The Hyaline conglomerate inclusions are found in some ALS patients and consist of large aggregates of neurofilaments associated with other cytoplasmic proteins and organelles.

Neurofilament (NF) proteins are components of the neuronal cytoskeleton and have important functions in axonal transport, maintenance of cell shape and the caliber of axons (Rowland et al. 2001; Shaw 2000). Abnormalities in NF protein expression has been observed in ALS and neuronal spheroids containing NF were found more numerous and larger in size in ALS spinal cord as compared to controls (Shaw 2000). Animal studies have shown transgenic mice that overexpress NF proteins to develop massive NF inclusions and clinical MND, resembling human ALS (Cote et al. 1993; Xu et al. 1993). Furthermore, mice overexpressing the human NF heavy gene showed NF accumulations sufficient to induce motor neuron degeneration (Julien et al. 1995). This mutation in the NF heavy gene has also been found in patients with sporadic and familial ALS (Al-Chalabi et al. 1999; Figlewicz et al. 1994).

The ability of the ubiquitin-proteasome system (UPS) to degrade proteins and keep the balance of protein synthesis and degradation is important to normal neuronal function, and alterations in the UPS have been linked to ubiquitylated NF protein aggregations in neurodegenerative disorders (Layfield et al. 2003). Recent studies suggest that mutant SOD1 proteins are, or become, misfolded and prone to form aggregates that are selectively toxic to motor neurons (Bruijn et al. 1998; Rakhit et al. 2002; Valentine et al. 2003). Accumulation of mutant SOD1 has also been proposed to disturb the UPS and thereby reduce the ability to degrade not only damaged proteins but also misfolded SOD1 molecules (Johnston et al. 2000).

Neurotrophic factors
Neurotrophic factors have the ability to prevent neuronal atrophy and death during the development of the nervous system, and are also considered to have essential functions in the adult nervous system (Hefti 1997). It has been assumed that a lack of neurotrophic factors could contribute to motor neuron disease if this coincides with other pathogenic processes (Sendtner 2000). Some neurotrophic factors that support motor neurons are:

• Nerve growth factor (NGF)
• Neurotrophins: brain-derived neurotrophic factor (BDNF), neurotrophins (NT-3, NT-4/5)
• Ciliary neurotrophic factor (CNTF) – leukemia inhibitory factor (LIF) family
• Hepatocyte growth factor (HGF)
• Insulin growth factors (IGF-I, IGF-II)
• Glial-derived neurotrophic factor (GDNF) and related factors such as neurturin (NTR) and persephin

In animal models, factors such as CNTF, BDNF, NT-3 and GDNF, have been shown to influence survival of motor neurons (Ernfors et al. 1995; Henderson et al. 1994; Mitsumoto et al. 1994; Yan et al. 1995).

Recent interest has focused on the vascular endothelial growth factor (VEGF) that besides control of growth and permeability in blood vessels also is proposed to have neuroprotective functions (Skene et al. 2001). A study in mice lacking the ability to respond to tissue hypoxia by VEGF expression showed progressive motor neuron degeneration in the spinal cord of the animals (Oosthuyse et al. 2001). A recent investigation also found increased levels of VEGF in serum but not in spinal cord from patients with ALS (Nygren et al. 2002).

Although clinical trials have been performed with neurotrophic factors such as CNTF, BDNF and IGF-1 in patients with ALS, the results have not shown any significant treatment effects (ALS CNTF Treatment Study Group 1996; Kalra et al. 2003; Lai et al. 1997; The BDNF Study Group 1999).

CNS Inflammation

Autoimmune mechanisms were many years ago proposed in ALS pathology although convincing evidence for the presence of inflammation was never presented. Use of immunosuppresant therapy has not been proved to halt the progression of ALS (Brown et al. 1986; Drachman et al. 1994).

In the last decade inflammatory mechanisms have again been reported in the process of motor neuron degeneration in ALS. Examinations of degenerating white matter from ALS spinal cord showed activated microglia and small numbers of T cells (Lampson et al. 1990). Significant numbers of T cells were later found by Kawamata et al (Kawamata et al. 1992) in spinal cord and brain tissue from ALS patients. The presence of IgG in ALS motor neurons and serum has also been reported (Apostolski et al. 1991; Appel et al. 2000). Furthermore, increased inflammatory mediators have been observed in ALS spinal cord and transgenic SOD1 mice (Almer et al. 2001).

Apoptosis

Apoptosis refers to an active, energy-requiring process of programmed cell death. The release of cytochrome c from mitochondria after an apoptotic stimulus plays a major part in the process of cell death. Released cytochrome
c activates proteolytic caspases that specifically cleave amino acids and trigger the apoptotic process (Rosse et al. 1998).

The morphological characteristics of apoptosis include cell shrinking, cytoplasmic and chromatin condensation, and nuclear fragmentation. These morphological features distinguish apoptosis from necrosis along with the fact that necrosis is not energy dependent (Sathasivam et al. 2001). Many factors are involved in the regulation of the apoptotic pathway, including the death receptor Fas (CD95), caspases, Bcl-2 family of oncoproteins and cytochrome c (Figure 2).

The Fas receptor is a low-affinity neurotrophin receptor known to participate in the death of embryonic motor neurons in primary cultures (Raoul et al. 2002). Caspases are a family of cysteine-aspartate proteases involved in programmed cell death both at upstream initiating levels and downstream at the effector levels. So far, 14 mammalian caspases have been reported.

The Bcl-2 family of oncogenes include both cell death suppressors such as Bcl-2 and Bcl-XL and cell death promoters such as Bax, Bad, Bak and Bcl-xS. The members of the Bcl-2 family are important in the regulation of programmed cell death and are involved in the mitochondria-dependent apoptotic pathway (Guegan et al. 2003). The interaction of the antiapoptotic Bcl-2 (B-cell lymphoma/leukemia 2) and the apoptosis stimulating Bax (Bcl-2-associated X protein) has been widely studied. Bcl-2 has been reported to inhibit glutamate-mediated cell death in neuronal cell populations (Zhong et al. 1993) and also to block cytochrome c release from mitochondria by forming heterodimers with Bax (Howard et al. 2002). The role of Bcl-2 is more complex than just preventing apoptosis by inhibiting the proapoptotic actions of Bax; indeed, reports are made that Bcl-2 helps maintain mitochondrial function and act as an antioxidant (Howard et al. 2002). Measurements of the ratio of Bcl-2:Bax in the cell might give an estimation of the cell vulnerability after an apoptotic stimulus (Korsmeyer et al. 1993).

Altered expressions of bcl-2 and bax mRNA were shown in ALS spinal cord motor neurons by Mu et al (Mu et al. 1996). Furthermore, increased levels of Bcl-2 and apoptosis have been detected in ALS brain tissue surrounding affected motor cortex (Troost et al. 1995a, 1995b). Activation of caspase-9, Bax translocation and release of cytochrome c observed in spinal cord of transgenic SOD1 mice together with findings of cytochrome c translocation in human ALS spinal cord indicate a mitochondria-mediated apoptotic pathway in ALS motor neurons (Guegan et al. 2001). Treatment with minocyclin, an inhibitor of cytochrome c release, was reported to delay progression of the disease in ALS transgenic mice (Zhu et al. 2002). Activation of caspase-1 has also been found in spinal cord samples of both transgenic SOD1 mice and humans with ALS (Li et al. 2000). Other pathways of cell death include the Fas-FADD/caspase-8 cascade (Figure 2) and a study by Raoul et al (Raoul et al. 2002) has shown motor neurons from
transgenic SOD1 mice to be extra sensitive to Fas- or NO-triggered cell death.

Figure 2. Illustration of possible apoptotic pathways. Abbreviations used: FADD (Fas-associated death domain), ASK1 (apoptosis signal-regulating kinase 1), NOS (nitric oxide synthase), NO (nitric oxide), Bcl-2 (B-cell lymphoma/leukemia 2), Bax (Bcl-2-associated X protein).

Studies of the antigen LeY, characteristic of cells undergoing apoptosis and TdT-mediated dUTP nick end labeling (TUNEL) indicating DNA fragmentation, have shown positive results in ALS spinal motor neurons (Yoshiyama et al. 1994). In contrast, an investigation of genes necessary for neuronal apoptosis (c-Jun, JNK/SAPK) and neuroprotection (NF-kappa B) showed no evidence for apoptotic involvement in ALS motor neurons (Migheli et al. 1997). Furthermore, a recent study by Adamek et al (in abstract, Adamek et al. 2003) investigating motor cortex, medulla and spinal cord from patients with ALS found no features of apoptosis in the material and the authors question the role of apoptosis in ALS.

However, a review on apoptosis in ALS (Sathasivam et al. 2001) concluded that there is good evidence for an apoptotic pathway in ALS.
based on morphological studies in postmortem tissue, findings of alterations in Bcl-2 family members and increased expression or activation of caspases in cellular- and tissue-based models of ALS.

Transmethylation reactions

The transmethylation reaction (also known as the one carbon cycle) has three important functions in the cell: (1) it provides S-Adenosyl-L-methionine (AdoMet, SAM, SAMe), the primary methyl group donor in the cell and necessary in the synthesis of the polyamines spermidine and spermine, (2) it triggers the transsulphuration pathway leading to the formation of glutathione, an important antioxidant required for the detoxification of various compounds and for scavenging of free radicals in the cell, and (3) it recycles 5-methyl-tetrahydrofolate into tetrahydrofolate, a necessary cofactor in the synthesis of DNA and RNA (Mato et al. 1997). The one carbon cycle is illustrated in Figure 4.

S-Adenosyl-L-methionine

The structure of AdoMet (Figure 3) was first described in 1953 by Cantoni (Cantoni 1953). AdoMet is synthesized in the cell cytosol and distributed in almost every human tissue and fluid. Most synthesis and degradation of AdoMet occurs in the liver (Lu 2000). It also crosses the blood-brain barrier and slowly accumulates in the CSF (Friedel et al. 1989; Placidi et al. 1977).

![Figure 3. Structure of S-Adenosyl-L-methionine (AdoMet).](image)

AdoMet is the methyl group donor in most transmethylation reactions being involved in the methylation of a variety of substrates including proteins, neurotransmitters, phospholipids, monoamines and nucleic acids. Besides, decarboxylated AdoMet (AdoMetDC) participates in the synthesis of the
putrescine-derived polyamines, spermidine and spermine, by donation of an aminopropyl group (Carney 1986; Lu 2000).

AdoMet is formed from methionine and adenosine triphosphate (ATP) in a reaction catalysed by methionine adenosyltransferase (MAT). The demethylated product of AdoMet, S-adenosylhomocysteine (AdoHcy) is recycled back to methionine and AdoMet via homocysteine and the enzyme methionine synthase (MS) (Mato et al. 1997).

Figure 4. Illustration of some metabolic pathways of the one-carbon cycle and the biosynthesis of polyamines. Abbreviations used: MAT (ATP:L-methionine-S-adenosyltransferase), AdoMet (S-Adenosyl-L-methionine), AdoHcy (S-adenosylhomocysteine), MS (methionine synthase), CH$_3$-THF (5-methyl-tetrahydrofolate), THF (tetrahydrofolate), GSH (glutathione), SO$_4$ (sulphur), MT (methyltransferase enzymes), AdoMetDC (S-Adenosyl-L-methionine decarboxylase), ODC (ornithine decarboxylase), 1 (spermidine synthase), 2 (spermine synthase), 3 (spermidine/spermine N-acetyltransferase), 4 (polyamine oxidase).
Methionine adenosyltransferase

Methionine adenosyltransferase (ATP:L-methionine S-Adenosyltransferase, EC 2.5.1.6, MAT, AdoMet synthetase) is found in all cells and shows a high degree of conservation between species. Mammalian MAT exists in three isoforms, MAT I, MAT II and MAT III. MAT I and MAT III are expressed exclusively in the liver. MAT II is also present in the liver, but represents the predominant form of MAT in tissues such as blood, kidney and the CNS (Kotb et al. 1993). The highest specific activity of MAT is found in the liver where most of the methionine metabolism occurs (Mato et al. 1997).

Abnormalities in transmethylation reactions

The demethylated product of AdoMet, AdoHcy, is a potent inhibitor of transmethylation reactions, and increased AdoHcy levels and decreased AdoMet levels (the AdoMet to AdoHcy ratio) are known to cause abnormalities in the transmethylation cycle (Mato et al. 1997). It is therefore essential for the cell to convert AdoHcy to homocysteine. The intracellular levels of AdoMet depend on methionine availability and the rate of polyamine synthesis (Lu 2000).

Abnormalities in the metabolism of AdoMet and transmethylation have been reported in liver diseases, heart disease, cancer and aging (Friedel et al. 1989; Hoffman 1997; Lu 2000).

Transmethylation reactions in the nervous system

Transmethylation reactions are essential in the CNS and an adequate supply of AdoMet is required for a normal function (Fava et al. 1994; Lu 2000). A protective effect of AdoMet has been suggested in a study of neuronal death induced by transient ischemia in rats (Matsui et al. 1987). AdoMet is also implicated in the process of nerve regeneration (Cestaro 1994). Animal models with mice treated with cycloleucin, an inhibitor of MAT, have shown abnormalities in the CNS of young animals such as myelin vacuolation, and distal motor axonal degeneration in the peripheral nervous system (Lee et al. 1992). Deficiencies in levels of MAT I/III and AdoMet have also been shown in patients with demyelination of the brain and spinal cord (Chamberlin et al. 1996; Surtees et al. 1991). However, a study by Hazelwood et al (Hazelwood et al. 1998) report a case with deficient MAT I/III activity and normal brain myelination.

Altered activities of MAT have been observed in patients suffering from neuropsychiatric and dementia disorders (Gomes Trolin et al. 1995, 1998). Defective transmethylation pathways have also been found in the neurodegenerative disorder Parkinson’s disease (Cheng et al. 1997; Hoffman 1997).
Clinical treatment with AdoMet

AdoMet has been commercially used in Italy in liver disease therapy since 1977. It has also been used for treatment of patients with schizophrenia, depression and Alzheimer’s dementia and shown beneficial results (Bottiglieri et al. 1990, 1994; Carney 1986; Friedel et al. 1989).

Polyamines

The three most common natural polyamines are spermidine (N-(3-aminopropyl)-1,4-diaminobutane), spermine (N,N’-bis(3-aminopropyl)-1,4-diaminobutane) and their precursor putrescine (1,4-diaminobutane). Although putrescine is a diamine it is usually included in the polyamine group. Polyamines are small, water-soluble, aliphatic molecules carrying a positive charge on each nitrogen atom at physiological pH (Morgan 1999; Wallace et al. 1990).

![Figure 5. Molecular and structural formula of the three most common natural polyamines.](image)

The history of polyamines began in 1678 when Anthonii van Leuwenhoek (Leuwenhoek 1678) described crystals formed in human semen, today known as spermine.
Biosynthesis

Putrescine, spermidine and spermine are synthesised in almost every eukaryotic and prokaryotic cell. In mammalian cells and fungi the biosynthesis of polyamines starts with the formation of putrescine from ornithine in a reaction catalyzed by the enzyme L-ornithine decarboxylase (ODC, EC 4.1.1.17).

Ornithine is present in the blood plasma and can also be derived from diet and the urea cycle. In cells lacking a complete urea cycle the first step of the polyamine biosynthesis might be the formation of ornithine from arginase (Morgan 1999). ODC and AdoMetDC are considered the most important enzymes in the biosynthetic pathway of polyamines and are also rate-limiting steps in the polyamine synthesis. ODC can be induced by a variety of stimuli and it has a very short turnover time in mammalian cells. The catalytic activity of ODC is controlled by a natural inhibitor, the antizyme, which stimulates the degradation of ODC and blocks polyamine transport into the cell (Coffino 2001; Suzuki et al. 1994).

Spermidine is synthesised from putrescine through the addition of an aminopropyl group donated from AdoMetDC and spermine is in the same way formed from spermidine by aminopropyl group addition. Spermine and spermidine can be converted back to putrescine through acetylation (the interconversion pathway) (Morgan 1999). The biosynthesis of polyamines is schematically illustrated in Figure 4.

Cellular functions

The positively charged polyamines can electrostatically interact with negatively charged cellular macromolecules and membrane structures, particularly phospholipids, proteins, DNA and RNA. These interactions involve the polyamines in essential cellular functions such as regulation of gene expression, cellular growth, proliferation and differentiation (Heby 1981; Tabor et al. 1984; Thomas et al. 2001). Polyamines can also stabilize ribosomes, membranes and nucleic acids and protect the cell from lipid peroxidation and free radicals (Drolet et al. 1986; Patocka et al. 2000). Furthermore, polyamines have been shown to modulate Ca\(^{2+}\)-flux at the cell membrane, neurotransmitter release and some ion channels (Paschen 1992; Williams 1997b). Polyamines are also studied in the process of apoptosis (Brooks 1995; Schipper et al. 2000).

Modulation of ion channels

The polyamines, in particular spermine, have been reported to block and modulate ion channels such as inward rectifier K\(^+\)-channels (Kir), some types of AMPA and kainate receptors, and the NMDA receptor (Scott et al. 1993; Williams 1997a, 1997b), see Figure 6.
Figure 6. Model illustrating the block and modulation of spermine on ion channels and receptors.

Kir-channels maintain the resting potential of the cell, and in neurons and muscle cells they control the excitability thresholds. Increased concentrations of intracellular polyamines have been found to increase the blockade of the Kir-channels and thereby increase cellular excitability (Williams 1997b). Kir-channels show the highest affinity for spermine which is the most charged polyamine.

Intracellular polyamines can also block some types of Ca\(^{2+}\)-permeable AMPA and kainate receptors. The AMPA/kainate receptors mediate fast excitatory synapses in the mammalian CNS and an overactivation of these receptors has been found to contribute to neuronal death. A decrease in polyamine concentrations would reduce the block of the AMPA/kainate channels and lead to increased cell excitability and increased Ca\(^{2+}\)-influx.

Spermine is thought to have multiple effects on NMDA receptors and is reported to affect the affinity of the receptor for both glycine and glutamate. An excessive release of extracellular polyamines, maybe from injured cells, might overactivate the NMDA receptor and lead to neuronal death (Scott et al. 1993; Williams 1997a, 1997b). Implications are made that the neurotoxic effect of spermine might be dose-dependent and that high concentrations of spermine, on the contrary, could be neuroprotective by blocking the NMDA receptor- and Ca\(^{2+}\)-channels (Ferchmin et al. 2000).
Polyamines found in spider and wasp venoms are suggested to act as neurotoxins by targeting the glutamate neuromuscular junction and paralysing the prey (Scott et al. 1993).

Polyamines and apoptosis
The involvement of polyamines in apoptosis has been extensively studied, and both increased and decreased levels have been reported in apoptotic cells. Significantly increased polyamine levels were found in cells undergoing apoptosis after treatment with hepatocyte growth factor (Yanagawa et al. 1998). Apoptosis was also induced in rodent cells from accumulated levels of putrescine (Xie et al. 1997). In contrast, findings in a murine cell line indicated apoptosis as a result of depletion in intracellular polyamine levels (Nitta et al. 2002).

Cytotoxic products - hydrogen peroxide and aminoaldehydes - produced by oxidation of the polyamines in the interconversion pathway have also been suggested to contribute to apoptosis (Maccarrone et al. 2001; Seiler 2000).

A report by Brooks (Brooks 1995) suggests that both too high and too low levels of polyamines can induce apoptosis by triggering mechanisms that lead to nuclease activity and chromatin condensation. It has also been proposed that polyamines might perform multiple actions in the cell and participate at several levels of the apoptotic process (Schipper et al. 2000).

A recent review by Thomas et al (Thomas et al. 2001) suggests a protective role of polyamines in apoptosis where increased levels of polyamines appear to drive cells into the proliferative pathway.

Bioavailability of polyamines
The intracellular pool of polyamines is maintained by the biosynthesis and interconversion of spermidine and spermine back to putrescine, but also regulated by extracellular uptake, degradation and excretion (Morgan 1999). The uptake is generally low but increases in response to proliferative stimuli such as growth factors, hormones and decreased levels of intracellular polyamines. Significantly increased polyamine uptake has been observed in cells treated with difluoromethyl ornithine (DFMO), a potent inhibitor of ODC and the polyamine synthesis (Cullis et al. 1999; Morgan 1998). The main uptake of polyamines is energy requiring, temperature dependent and Na⁺-activated, suggesting a carrier-mediated transport (Seiler et al. 1990; Urdiales et al. 2001).

The majority of polyamines in the cell are conjugated to other cellular constituents, which implies that only the free pool of polyamine is physiologically active (Morgan 1999; Urdiales et al. 2001).

Polyamines can be derived from diet and are found in a variety of foods and beverages such as meat, fruits, cheese, vegetables and red wine. A daily
polyamine intake for an adult has been estimated to be around 350 - 550 micromol (Bardocz et al. 1995; Deloyer et al. 2001). Studies in rats and humans have shown that dietary polyamines are rapidly taken up from the gut lumen, probably by passive diffusion, and metabolised in the intestinal wall and/or the liver before reaching the systemic circulation (Bardocz et al. 1995; Milovic 2001). It was reported that only 20% of dietary putrescine reaches the systemic circulation (Milovic 2001). In the blood compartment, the erythrocytes play an important role in the uptake, storage and transport of polyamines (Moulinoux et al. 1984; Seiler 1991). Circulating polyamines may also be released from various organs through excretion or as a consequence of cell death (Seiler 1990).

Polyamines can cross the blood-brain barrier. Although some studies report the transport to be limited and restricted (Carter 1994; Seiler 2000) other investigations have shown that proteins covalently bound to polyamines get increased permeability over the blood-brain barrier (Poduslo et al. 1998).

Distribution of polyamines

In humans, high levels of polyamines are found in the early stages of the development of the CNS (10 - 15th week of gestation) and the levels gradually decrease during the later stages (Chaudhuri et al. 1983). It has also been reported that higher concentrations of polyamines are found in the early development of the rat brain (Seiler et al. 1975a).

Polyamine levels in blood plasma, urine and CSF are low (Seiler 1977). However, mature tissues with highly active protein synthesis, such as prostate gland and pancreas, show high concentrations of polyamines (Williams-Ashman et al. 1979). Rapidly growing cells and tumors also require higher polyamine concentrations than normal cells (Heby 1981; Janne et al. 1978; Weiss et al. 2002).

The effect of aging on polyamine levels studied in human brain has shown slowly decreasing concentrations of spermidine after the fifth decade of life, but no significant changes in levels of putrescine and spermine (Morrison 1995a; Vivò 2001). Decreasing levels of putrescine and spermidine were found in CSF from newborn children to the age of 1 year and thereafter the concentrations remained stable, although at much higher levels than concentrations found in adult CSF (Albright et al. 1983).

Polyamines in the central nervous system

Polyamines have been detected in the CNS of all kind of mammalian species (Chaudhuri et al. 1983; Morrison et al. 1995a; Seiler et al. 1975b; Shaw et al. 1973). They have been localized both in neurons and glial cells (Carter 1994). There have been reports showing that spermidine is the most
expressed polyamine in regions rich in white matter in human brain (Morrison et al. 1995a; Shaw et al. 1973) and in brain and spinal cord from rats (Seiler et al. 1975a; Virgili et al. 2001).

It has been suggested that spermidine and spermine can be cleared from CSF by an active transport system in the choroid plexus (Halliday et al. 1978). The ODC activity is low in normal adult brain and levels of putrescine have been estimated to only 2 - 3 percent of spermidine concentrations in mammals (Shaw 1979).

The high levels of polyamines, in particular of putrescine in developing spinal cord, indicate their involvement in nucleic acid metabolism and myelination (Chaudhuri et al. 1983; Shaw 1979). An investigation performed in CNS of rats showed that the highest concentrations of polyamines are found in spinal cord and peripheral nerves (Seiler et al. 1975b).

The ODC-like immunoreactivity found in rat spinal cord indicates that polyamines can be synthesized both in the motor neuron somata and in the nerve endings. Possibly, polyamines exert trophic effects on Schwann cells and/or muscle cells (Junttila et al. 1993). Findings in the rat sciatic nerve indicate that polyamines are transported axonally in both intact and regenerating motor axons (Lindquist et al. 1985).

Polyamines in traumatic injuries of brain and spinal cord

Increased ODC synthesis and putrescine concentrations have been found in trauma and injury of the brain and spinal cord of rats and gerbils (Adibhatla et al. 2002; Henley et al. 1996; Mautes et al. 1999), but no significant increase in AdoMetDC activity and spermidine or spermine levels has been found. This overactivation of ODC and increase of putrescine levels is thought to contribute to ischemia-related delayed neuronal death by triggering increased Ca\(^{2+}\)-influx to the cell (Komulainen et al. 1987; Paschen 1992; Porcella et al. 1991). Studies performed in rats have shown a correlation between the increase in putrescine in the brain and the extent of ischemia-induced neuronal damage (Camon et al. 2001; Rohn et al. 1990).

The toxicity of the polyamines, in particular spermine, is mainly induced by overactivation of the NMDA receptor leading to glutamate-mediated cell death (Otsuki et al. 1995; Porcella et al. 1991; Sparapani et al. 1997). However, it has been observed in rat and gerbil that exogenous polyamines given immediately after axonal injuries and trauma rescues peripheral and central neurons (Gilad et al. 1988; 1991) and high concentrations of spermine have been reported as neuroprotective (Ferchmin et al. 2000). In accordance with the neuroprotective effect of AdoMet in ischemic rat brain (Matsui et al. 1987), spermidine and spermine have been found to enhance recovery after sciatic nerve trauma in rats (Kauppila 1992). Some neuroprotective effects of the polyamines are thought to be:
• Regulation of the cellular ionic environment by counteracting potassium loss and preventing acid stress.
• Formation of gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter that may prevent the neurotoxic effects of excessive excitatory aminoacids. GABA is metabolised from putrescine and acetylcoenzymeA.
• Regulation of lipid peroxidation by binding to phospholipids.
• Interaction with nucleic acids to initiate processes of defence and survival in the cell.


**Therapeutic application of polyamines in traumatic injuries**

Extracellular polyamine levels have been investigated as biomarkers of enhanced protein mobilization and synthesis in response to severe trauma. There are indications that urinary levels of putrescine and spermidine correlate to the rate of protein breakdown and synthesis and that these levels may be applied as valid biomarkers of severe trauma and for measuring the response to therapy in the patients (Jeevanandam et al. 1989, 1991, 2001). Other authors also confirm the therapeutic potential of polyamine derivatives as neuroprotective agents in models of neurotrauma (Gilad et al. 1999; Munir et al. 1993).

**Polyamines in chronic neurological diseases**

Altered polyamine metabolism has been observed in neuromuscular diseases, such as Duchenne muscular dystrophy, Becker dystrophy, polymyositis and myasthenia gravis (Kaminska et al. 1981; Russell et al. 1981; Szathmary et al. 1994).

In brain specimens of patients with Alzheimer’s disease there have been reports on increased levels of AdoMet and spermidine (Morrison et al. 1993, 1995b) while other authors found decreased levels of spermidine and spermine (Seidl et al. 1996). Significantly decreased levels of spermine were also found in putamen from individuals with Huntington’s disease whereas no changes in polyamine levels could be found in basal ganglia tissues from patients with Parkinson’s disease and supranuclear palsy (Vivo et al. 2001).

Recently, increased concentrations of spermidine and spermine were found in red blood cells from patients with ALS and Parkinson’s disease (Gomes-Trolin et al. 2002).
AIMS OF THE PRESENT STUDY

The principal aims of the studies described in the present thesis were:

- To study the process of neuronal cell death in ALS by investigation of apoptosis-regulating factors and signs of apoptosis in spinal cord tissue.

- To investigate whether basic mechanisms of cellular function such as transmethylation and polyamine metabolism are altered in patients with ALS.

- To study the regional distribution of MAT activity in mammalian spinal cord and the distribution of polyamines in spinal cord from ALS patients and controls.

- To develop a selective analytical method with precision for determination of polyamines in human tissues and fluids of different characteristics.

Paper I

The objective of this study was to investigate whether degeneration of motor neurons in ALS spinal cord is related to altered levels of the apoptosis-regulating proteins Bcl-2 and Bax, and increased expression of the cysteine protease ICH-1L (Ice and ced-3 homolog, the long protein). The number of motor neurons with DNA fragmentation, indicative of apoptosis were also studied.
Paper II

The aim of this study was to investigate the role of transmethylation mechanisms in ALS. The activity of MAT was analysed in erythrocytes and spinal cord from patients with ALS and matched control subjects.

Paper III

The aim was to provide a background for future studies on neurodegenerative changes in the spinal cord. Because MAT shows a high degree of conservation between species this study investigated MAT activity in spinal cord regions of different animal species and compared those to the MAT activity found in human spinal cord (paper II). The postmortem stability of MAT was also investigated in rat spinal cord.

Paper IV

The main purpose of this study was to make an adaptation of already described methods for quantitative determination of polyamines and to show that this adaptation could be applied for analysis in human materials of different characteristics without complicated preparations. The method is based on precolumn derivatization with 9-fluorenymethyl chloroformate (FMOC) followed by high performance liquid chromatography (HPLC) assay with fluorescence detection.

Paper V

The aim of this study was to investigate the levels and distribution of polyamines in the spinal cord from ALS patients. The study was preceded by an investigation of the postmortem stability of polyamines in rat spinal cord.
MATERIAL AND METHODS

Experiments performed in animals

Postmortem stability of MAT and polyamines in rat spinal cord (Studies III and V)

Male Spraque-Dawley rats weighing 220 - 250 g (N=45) were purchased from BK Laboratories, Stockholm, Sweden. The animals had free access to food and water. The study was approved by the local Ethics Committee for Animal Research. After decapitation the spinal cords were left in situ at room temperature (23°C) for 0, 2, 4 and 8 h postmortem and at +4°C for 16, 24, 36, 48 and 72 h postmortem before removal of the spinal cord. The spinal cords were then divided into two pieces, one for the study of the postmortem stability of MAT (Study III, spinal cords of 5 rats/time point, 0 - 72 h) and the other for the polyamine stability test (Study V, spinal cords of 3 rats/time point, 0 - 36 h). The tissue samples were immediately frozen in liquid nitrogen and stored at -72°C until analysis.

MAT activity in mammalian spinal cord (Study III)

Spinal cords from 7 pigs (castrated males) and 13 bovine animals (7 females and 6 males) were obtained from a local slaughterhouse. Tissues were kept on ice until cut into 10-mm sections and frozen on a metal plate in liquid nitrogen before storage at −75°C. The dorsal horn, ventral horn and white matter regions were dissected from frozen tissue and kept in liquid nitrogen before homogenisation and analysis. Spinal cords of one cat and one Rhesus monkey were also kept at −75°C.
Studies in human tissues and fluids

Postmortem tissues
Specimens of spinal cord (Study I, II, IV and V), brain and skeletal muscle (Study IV) were obtained postmortem from autopsies. The spinal cord was immediately removed of dura mater and sectioned into 5-mm segments, and cerebellum was dissected from the brain and sliced before freezing of the tissues. All tissues were then frozen on a metal plate in liquid nitrogen and stored at −72°C until analyses.

The postmortem delay is defined as the interval between death and freezing of the tissue. Spinal cord tissue from patients with ALS and control subjects were used. All patients suffered from sporadic ALS and were diagnosed according to the El Escorial World Federation of Neurology Criteria for the diagnosis of ALS. The cause of death in the control group was cardiac failure and myocardial infarction.

In Study I, spinal cord samples of thoracic level, from five ALS patients and five control subjects were used. In Study II and Study V, frozen spinal cord (third cervical to fourth thoracic level) from 7 patients with ALS and 7 control subjects were dissected into ventral horn, dorsal horn and white matter. The tissues were kept in liquid nitrogen until homogenisation. Tissue of spinal cord white matter from 3 control individuals were also used in Study IV.

Blood samples
Venous blood samples from patients with ALS (N = 21) and healthy control subjects (N = 20) were used in Study II. The study was approved by the Ethics Committee of the Faculty of Medicine in Uppsala, Sweden. Blood samples from control individuals were obtained from the Dept. of Clinical Immunology and Transfusion Medicine, Uppsala University Hospital, Sweden. Normal health status of controls were previously established by clinical, biochemical and haematological screenings and all ALS patients were diagnosed according to the El Escorial World Federation of Neurology Criteria for the diagnosis of ALS.

The blood samples were collected in Vacutainer® tubes containing sodium citrate (3.1%) as an anticoagulant and coded to conduct blind assays. After centrifugation at 470 g for 10 min (+4°C), the erythrocyte pellet was washed twice with 3 volumes of 0.14M NaCl, and centrifuged at 2400 g for 20 min (+4°C). The erythrocytes were then lysed in 3 volumes of water (Millipore grade) in an ice-bath for 30 min and the cytosol fraction stored at −72°C.
CSF samples
CSF was obtained from three control individuals by lumbar puncture and immediately frozen at –72°C (Study IV). Sampling of CSF was approved by the Ethics Committee of the Faculty of Medicine at Uppsala University, Sweden.

Microdialysate samples
In Study IV, muscle microdialysates were obtained from two consecutive 60-min microdialysis samplings from the muscle tibialis anterior. The method used was described by Axelson et al (Axelson et al. 2002). Five healthy control individuals were included in the study. The samples were frozen at –72°C until analysis. The study was approved by the Ethics Committee of the Faculty of Medicine at Uppsala University, Sweden.

Preparation of homogenates for analysis of MAT activity

Spinal cord tissue
For the analyses of MAT activity in spinal cord (Studies II and III), tissues were homogenised with a Polytron PT2100 homogeniser in 3-4 volumes of TRIS-DTT-EDTA buffer, pH 8.0 on ice. This was followed by centrifugation at 15 000 rpm for 20 min (+4°C). The supernatant was separated, the concentration of protein was measured and aliquots of the homogenate were stored at –72°C until analysis.

Erythrocyte samples
Lysed erythrocytes (Study II) were centrifuged at 15 000 rpm for 20 min (+4°C). The supernatant was separated, the concentration of protein was measured and aliquots of the erythrocyte fraction were stored at –72°C until analysis.
Preparation of homogenates for analysis of polyamines

Spinal cord and brain tissue
Spinal cord tissues from rats (Study V), human spinal cord (Study IV and V) and human brain (Study IV) were homogenized with a glass tissue grinder in perchloric acid 0.3M, 1.0 mL/100 mg tissue, on ice. A 20-µL aliquot was taken for protein measurement. Deproteinisation was performed by mixing equal volumes of homogenate and 10% trichloroacetic acid. After vortex mixing and centrifugation at 12 000 rpm for 30 min (+4°C), the supernatant was separated and stored at –72°C until analysis.

Muscle
Homogenates of muscle (Study IV) were prepared according to the same procedure described above but using the Polytron PT2100 homogenizer.

CSF
The free fraction of the polyamines in CSF was analysed in Study IV. The samples were thawed in an ice-bath and the internal standard (1.6-DAH) was added. The samples were then lyophilized for 2 h in a vacuum chamber provided with a cooling trap. The lyophilizate was resuspended in a solution of water/acetonitrile 50/50 (v/v).

Muscle microdialysate
The muscle microdialysate (Study IV) was thawed in an ice-bath and the internal standard (1.8-DAO) and acetone were added to the sample.

Determination of protein
Protein concentrations were measured by a modification of the Lowry procedure (Markwell et al. 1978) using bovine serum albumin as a standard.
Biochemical methods

Immunohistochemistry (Study I)

To investigate the immunoreactivity of Bcl-2, Bax, ICH-1L and the amount of motor neurons with DNA fragmentation, 8-µm sections of the spinal cords were cut in a cryostate and thaw mounted on to poly-L-lysine coated slides.

Three series of slides were prepared, one series for Bcl-2, Bax and ICH-1L respectively, with three adjacent spinal cord sections in each. One additional series of slides was also prepared for the TUNEL stainings. The sections were cryopreserved and stored at -20°C. The first section in each series was stained with cresyl violet for morphological identification of nerve cells. The second section was stained with primary antibodies specific to the acetylcholine synthesizing enzyme choline acetyl-transferase (ChAT) for detection of cholinergic motor neurons. The third section was double-stained with primary antibodies specific to ChAT together with antibodies specific to either Bcl-2, or Bax or ICH-1L.

For visualization, fluorescently labelled secondary antibodies were used. Briefly, frozen sections were fixed for 15 min using 4% paraformaldehyde in phosphate buffer and then rinsed in Tris-buffered saline. Nonspecific antibody binding was blocked in 3% normal goat serum before addition of the primary antibody and incubation at +4°C overnight. After another wash the secondary antibody was added and slides incubated for 90 min (RT). Finally, coverslips were applied using Entellan mounting medium.

TUNEL staining and conversion of TUNEL-positive cells by an antifluorescein antibody was mainly performed according to the product description for TUNEL-POD. Counterstaining was achieved using cresyl violet prior to the mounting of coverslips with Entellan mounting medium.

For a more detailed description of the immunohistochemistry, see the Material and Methods section in Paper I.

Assay of MAT activity (studies II and III)

MAT activity was assayed by measuring the rate of formation of S-Adenosyl-L-[methyl-3H]methionine from L-[methyl-3H]methionine and ATP using the Oden & Clarke procedure (Oden et al. 1983) modified by Gomes-Trolin et al (Trolin et al. 1994). The separation of [methyl-3H]-AdoMet was performed by ion-exchange chromatography using columns containing 0.4 mL of Dowex AG 50W-X8 resin in the NH₄⁺ form.

The reaction mixture (0.1 mL, pH 7.7) contained final concentrations of: 35mM HEPES, 30mM MgCl₂, 26mM KCl, 10mM ATP, L-[methyl-3H]methionine containing unlabeled methionine and tissue samples.
In Study II, MAT activity was assayed in samples containing 150 µg and 20 µg protein for erythrocytes and spinal cord, respectively. The radioactively labeled methionine (L-[methyl-\(^{3}\)H]methionine, 12 000 cpm/nmol) contained unlabeled methionine in the range of 1 - 16 µM for erythrocytes, and 1 - 60 µM methionine for spinal cord extracts.

In Study III, tissue extracts of 20 µg protein were analysed with L-[methyl-\(^{3}\)H]methionine (191 mCi/mmol) and unlabeled methionine in the range of 1 - 60 µM. Blanks were prepared with water instead of ATP.

After a 60-min incubation (+37°C) the reaction was interrupted with 1.0 mL ice-cold citric acid (1.6mM) in 50% ethanol. The reaction mixture (1.0 mL) was then applied to the ion-exchange column and kept in darkness for 30 min. Unreacted L-[methyl-\(^{3}\)H]methionine was eluted with citric acid (2.0mM, pH 6.0) and the columns rinsed with 3 x 10 mL water. Elution of [methyl-\(^{3}\)H]-AdoMet was performed with 2.0 mL NH\(_4\)OH (3.0 M) into scintillation vials containing 10 mL of scintillation fluid. The radioactivity was counted in a liquid scintillation counter.

The MAT activity was measured in duplicate for each sample and the apparent values of \(V_{\text{max}}\) (pmol [methyl-\(^{3}\)H]-AdoMet/mg protein/minute) and \(K_{\text{m}}\) (µM methionine) were determined by linear regression of the double reciprocal plots.

Analyses of polyamines by precolumn derivatization with FMOC and HPLC assay (Studies IV and V)

**Brain, spinal cord and muscle homogenates (Studies IV and V)**

The derivatization was performed in aliquots containing 100 µg protein. A 60 µl of 0.2M borate buffer pH 9.0 and a 5 µl of 30 nmol/mL 1.6-DAH were added to the samples. After vortex mixing, 200 µL of FMOC (0.01M in acetone) was added to the mixture and the derivatization reaction was allowed to proceed for 90 s with the first 45 s under mixing. The reaction was then interrupted by the addition of 240 µL of 0.04M glycine solution (EVE) and mixing for 45 s. Glycine solution was prepared by dissolving in 0.2M borate buffer pH 9.0:acetone 50/50 (v/v) made fresh. This was followed by the addition of dilution buffer (0.05M sodium acetate buffer, pH 4.2:acetonitrile 30/70, v/v) and mixing for 45 s. The final volume was adjusted to 800 µL with 200 µL of water (Millipore grade). A 20-µL sample was injected into the HPLC system.

**CSF samples (Study IV)**

The derivatization proceeded as described above. The volumes used for CSF samples and reagents were 20 µL CSF, 60 µL borate buffer, 100 µL FMOC, 120 µL EVE and 100 µL dilution buffer.
Microdialysate samples (Study IV)
The same procedure as described above for tissues and CSF was used for derivatization of muscle microdialysates. The volumes used for samples and reagents were 2 µL microdialysate + 10 µL 1.8-DAO + 12 µL acetone, 60 µL borate buffer, 80 µL FMOC, 100 µL EVE and 140 µL dilution buffer.

High performance liquid chromatography (HPLC) assay
The HPLC system consisted of a multiple solvent delivery pump (LDC analytica CM 4000), an autosampler injector with a cooling tray kept at +4°C (Midas Spark, Holland), a C8-column (250 x 4 mm I.D., 5 µm) protected by a 5-µm guard column (Reprosil polyamine-1, A. Maisch, Germany) maintained at +40°C and a fluorescence detector (Jasco 821-FP). The excitation and emission wavelengths of the detector were set at 264 nm and 310 nm, respectively.

The gradient procedure for cerebellum, spinal cord, CSF and muscle samples (Studies IV and V) were as follows: the separation was carried out at a flow rate of 1.2 mL/min. Buffer A consisted of sodium acetate buffer 0.05M, pH 4.2:acetonitrile 80/20, v/v. Buffer B consisted of sodium acetate buffer 0.05M, pH 4.2:acetonitrile 5/95, v/v. The gradient used was at 0 - 5 min, 50% buffer A:50% buffer B. This was followed by a linear increase of buffer B reaching 100% at 28 min, at 28 - 28:30 min 100% buffer B, at 28:30 - 38 min 50% buffer A:50% buffer B.

Gradient procedure for microdialysate sample (Study IV): Buffers A and B were of the same composition as described above. The flow rate was 1.3 mL/min. The gradient used was at 0 - 5 min 50% buffer A:50% buffer B, linear increase of buffer B reaching 100% at 20 min, at 20 - 25:30 min 100% buffer B, at 25:30 - 30 min 50% buffer A:50% buffer B.
RESULTS AND DISCUSSION

Signs of apoptosis in ALS spinal cord motor neurons (Study I)

This study was conducted to investigate the levels of Bcl-2, Bax and ICH-1L and to search for signs of apoptosis in ALS spinal cord motor neurons. The results demonstrate increased apoptotic activity in motor neurons from patients with ALS. This is supported by data showing an upregulation and increased expression of the cell death-promoting protein Bax, as well as increased DNA degradation in ALS motor neurons as compared to controls, see Figure 7.

Figure 7. Sum of motor neurons (MN’s) in spinal cord sections from five ALS (A) and five control (B) subjects. Bars present total number of MNs in each serie and the filled parts indicate those MNs immunoreactive for Bcl-2-, Bax-, ICH-1L- and TUNEL-stainings, respectively. (*) significantly higher values of immunoreactive MNs in Bax- and TUNEL-stainings in ALS as compared to controls (p < 0.01).
The Bcl-2 to Bax ratio in Figure 8 shows that the Bcl-2 expression is favored relative to Bax in the control material, giving a ratio of 1.98. In contrast, the ratio of 0.72 obtained in ALS neurons indicate preponderance for Bax in this disease.

Figure 8. This graph shows the Bcl-2/Bax ratio, indicative of vulnerability to apoptotic cell death, in ALS and control spinal cord motor neurons. Number of motor neurons immunoreactive for Bcl-2 and Bax are calculated as quotients of total number of motor neurons in respective series. The Bcl-2 to Bax ratio for ALS is 0.72 and for controls 1.98.

The reduction in number of spinal motor neurons staining positive for ChAT in the ALS material demonstrates the process of extensive nerve cell death. However, no significant changes were observed in the expression of the anti-apoptotic protein Bcl-2 and the cysteine protease ICH-1L. These results are in agreement with studies showing unchanged Bcl-2 protein levels and increased DNA degradation (Troost et al. 1995b) and decreased ChAT activity (Nagata et al. 1982; Rothstein et al. 1990) in ALS spinal cord. Furthermore, DNA fragmentation characterised by TUNEL-technique and a strong expression of Bax has been reported in muscle cells in patients with polyneuropathy and ALS (Tews et al. 1997).

Upregulated bax mRNA and decreased bcl-2 mRNA expressions have also been presented in ALS spinal cord motor neurons (Mu et al. 1996). In contrast, recent studies of genes necessary for apoptosis and neuroprotection (Migheli et al. 1997) and TUNEL stainings of motor cortex, medulla and spinal cord from patients with ALS (Adamek et al. 2003, in abstract) report no evidence for an apoptotic involvement in ALS.

Conclusive evidence of apoptosis is likely to be difficult to detect in postmortem tissue from patient with ALS since the expected time for visible signs of an apoptotic cell range from a few to a maximum of 24 h (Sathasivam et al. 2001).
MAT activity in erythrocytes and spinal cord of patients with ALS (Study II)

This study was undertaken to investigate the role of transmethylation mechanisms in ALS. The pattern of distribution of MAT activity in the spinal cord was studied in dorsal horn, ventral horn and white matter regions. The results were compared with those obtained in spinal cord from controls.

Significantly higher values ($p < 0.05$) of MAT $V_{\text{max}}$ and $K_{\text{M}}$ were found in erythrocytes from patients with ALS in comparison to controls. The analysis of the effect of gender showed that the significant difference between ALS and controls was specific for the male group. No significant difference in MAT activity was observed in the female groups (Fig. 9).

![Figure 9](image)

*Figure 9.* Data of MAT activity in erythrocytes of control subjects (Ctrl) and patients with ALS. Figures 1A and 1B show the results of MAT $V_{\text{max}}$, and Figures 1C and 1D show the results of MAT $K_{\text{M}}$. N=20 controls (10 male + 10 female) and 21 ALS cases (11 male + 10 female). Figure 1B and 1D shows gender differences. Mean values ± SEM are indicated by bars. (*) significantly different in comparison with the respective control ($p < 0.05$).
This was the first study to provide data on the activities of MAT in tissue from patients with ALS. The decrease of the affinity of MAT for methionine in erythrocytes of male ALS patients seems to be compensated by an increase in the amount of the enzyme ($V_{\text{max}}$). This is in accordance with the suggestion that AdoMet exerts a negative feedback effect on MAT catalytic activity (Caboche 1977) and gene transcription (Gomes Trolin et al. 1998). This difference in MAT activity between male- and female-ALS patients might be interesting to explore since it is known that there is a male preponderance for the disease with a male to female ratio of 3:2. A tissue-specific positive effect of testosterone on MAT catalytic activity and polyamine biosynthesis has been demonstrated in mice (Manteuffel-Cymborowska et al. 1992).

With regard to spinal cord MAT, a mean $K_m$ value of approximately 10.0µM methionine is the same as that reported for the human brain ($K_m \approx 10.0\mu\text{M}$ (Trolin et al. 1994)) although the maximum activity was found much lower in the spinal cord. Comparisons between the MAT activities from ALS and control group revealed no significant difference. However, an effect of postmortem time might be considered.

The stability of MAT investigated in rat spinal cord (Study III) showed a 39% decrease of the enzyme activity at 8 h postmortem. In the human cases the spinal cords were removed in the mean interval of 18 - 26 h postmortem. Thus, it cannot be ruled out that a long postmortem delay has made it difficult to detect any difference between the activities of MAT in spinal cord tissues from controls and ALS patients.

In summary, this study demonstrates a sex-linked effect on the activity of MAT in erythrocytes of patients with ALS. The studies performed in spinal cord tissues showed no difference in the regional distribution or changes in the activities of MAT between controls and ALS patients.

MAT activity and regional distribution in mammalian spinal cord (Study III)

This comparative study analysed MAT activity in dorsal horn, ventral horn and white matter of spinal cord from bovine, pigs, one cat and one monkey, and compared the results with those of human spinal cord (Study II). The stability of the MAT enzyme was also investigated in postmortem rat spinal cord.

The MAT $V_{\text{max}}$ was found quite homogeneously distributed between spinal cord regions and species investigated. However, significantly higher values were found in all regions of the male bovine group ($p < 0.0001$) as compared to corresponding regions of bovine females and all other species, except white matter of pig spinal cord. MAT $V_{\text{max}}$ found in white matter of
pig spinal cord showed significantly higher values in comparison to pig dorsal horn (p < 0.005), pig ventral horn (p < 0.001) and white matter from bovine female and human (p < 0.0001). The lowest affinity for methionine (highest $K_M$) was found in human spinal cord (p < 0.0001) as compared to all other species and regions.

The stability of the enzyme was found to significantly decrease during the first 8 h postmortem (p < 0.0001) in rat spinal cord. Thereafter the enzyme remained at a mean level of 60% of the initial value until 72 h postmortem (Fig. 10).

![Figure 10](image)

Figure 10. Postmortem changes in MAT activity in rat spinal cord. The spinal cords were left in situ at 23°C for 0 - 8 h postmortem and the remaining time at +4°C. N = 5, except at 24 h and 72 h (N = 4). All data are Mean ± SEM. (*) p < 0.0001.

The stability of MAT investigated in the rat spinal cord is in accordance with a study of MAT stability in the rat brain, presenting a steady decrease of activity at room temperature but no significant change at +4°C (Trolin et al. 1994).

The levels of MAT activity were found quite homogeneous in the mammalian spinal cords investigated although the male bovine group showed a 60% higher activity than the bovine females. Gender differences in MAT activity have also been observed in erythrocytes of patients with ALS (Study II). Furthermore, it has been demonstrated that the treatment of female mice with testosterone induces a significant increase of MAT activity in their kidneys (Manteuffel-Cymborowska et al. 1992).

The significantly higher MAT activity in the white matter region of the spinal cord of castrated pigs as compared to the grey matter might be related to an increased lipid metabolism in that area. It has been shown that castrated male pigs have a higher adipose tissue lipogenesis in comparison to
normal males and females (Mersmann 1984) and AdoMet is known to be involved in the transmethylation of phospholipids.

In conclusion, this comparative study showed a quite homogeneous distribution of MAT activity among the species and regions investigated. The highest enzyme activity was observed in the male bovine group and the lowest affinity for methionine in human spinal cord.

Determination of polyamines in human tissues and fluids (Study IV and V)

HPLC assay for polyamine analysis (Study IV)

An adaptation of already described analytical methods for determination of polyamines was made to be applied in human materials of different characteristics without complex preparation procedures (Study IV). The method was then utilized in the study of polyamines in ALS spinal cord (Study V).

The method consisted of precolumn derivatization with FMOC followed by HPLC with fluorescent detection. The method was validated by analyses of linearity, within- and between-assay precision, recovery and stability test of the derivatives. All results are presented in Table 1.

Table 1. Parameters of method validation

<table>
<thead>
<tr>
<th>Test</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equations of linearity</td>
<td>y = -51.504+3241.6x</td>
<td>y = -8.175+432.8x</td>
<td>y = -22.421+3630.6x</td>
</tr>
<tr>
<td>(r=0.995)</td>
<td>(r=0.999)</td>
<td>(r= 0.991)</td>
<td></td>
</tr>
<tr>
<td>Within-assay (N=3)</td>
<td>Accuracy (%)</td>
<td>Accuracy (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td></td>
<td>-19.5 to +3.9</td>
<td>-2.9 to –0.2</td>
<td>-18.6 to 0</td>
</tr>
<tr>
<td></td>
<td>C.V. (%)</td>
<td>1.0 to 3.8</td>
<td>3.3 to 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.9 to 4.5</td>
</tr>
<tr>
<td>Between-assay (N=6)</td>
<td>Accuracy (%)</td>
<td>Accuracy (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td></td>
<td>+3.6</td>
<td>+1.0</td>
<td>-1.2</td>
</tr>
<tr>
<td></td>
<td>C.V. (%)</td>
<td>5.3</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Recovery (N=3)</td>
<td>(%) Mean ± SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>83.7 ± 5.1</td>
<td>89.3 ± 3.6</td>
<td>93.5 ± 1.0</td>
</tr>
<tr>
<td>Stability of the derivatives (N=8)</td>
<td>(nmol/mL, Mean ± SEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22 ± 0.001</td>
<td>0.22 ± 0.001</td>
<td>0.24 ± 0.001</td>
</tr>
</tbody>
</table>
The versatility of the method was demonstrated by analysing samples of different characteristics such as human cerebellum, spinal cord, CSF, skeletal muscle and muscle microdialysate (Fig. 11).

Figure 11. Chromatographic separation of putrescine (Put), spermidine (Spd), spermine (Spm) and internal standards (1.6-DAH and DAO) in human tissues and fluids. a) polyamin standard solution, b) cerebellum, c) spinal cord (white matter), d) cerebrospinal fluid, e) muscle (m abductor poll. brevis) and f) muscle microdialysate (tibialis anterior).

The linearity of the method was tested for polyamine standards at a minimum of 5 concentrations of putrescine and spermine (N=5/level) and 7 concentrations of spermidine (N=5/level). For each polyamine the regression line (y=a+bx) was calculated. The intercept (a) was determined in units of mV and the slope (b) in units of nmol/mL.
The within-assay precision was determined by three successive injections into the HPLC system of standard solutions of polyamines at three different concentrations. The between-assay precision was analyzed by derivatization of standard solutions containing 0.2 nmol/mL of putrescine and spermidine and 0.24 nmol/mL of spermine at 6 different occasions within 37 days. The accuracy% and C.V.% were calculated for both within- and between-assay precision.

Recovery of the polyamines were tested in homogenates of spinal cord added with 0.10nmol of standard polyamine solution.

The stability of FMOC-polyamine derivatives was investigated by keeping derivatized standard samples of polyamines at +4° for up to 14 h. The samples (0.2 nmol/mL for putrescine and spermidine; and 0.24 nmol/mL for spermine) were analysed in a total of 8 times at intervals of 2 h and found stable for at least 14 h.

Polyamines in ALS spinal cord (Study V)

Analysis of polyamine concentrations and regional distribution in spinal cord from ALS patients and healthy control subjects was performed with the method described in Study IV.

The stability of polyamines was examined in rat spinal cord during the interval of 0 - 36 h postmortem. The levels of putrescine, spermidine and spermine increased by 32%, 15% and 2%, respectively, see Fig 12.

Figure 12. Stability of polyamines in postmortem whole spinal cord of rat (N = 3). After decapitation the spinal cords were left in situ at +23°C for 8 h postmortem and the remaining time at +4°C. Bars indicate SEM. (*) significantly higher levels at 2, 4, 8, 16, and 24 h postmortem (p < 0.05).
The increase in both putrescine and spermidine levels suggests that during the first 4 h postmortem the mechanism controlling their synthesis is still working. Increases in putrescine and spermidine levels were described in rats after traumatic injuries of the spinal cord and the brain. Thus, there have been reports showing significantly increased concentrations of putrescine 2 - 6 h post injury of spinal cord (Gonzalez et al. 1995; Mautes et al. 1999) and a slight increase in spermidine levels after traumatic brain injury (Henley et al. 1996).

This is, to our knowledge, the first study of polyamines in adult human spinal cord and nervous tissue from patients with ALS. The level of spermidine in white matter from ALS patients was significantly higher in comparison to ventral and dorsal horn regions (p < 0.005 and p < 0.05). Higher levels of spermidine in white matter areas were also found in the control group as compared to ventral horn (p < 0.05).

Spermidine has been reported as the mostly expressed polyamine in regions rich in white matter in both fetal human spinal cord and human brain (Chaudhuri et al. 1983; Morrison et al. 1995a; Shaw et al. 1973).

No significant differences in polyamine levels were found between the ALS group and the control group. However, in female patients with ALS a significantly higher concentration of spermidine was found in ventral horn as compared to ventral horn of male ALS patients and control females (p < 0.05, Fig. 13).

Figure 13. Spermidine levels in ventral horn tissue from ALS patients and control group. Female (N=3), Male (N=4). Black dots indicate Mean ± SEM. (*) significantly higher values of females with ALS as compared to male ALS patients and the control female group (p < 0.05).
Levels of spermine were also found significantly increased in ventral horn areas of the female ALS group in comparison to the male ALS group (p < 0.05).

It is interesting to note that the females with ALS included in this study had a shorter duration and a more rapid progression of the disease than the male ALS patients. It is possible that an involvement of the polyamine metabolism is transient and more pronounced in the early phases of the disease. A recent study in red blood cells from patients suffering from ALS found increased levels of spermidine and spermine indicating a general involvement of the polyamine metabolism in this neurodegenerative disorder (Gomes-Trolin et al. 2002).

In conclusion, no major differences in polyamine levels were found between spinal cord from patients with ALS and control subjects, indicating a maintained regulation of polyamines at the end stage of this neurodegenerative disorder.
SUMMARY AND CONCLUSIONS

The main findings of this study may be summarized as follows:

I) The increased levels of the proapoptotic protein Bax together with signs of DNA fragmentation in ALS spinal cord indicate an apoptotic process of neuronal cell death in the tissue.

II) Altered MAT activity was observed in erythrocytes from male ALS patients but not in ALS spinal cord as compared to controls. The influence of postmortem delay might have made it difficult to detect differences in MAT activity in spinal cord tissues, as reported in Study III.

III) The comparative study on transmethylation mechanisms, i.e the MAT activity, in spinal cord from different mammalian species showed a quite homogeneous distribution of MAT activity among the species and regions examined. Postmortem changes in MAT activity investigated in the rat spinal cord showed significantly decreased levels during the first 8 h postmortem (p < 0.0001). Thereafter the enzyme remained at a mean level of 60% of the initial value until 72 h postmortem.

IV) The modification of earlier described polyamine assays resulted in a sensitive and selective method applicable to polyamine analysis in human materials of different characteristics without complicated preparations. The method was validated by analyses of linearity, within- and between-assay precision, recovery and stability test of the derivatives. All polyamines were separated within 35 min.

V) Polyamine levels in the spinal cord did not differ significantly between the ALS group and the control group, suggesting a maintained regulation of polyamines at the end stage of this neurodegenerative disorder. However,
increased spermidine levels were found in spinal cord regions of female ALS patients as compared to male ALS patients and female controls. Increased levels of spermine were also found in the female ALS group. These findings together with previous results by Gomes-Trolin et al (Gomes-Trolin et al. 2002) showing increased levels of spermidine and spermine in erythrocytes of ALS patients, might indicate a transient and more pronounced polyamine metabolism in the early phases of the disease.
SVENSK SAMMANFATTNING

Varje år drabbas ca 170 personer i Sverige av sjukdomen amyotrofisk lateralskleros (ALS). Sjukdomen debuterar oftast i 50-70 års ålder, men kan ses i alla åldrar. De flesta ALS-fall är av sporadiskt ursprung och endast hos 5-10% av patienterna kan en ärfilig form konstateras (familjär ALS).

ALS kännetecknas av specifik celldöd i motoriska hjärnbarken, hjärnstam och ryggmärg vilket resulterar i försvagning och förtvivning av muskler. Sjukdomsförloppet är snabbt och de flesta patienter avlider inom 3-5 år efter symtomdebuten.

Trots att ALS beskrevs och namngavs redan på 1860-talet av den franska neurologen Charcot är bakomliggande faktorer till sjukdomen fortfarande i stort sett okända. Många hypoteser har presenterats genom åren för att belysa orsak och verkan bakom sjukdomsförloppet och områden som studerats berör bland annat genetiska defekter, påverkan av fria radikaler som kan skada celler, förhöjda nivåer av aminosyran glutamat, ackumulering av icke-fungerande protein, inflammationer, brist på tillväxtfaktorer och apoptos (genetiskt programmerad celldöd).

Behandling med riluzole, en substans som dämpar höga nivåer av glutamat, har visat en gynnsam effekt på överlevnad vid ALS.

Få studier hittills har undersökt basala mekanismer för cellers överlevnad och funktion såsom transmetylering och polyaminmetabolism i samband med ALS.


De tre vanligaste polyaminerna, putrescin, spermidin och spermin är involverade i livsnödvändiga funktioner i cellen såsom kontroll av gener avläsning till fungerande proteine, celltillväxt, celldelning och specialisering.

I den första studien i denna avhandling undersökt processen av celldöd i ryggmärg från patienter med ALS och individer utan tecken på neurologisk sjukdom (kontrollindivider). Resultaten visar ökade nivåer av Bax, ett protein som stimulerar programmerad celldöd. Förhöjda nivåer av DNA-
sönderfall observerades också vilket tyder på en process av programmerad celldöd i vävnaden.

För att lågga en grund till fortsatta studier av transmetylering vid processer av celldöd i ryggmärg undersöktes aktiviteten av enzymet MAT i ryggmärg från olika djur. Studien visade att MAT aktiviteten låg på en likartad nivå hos de olika arterna. För att få en uppfattning om hur MAT-enzymet bevaras i död vävnad som t.ex ryggmärg från patienter med ALS, studerades ryggmärg från rätta. Rätorna avlivades och med jämna intervall under 0 - 72 timmar efter det att rätten avlivats analyserades MAT aktiviteten. Enzymaktiviteten visade sig sjunka med ca 40% under de första 8 timmarna för att sedan ligga kvar på en konstant nivå.

En undersökning av MAT aktivitet i röda blodkroppar vid ALS visade förändringar hos manliga patienter men inte hos kvinnliga patienter. Inga skillnader i MAT aktivitet upptäcktes i ryggmärgsvävnad från ALS-patienter jämfört med kontrollindivider. Det kan inte uteslutas att detta kan bero på att MAT aktiviteten blivit låg i ryggmärgsmaterialet, då patienterna varit avlidna mellan 18 - 26 timmar innan ryggmärgen frystes.

Inför studierna av polyaminer i ALS-ryggmärg utvecklades en analysmetod för att noggrant och effektivt kunna mäta nivåer av putrescin, spermidin och spermin. Metoden baseras på tidigare beskrivna analyser av polyaminer och aminosyror. Metoden testades med validerings-parametrar och applicerades på mänskliga vävnader och vätskor såsom hjärna, ryggmärg, muskel, microdialysat från muskel, och ryggmärgsvätska.

Hur polyaminer bevaras i död vävnad undersöktes också i ryggmärg från rätta i intervallet 0 - 36 timmar efter att rätten avlivats. Resultaten visade att polyaminnivåerna ökade med 32% (putrescine), 15% (spermidine) och 2% (spermine) under de första 4 timmarna efter avlivning.

I studien av polyaminer i ALS- och kontrollryggmärg upptäcktes inga större skillnader mellan grupperna. Dock kunde ökade nivåer av spermidin observeras i en ryggmärgsregion från kvinnor med ALS i jämförelse med samma ryggmärgsregion hos män med ALS och kvinnliga kontroller. Även ökade nivåer av spermin hittades i samma ryggmärgsregion från kvinnor med ALS. En intressant iakttagelse är att kvinnorna med ALS i denna studie hade en kortare sjukdomstid och ett snabbare sjukdomsförlopp än männen med ALS. Observationen av ökade spermidin- och spermin-nivåer hos kvinnor med ALS kanske kan tyda på att ökade polyaminnivåer vid ALS förekommer i tidigare skeden av sjukdomen. Detta stöds av resultat från en undersökning av blodprov från patienter med ALS som visade ökade nivåer av både spermidin och spermin vid jämförelse med blod från friska personer (Gomes-Trolin et al. 2002).

Dessa könsrelaterade förändringar i transmetylering och polyaminförekomst är intressanta eftersom ALS oftare drabbar män än kvinnor. Att inga större avvikelse i polyaminnivåer kunde upptäckas i
ryggmärg från patienter med ALS talar för att bildandet av polyaminer finns bevarat även i slutfasen av denna nedbrytande sjukdom.

Förhoppningen med detta projekt är kunna tillföra kunskap om några av de många bakomliggande faktorerna vid sjukdomen ALS och i ett längre perspektiv hoppas vi att dessa resultat ska bidraga till en bättre behandling av sjukdomen.
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Amanda, my princess, for joy and happiness.

And Michael, my husband and best friend, for all your love, encouragement and believing in me – Jag älskar dig.
REFERENCES


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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 *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine*. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)