Alma Mater Studiorum • University of Bologna

PhD Program in
Cell Biology and Physiology
XIX cycle- scientific area code BIO09

Neurochemical analysis and identification of potentially involved proteins in a mouse model of Amyotrophic Lateral Sclerosis

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April 2007

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CHAPTER 1: INTRODUCTION

The motoneuron

In vertebrates, the term motoneuron (motor neuron) classically applies to neurons located in the central nervous system (CNS) which project their axons outside the CNS and directly or indirectly control muscles. Motoneurons can be divided into somatic motoneurons and visceral motoneurons.

Alpha motoneurons innervate extrafusal muscle fibers (typically referred to simply as muscle fibers) located throughout the muscle. They posses large-caliber, heavily myelinated axons that conduct action potentials rapidly. Gamma motoneurons innervate intrafusal muscle fibers found within the muscle spindle and posses slender, lightly myelinated axons that conduct less rapidly.

In addition to voluntary skeletal muscle contraction, alpha motoneurons also contribute to muscle tone, the continuous force generated by noncontracting muscle to oppose stretching. When a muscle is stretched, sensory neurons within the muscle spindle detect the degree of stretch and send a signal to the CNS. The CNS activates alpha motoneurons in the spinal cord which cause extrafusal muscle fibers to contract and thereby resist further stretching. This process is also called the stretch reflex. Gamma motoneurons regulate the sensitivity of the spindle to muscle stretching. With activation of gamma neurons, intrafusal muscle fibers contract so that only a small stretch is required to activate spindle sensory neurons and the stretch reflex.

Lower motoneurons are located in the ventral horns in the spinal cord whereas upper motoneurons are located in the motor cortex in the brain (Fig.1 and 2). Usually upper motoneurons synapse lower motoneurons in the spinal cord and then lower motoneurons contact voluntary muscles at the neuromuscuar junction.

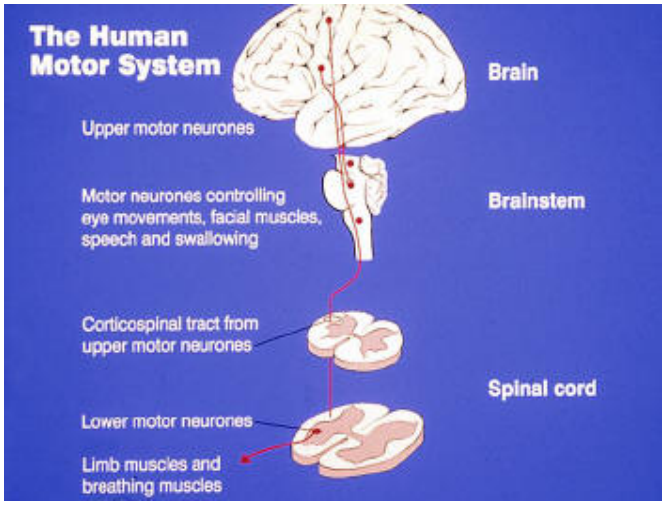


Fig 1. Motoneuron organization in the central nervous system

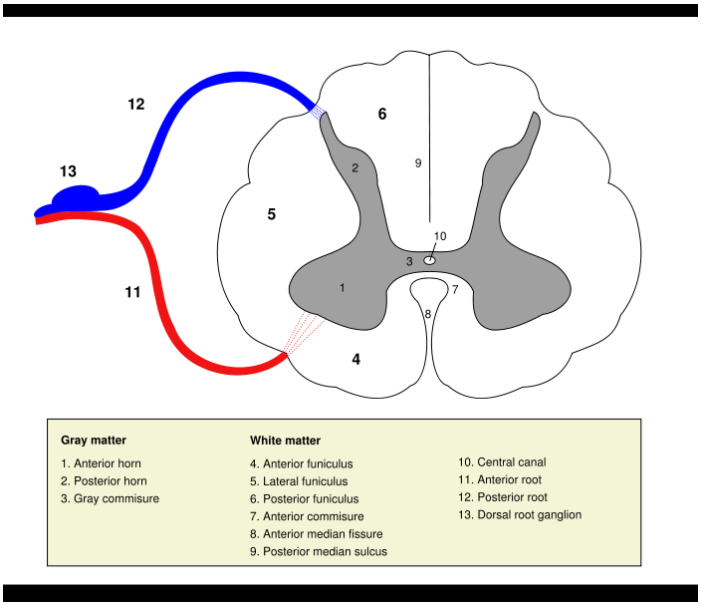


Fig 2. Spinal cord anatomy and organization

The neuromuscular junction

A neuromuscular junction (NMJ) is the synapse or junction of the axon terminal of a motoneuron with the motor end plate, the highly-excitable region of muscle fiber plasma membrane responsible for initiation of action potentials across the muscle's surface, ultimately causing the muscle to contract (Fig. 3). The signal passes through the neuromusclar junction via the neurotransmitter acetylcholine.

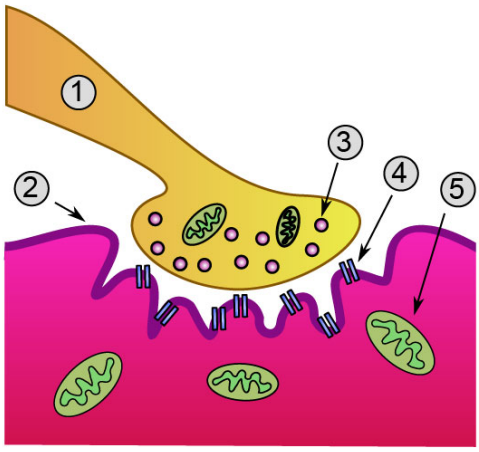


Fig 3. Schematic representation of the neuromuscular junction: 1.Presynaptic terminal. 2.Sarcolemma. 3.Synaptic vescicles. 4.Nicotinic acetylcholine receptor. 5.Mitochondrion

Upon the arrival of an action potential at the axon terminal, voltage dependent calcium channels open and Ca^{2+} ions flow from the extracellular fluid into the motor neuron's cytosol. This triggers excitation-contraction coupling, a biochemical cascade that causes neurotransmitter-containing

vesicles to fuse to the motor neuron's cell membrane and release acetylcholine into the synaptic cleft. Acetylcholine crosses the synaptic cleft and binds to the nicotinic acetylcholine receptors that dot the motor end plate. The receptors are ligand-gated ion channels, and when bound by acetylcholine, they open, allowing sodium and potassium ions to flow in and out of the muscle's cytosol, respectively. Because of the differences in electrochemical gradients across the plasma membrane, more sodium moves in than potassium out, producing a local depolarization of the motor end plate known as an end plate potential (EPP). This depolarization spreads across the surface of the muscle fiber into transverse tubules eliciting the release of calcium from the sarcoplasmic reticulum, thus initiating muscle contraction. The action of acetylcholine is terminated when the enzyme acetylcholinesterase degrades the neurotransmitter.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS), also known as motoneuron disease or Lou Gherigh's disease, is a fatal neurodegenerative disease that primary affects upper and lower motoneurons in the central nervous system. Interestingly, only alpha-motoneurons appear to be affected during the disease (Conradi et al., 1993). It was first described by the French biologist Jean-Martin Charcooth in 1869. The word amyotrophic is Greek in origin. A means no or negative, myo refers to muscle and trophic means nourishment, meaning "no-muscle-nourishment." Lateral identifies the areas of the spinal cord where portions of the nerve cells that signal and control the muscles are located. As this area degenerates it leads to scarring or hardening (sclerosis) in the region. The degeneration of the motoneurons leads first to muscle denervation and eventually to muscle atrophy. Motoneuronal degeneration is distal, that is from the synapse to the cell body, so there is a dying back process (Fischer et al., 2004).

Epidemiology of ALS

Rough average world prevalence and incidence of the disease are of 4-6 and 1-2 cases per 100,000 (Kahana et al., 1976; Murros et al., 1983; Murray et al., 1987; Traynor et al., 1999; Beghi et al., 2007) individuals respectively whereas the age of onset is usually after the fourth decade, but there are also early age onset variants. The worldwide prevalence of ALS seems to be approximately the same but from some statistical studies, some populations seemed to have a higher prevalence than others (UK 8/100,000; in Italy 6/100,000; in Mexico 2/100,000). It seemed as if the Mexican population had some kind of resistance or protective factor against ALS (Olivares et al., 1972).

However these results were not successfully confirmed in following studies, suggesting that probably the results were biased because of an inadequate pool of individuals studied (Kurtzke 1982; Otero-Siliceo et al., 1997).

ALS incidence seems to be higher than the rest of the world in the Kii Peninsula (Japan) and the island of Guam in the pacific (Reed et al., 1975a, 1975b; Kurtzke et al., 1982) Some risk factors seem to have been identified. In the island of Guam, the Chamorro population, a native population, seems to develop an ALS-similar disorder (Reed et al., 1975a, 1975b; Brody et al., 1995), the ALS-Parkinson's Disease complex (ALS-PD). It has been found that these populations include cycad nuts in their diets. Steryl glycosides have been identified in cycad nut flour and they seems to exert an excitotoxic mechanism in culture and mice (Ly et al., 2007).

A possible association between ALS and sport has been proposed. Football players, especially in Italy, seem to have a higher incidence of ALS, when compared to the mean population (Belli et al., 2005; Chio et al., 2005). It is not yet understood what could cause ALS in football players. Whether it is due to a genetic predisposition, environmental risk factors or substances that football players are administered during their sport career is not yet understood.

Other studies have proposed exposure to lead and pesticides as risk factors (Qureshi et al., 2006, Kamel et al., 2005). On the other hand risk factors like cigarette smoke have given controversial results. In one study cigarette smoke was found to be not a risk factor for developing ALS (Fang et al., 2006) but in another one it was (Veldink et al. 2005a). Another proposed risk factor would be constant stress applied to motoneurons, that is from excessive exercise. Again, controversial results were obtained regarding physical activity as a risk factor. In one study, high physical activity was associated with an earlier age of onset (Veldink et al., 2005a) . In another study however, sport practice was not associated (Valenti et al., 2005). Care should be taken when risk factors are involved, because risk factors can behave differently in various populations and the studies described before were performed in different populations.

Requierements for the diagnosis of ALS

Because symptoms of ALS can be similar to those of a wide variety of other, more treatable diseases or disorders, appropriate tests must be conducted to exclude the possibility of other conditions. Infectious diseases such as human immunodeficiency virus (HIV) infection (Verma et al., 2006; MacGowan et al., 2001), human T-cell leukemia virus (HTLV) (Silva et al., 2005; Matsuzaki et al., 2000), Lyme disease (Hansel et al., 1995), syphilis (Malin et al., 1986) and tick-

borne encephalitis (Müller et al., 1975) viruses can in some cases cause ALS-like symptoms. Neurological disorders such as multiple sclerosis, post-polio syndrome, multifocal motor neuropathy, and spinal muscular atrophy can also mimic certain facets of the disease and should be considered by physicians attempting to make a diagnosis.

The diagnosis of Amyotrophic Lateral Sclerosis (ALS) is based on El Escorial criteria (Fig. 4) and requires the presence of:

1. Evidence of lower motor neuron (LMN) degeneration by clinical, electrophysiological or neuropathologic examination.
2. Evidence of upper motor neuron (UMN) degeneration by clinical examination.
3. Progressive spread of symptoms or signs within a region or to other regions, as determined by history or examination.

Together with the absence of:

1. Electrophysiological and pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration.

2. Neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.

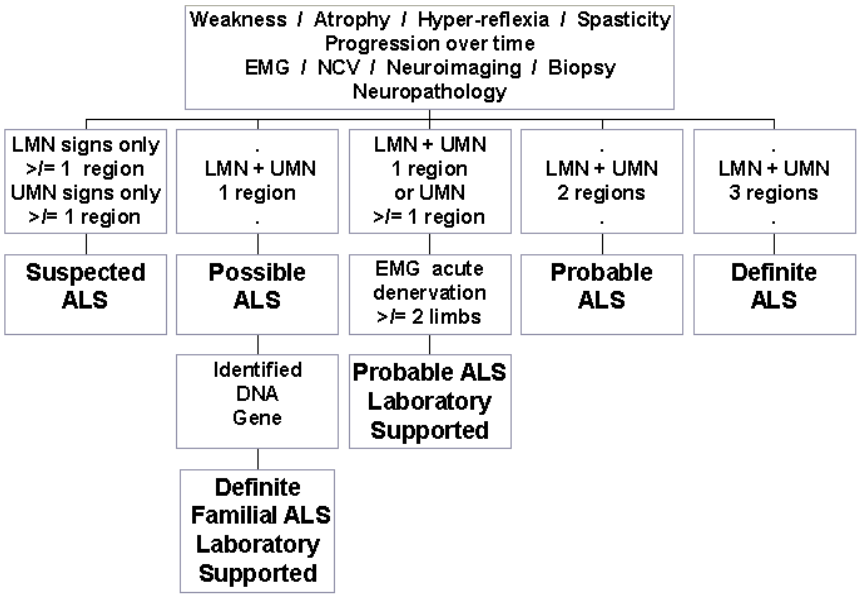


Fig 4. From Subcommittee on Motor Neuron Diseases of World Federation of Neurology Research Group on Neuromuscular Diseases, El Escorial "Clinical Limits of ALS" Workshop Contributors (1994). El Escorial World Federation of Neurology criteria for the diagnosis of amyotrophic lateral sclerosis, Journal of the Neurological Sciences 124 : 96-107.

Recently, through proteomic studies, three proteins were found in significantly lower concentration in the cerebral spinal fluid of patients with ALS than in healthy individuals

(Pasinetti et al, 2006). These proteins were identified as a 13.4kDa protein (cystatin C), a 4.8kDa protein (a proteolytic fragment of the neuroendocrine specific protein VGF) and an additional 7.6kDa protein that could not be identified because of low-abundance.. Evaluating the levels of these three proteins proved 95% accurate for diagnosing ALS. Elevated angiogenin serum levels (Cronin et al. 2006) have also been linked to ALS diagnosis but they need further validation.

Other reports show increased NOGO-A levels and decreased NOGO-C levels in ALS diagnosed patients from muscle biopsies and post-mortem samples (Dupuis et al, 2002) NOGO-A is a potent inhibitor of neurite outgrowth in the central nervous system (Chen et al. 2000). It gives rise also to isoforms B , C, D and E through alternative splicing and is not normally expressed in muscle. Why NOGO-A is expressed in muscle from ALS patients is not understood but it can serve as another diagnosis biomarker that can be evaluated from a muscle biopsy. With current methods, the average time from onset of symptoms to diagnosis is around 12 months. Since once the disease has been diagnosed, there is a life expectancy of six to twelve months, a fast diagnosis would allow patients to receive relief from symptoms at an earlier stage.

About 75% of patients experience limb onset ALS. In some of these cases, symptoms initially affect one of the legs, and patients experience awkwardness when walking or running or they notice that they are tripping or stumbling more often. Other limb onset patients first see the effects of the disease on a hand or arm as they experience difficulty with simple tasks requiring manual dexterity such as buttoning a shirt, writing, or turning a key in a lock. About 25% of cases are bulbar onset ALS. These patients first notice difficulty speaking clearly. Speech becomes garbled and slurred. Nasality and loss of volume are frequently the first symptoms. Difficulty swallowing, and loss of tongue mobility follow. Eventually total loss of speech and ability to protect the airway when swallowing are experienced.

Regardless of the part of the body first affected by the disease, muscle weakness and atrophy spread to other parts of the body as the disease progresses. Patients have increasing problems with moving, swallowing (dysphagia), and speaking or forming words (dysarthria). Symptoms of upper motor neuron involvement include tight and stiff muscles (spasticity) and exaggerated reflexes (hyperreflexia) including an overactive gag reflex. Symptoms of lower motor neuron degeneration include muscle weakness and atrophy, muscle cramps, and fleeting twitches of muscles that can be seen under the skin (fasciculations). Around 15–45% of patients experience pseudobulbar effect, also known as "emotional lability", which consists of uncontrollable laughter or crying.

The disease usually does not affect cognitive abilities and patients are aware of their progressive

loss of function and may become anxious and depressed though. A small percentage of patients go on to develop frontotemporal dementia characterized by profound personality changes (Valdmanis et al., 2007; Yokota et al., 2006). A larger proportion of patients experience mild problems with word-generation, attention, or decision-making. Cognitive function may be affected as part of the disease process or could be related to poor breathing at night (nocturnal hypoventilation). Because of diaphragm and intercostal muscle atrophy, most people with ALS die of respiratory failure or pneumonia, not the disease itself.

Unfortunately no cure has been developed so far. The only drug that has been approved for human use is riluzole, which seems to extend survival by two months (Bensimon et al., 1994; 1996; Miller et al. 2007). Patients can benefit from supportive care therapy that aims to relieve symptoms and increase quality of life. In the end stage of the disease, patients are forced to live with a respirator to support ventilation.

Anatomic-pathologic-morphologic overview

From the anatomic-pathologic-morphologic point of view, protein aggregates (Bruijn et al., 1997, 1998; Gurney et al., 1994; Watanabe et al., 2001; Wong et al., 1995), neurofibrillary tangles ((Hirano et al., 1984; Mendonça et al., 2005; Mizusawa et al., 1989; Rouleau et al., 1996; Sobue et al., 1990; Wong et al., 2000), and SOD1 and/or ubiquitin immunoreactive inclusions (Deng et al., 2006; Furukawa et al., 2006; Johnston et al., 2000; Jonsson et al., 2004, 2006; Wang et al., 2003, 2005) have been reported in post-mortem samples of ALS patients and animal models (Fig. 5).

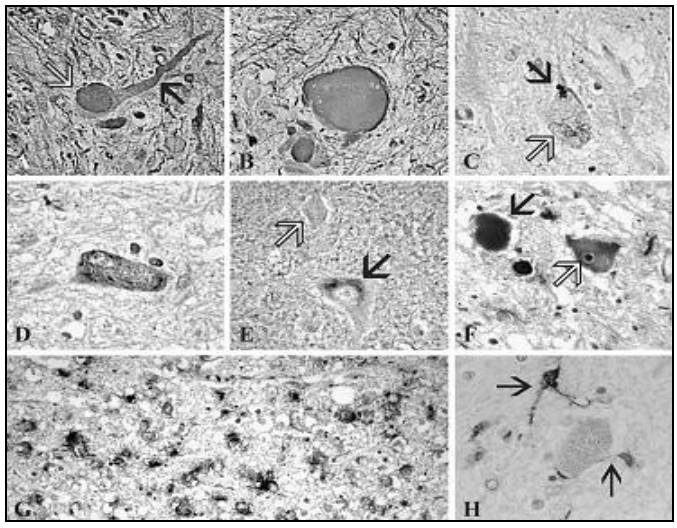


Fig. 5. Immunohistochemical features of pathology in ALS. (A) Ventral horn motor neuron immunoreactive to phosphorylated NF; (B) Neuroaxonal spheroids immunoreactive for neurofilament; (C) Degenerating motor neurons can be identified by ubiquitin immunoreactivity, either as skeins (white arrow) or as dense aggregates (black arrow); (D) Motor neurons are also immunoreactive to nitrotyrosine; (E) ALS motor neurons demonstrate diffuse perikaryal staining of α-internexin (black arrow); (F) Another diffuse perikaryal immunoreactivity for peripherin in a neuron containing an ubiquitin-immunoreactive Lewy-like body (white arrow) together with intense neuroaxonal spheroid immunostaining (black arrow); (G) Microglial activation observed as lipid-laden phagocytic microglia in degenerating corticospinal tracts

Protein aggregation is common a common feature of other neurodegenerative diseases such as

Alzheimer's disease (Kosik et al., 1984), Huntignton's disease (DiFiglia et al., 1997) and Kennedy's disease (Merry et al., 1998).The role of these protein aggregates in ALS is not understood.

A strong gliosis is also observed in the late stage of the disease in the spinal cord of animal models and ALS human patients (Kushner et al., 1991; Nagy et al., 1994; Schiffer et al., 1996; Howland et al. 2002). This gliosis could result from the activation from glial cells attempting to repair the damaged motoneuron sites by creating scars (Kalderon et al., 1990).

Fragmented Golgi apparati have been reported both in asymptomatic transgenic ALS mice (Mourelatos et al., 1996) and ALS patients (Gonatas et al., 1992; Fujita et al., 2000) as well as swollen and vacuolized (Fig. 6) mitochondria (Afifi et al. 1966; Xu et al., 2004). Muscle biopsies from transgenic animal models and human patients also reveal abnormalities such as elevated amyloid-precursor protein levels (Koistinen et. al, 2006). No other tissues/organs seem to develop any kind of abnormalities.

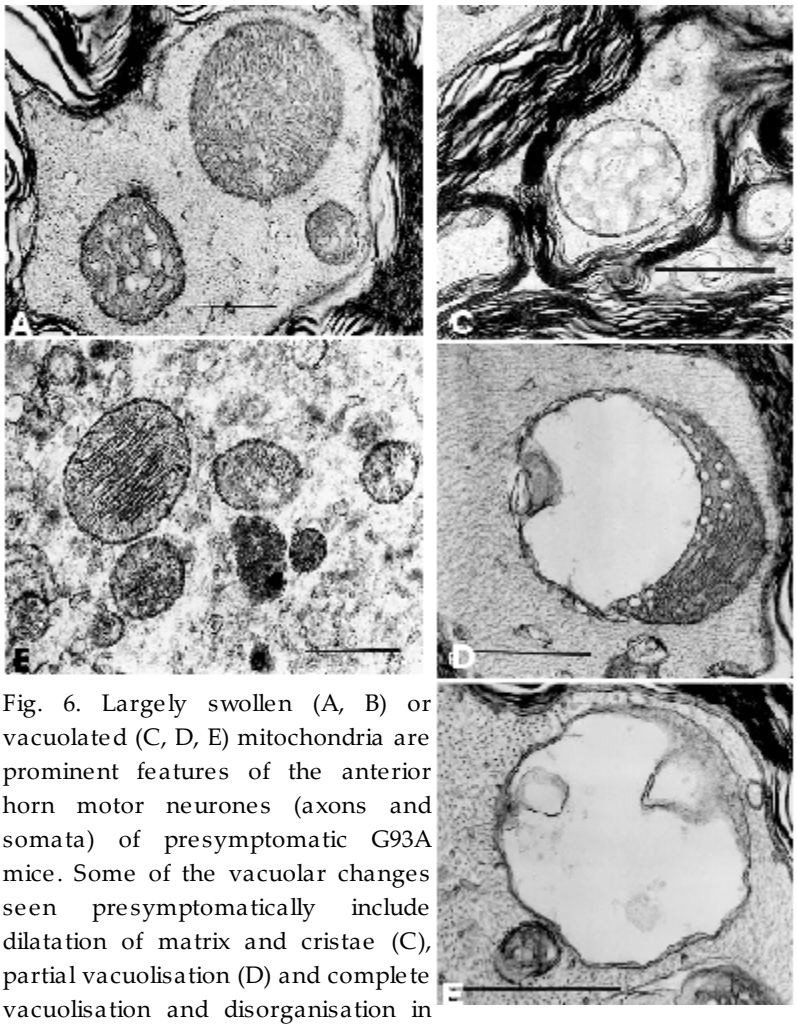


Fig. 6. Largely swollen (A, B) or vacuolated (C, D, E) mitochondria are prominent features of the anterior horn motor neurones (axons and somata) of presymptomatic G93A mice. Some of the vacuolar changes seen presymptomatically include dilatation of matrix and cristae (C), partial vacuolisation (D) and complete vacuolisation and disorganisation in the inner compartment

Genetics of ALS

When we speak about ALS, we are actually speaking about a heterogeneous group of motoneuron degenerative disorders. Both sporadic and hereditary cases have been reported. Sporadic cases are those considered when no other family member is affected by the disease, these account for about 90% of total cases. Hereditary cases on the other hand account for about 10% of total cases. Several loci have been associated to the disease from genetic studies and the causative genes for few of these loci have been reported:

Disease	Protein	Locus	Transmission	References
Typical ALS				
ALS1 (SOD1)	cytoplasmic superoxide dismutase	21q22.1	AD/AR	Rosen et al.,1993
ALS3	?	18q21	AD	Hand et. al, 2002
ALS6	?	16q12	AD	Abalkhail et al., 2003; Ruddy et al., 2003; Sapp et al., 2003
ALS7	?	20p13	AD	Sapp et al. 2003
ALS with dementia				
ALS-FTD	?	9q21-22	AD	Hosler et al. 2000; Ostojic et al. 2003
ALS-FTDP	MAPT	17q21.1	AD	Clark et al., 1998; Hutton et al., 1998
Atypical ALS				
ALS8	VAPB	20q13.3	AD	Nishimura et al. 2004
Progressive lower motoneuron disease	DCTN1	2p13	AD	Puls et. al. 2003
Other MNDs sometimes referred as ALS				
ALS2	ALSin	2q33	AR	Hentati et al. 1994; Hadano et al. 2001; Yamanaka et al. 2006
ALS4	SETX	9q34	AD	Chance et al. 1998; Chen et al. 2004
ALS5	?	15q15.1-q21.1	AR	Hentati et al. 1998
Mitochondrial genes				
ALS-M	COX1	mtDNA	Maternal	Comi G.P., 1998
ALS-M	IARS2	mtDNA	Maternal	Borthwick G.M., 2006

Players in Amyotrophic Lateral Sclerosis

Cytosolic superoxide dismutase (SOD1)

Superoxide ions are normally produced in the cell as a byproduct of metabolism (including mitochondria as a by-product of oxidative phosphorylation). Superoxide ions like other free

radicals, if not scavenged, can damage the cellular DNA, cell membranes and other cellular organelles. Cytosolic superoxide dismutase (SOD1) is an enzyme in charge of scavenging free superoxide ions and transforming them into peroxide (McCord and Fridovich, 1969) through the following reaction:

$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

Peroxide is then transformed into water by catalase according to the following reaction:

$2H_2O_2 + O_2 \rightarrow 2H_2O + 2O_2$

SOD1's biologically active form is actually a dimer (Fig. 7) and has a catalytic core formed by a copper and a zinc atom. The catalytically active atom is copper, whereas the zinc atom seems to have a structural role. (Carrico and Deutsch, 1970; Keele et al., 1971; Richardson et al., 1972; Hartz and Deutsch, 1972). Disulphide bonds are crucial in keeping the dimeric structure (Keele et al., 1971; Hartz and Deutsch, 1972; Arnesano et al., 2004).

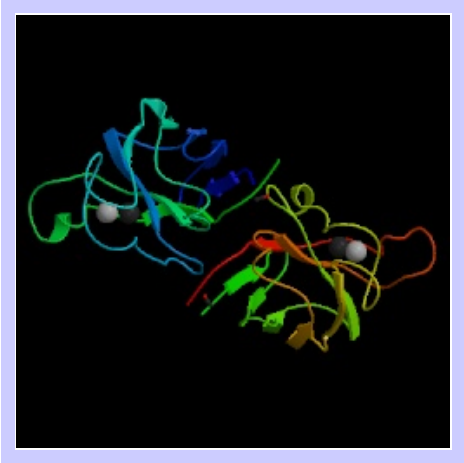


Fig. 7. SOD1 structure. Protein Data Bank entry 1SPD (1 copper and 1 zinc atom are outlined in each monomer)

SOD1 was shown to be the first proven cause of ALS when mutated, as described by Rosen (Rosen et al., 1993), through a post-mortem genetic, pathological and biochemical study on ALS patients. SOD1 mutations (ALS1) are responsible for 20% of all hereditary cases. Over 114 mutations in the human SOD1 gene have been reported and can be viewed on the ALSOD database (www.alsod.org). These mutations are spread all over the protein sequence including the metal binding residues (four histidines coordinate the copper atom). It is surprising that so many mutations can be found in such a small protein, consisting of 153 aminoacids. Most mutations are aminoacidic substitutions while others are insertions and truncations. Whilst most mutations are dominant, the D90A mutation shows to be recessive in Scandinavian populations (Robberecht et al., 1996). In fact in other world populations, the same D90A mutation is transmitted in a dominant

fashion. Therefore the environment or other factors must play a crucial role in developing the disease.

Recently, it has been reported that mutant cytosolic SOD1 can also be secreted with chromogramins in vescicles by motoneurons that can activate microglia (Urushitani et al., 2006). Activated microglia could then release factors that are toxic for motoneurons.

Two other superoxide dismutases are known: a mitochondrial manganese superoxide dismutase (SOD2) and an extracellular superoxide dismutase (SOD3) but they have not been associated with ALS.

Tau and ApoE

Tau is a protein involved in neurofilament assembly and organization and therefore also contributes to cytoskeletal stability. Another protein, ApoE, binds and protects Tau from hyperphosphorylation. In Alzheimer's Disease, tau might be hyperphosphorylated when bound by the ε4 polymorphism which seems to bind tau less tightly (Thaker et al., 2003) Hyperphosphorylated tau would then form aggregates with other proteins and would no longer bind to neurofilaments. Neurofilaments lacking tau binding would become disorganized and form tangles inside the cell. It is interesting to note that in transgenic ALS models, increased ApoE ε4 levels have been reported which could suggest a link with ALS (Haasdijk et al., 2002)

VAPB

Vesicle-associated membrane protein-associated protein B is a type IV 33 kD membrane protein found in plasma and intracellular vesicle membranes and can associate with microtubules. It has been implicated in endoplasmic reticulum to Golgi transport, while other functions in membrane transport have been postulated, in particular its role in axonal transport of membrane components (from previous evidence Skehel et al., 2000). A P56S mutation was identified in Brazilian families of Portuguese origin (Nishimura et al., 2004). The phenotype is characterised by slowly progressive lower motor neuron symptoms in all patients, accompanied, in most instances, by postural tremor, cramps and fasciculations, representing an atypical ALS. One fourth of the affected individuals in each family displayed a late-onset type of Spinal Muscular Atrophy (SMA), whilst a small proportion of patients developed symptoms of classic ALS (Nishimura et al., 2004)

Dynactin

Motoneurons are particular cells because they can have very long axons, (up to one meter when we talk about lower motoneurons that contact leg muscles from the spinal cord). Therefore the motoneuronal cytoskeleton must be very well organized so that trophic factors can be transported to the cell body, vescicles can be secreted, and cellular organelles can be transported all along the axon, especially mitochondria. The dynein-dynactin complex is one of the most important cargo complexes in motoneurons and is part of the major retrograde transport motor. Mutations in the p150 subunit of dynactin, a component of the dynein complex, have been reported and cause a lower motoneuron disorder (Puls et al. 2003). Since this complex is only expressed by motoneurons, in this case we can talk about pure ALS, because only motoneurons express the mutant protein. For all other cases, most mutant proteins that cause ALS seem to be ubiquitary or at least to be expressed by several cell types.

ALSin

ALSin is a 184kD protein associated with a recessive form of ALS (ALS2), first reported in a Tunisian family (Yang et al. 2001). Linkage analyses associated this variant to the ALS2 locus. Bioinformatic analyses identified three domains in this protein conserved in other GEF proteins: RCC1-like (chromatin condensation), RhoGEF and VPS9 (protein vacuolar traffic). All these GEF members interact with Ras GTPases and RhoGEF has been shown to interact with the cytoskeleton. An additional domain, a pleckstrin-like domain, has been identified in ALSin. Pleckstrin is a protein involved in signal transduction and cytoskeletal remodelling. Alsin seems to interact with mutant (Kanekura et al. 2004) but not wild-type SOD1 through its RhoGEF domain, which could suggest a link between the two types of ALS. It is therefore possible that ALSin interacts with the motoneuronal cytoskeleton. However, because a transgenic mouse model that expresses ALSin mutations, develops a disease very similar to spinal muscular atrophy (SMA) (Yamanaka et al. 2006), the ALSin model should rather be used to study SMA rather than ALS.

Senataxin

Senataxin is a 303 kDa protein with a DNA/RNA helicase domain with homology to human RENT1 and IGHMBP2 — two genes that encode proteins involved in RNA processing (Chen et al. 2004, 2006). Affected individuals present missense mutations and develop ALS-like symptoms.

Mitochondrial genes

Defects in two mitochondrial genes cause motor neuron disorders with clinical features that are suggestive of ALS.. A 5 bp deletion in the mitochondrial gene cyclooxygenase 1 (COX1) results in early adult onset of corticospinal motor neuron loss (Comi et al. 1998). By contrast, a 4272T->C mutation in mitochondrial transfer RNA (isoleucine) causes a late onset, slowly progressive, predominantly lower motor neuron disease (Borthwick et al., 2006)

More players in ALS?

Other genes seem to cause sporadic cases of the disease or could potentially be involved in ALS pathogenesis but no genetic association studies have reported any hereditary cases:

Gene	Protein	Localization
NF-H	neurofilament heavy chain	22q12.2
NF-L	neurofilament light chain	8q21
ANG	angiogenin	14q11.1
PRPH	Peripherin	12q12-13
EAAT2 (GLT-1)	glial glutamate transporter	11p12-13
AMPA	kainic glutamate transporter	5p33
ApoE	Apolipoprotein E	19q13.2
CNTF	Ciliary neurotrophic factor	11q12.2
VEGF	Vascular endothelial growth factor	6p12
Proteasome complex	Several subunits	Several chromosomes
Chaperones	Several proteins	Several chromosomes
SMN	Survival motoneuron	14q13
NAIP	Neuronal apoptosis inhibiroty protein	5q13.1

Neurofilaments, peripherin and interleukin-6

Neurofilaments are constituted of 3 different kinds of chains: heavy (NF-H), intermediate (NF-M) and light (NF-L). They were among the first genes suspected of causing ALS, since neurofilament accumulations are a common hallmark of both familial and sporadic ALS cases (Hirano et al. 1984) and are also present in transgenic mice that overexpress mutant SOD1 (Gurney et al., 1994; Bruijn et al. 1997). Transgenic mice that overexpress NF-H and NF-L subunits develop an age-dependent motoneuron disorder (Cote et al. 1993; Cleveland et al. 1996). Despite this evidence, exhaustive screening of the NF genes in patients has not found any mutation linked to the disease (Garcia et al 2006) eventhough dominant point mutations in NF-L have been linked to Charcot-Marie-Tooth disease (De Jonghe et al. 2001; Jordanova et al. 2003), a milder motoneuron disorder. NF-H in-frame insertions or deletions within the normal 44-45 KSP (lysine-serine-proline) repeats in the tail domain have been reported in about 1% of sporadic cases (Figlewicz et al., 1994; Tomkins et al.,

1998; Al-Chalabi et al., 1999).

Another player that could involve motoneurons in ALS is peripherin. Peripherin is another neuronal intermediate filament protein. A frameshift mutation that disrupts correct peripherin assembly has been reported in one sporadic ALS case (Gros-Louis et al., 2004). Transgenic mice that overexpress peripherin develop an ALS-like phenotype such as motoneuronal degeneration, muscle atrophy and fibrillary tangle accumulation inside motoneurons (Beaulieu et al. 1999). A peripherin neurotoxic splice variant has been reported in ALS mice (Robertson et al., 2003). Peripherin levels could be increased due to the action of inflammatory cytokines such as IL-6 (Sterneck et al., 1996; Beaulieu et al., 2002). It has been reported that IL-6 levels seem to increase with aging in healthy subjects (Wei et al., 1992) and mice (Ye et al., 1999). Therefore IL-6 could be another risk factor for ALS. It seems that mutations in neurofilament chains do not directly cause the disease but could be considered as risk factors that increase the chance of developing ALS.

Angiogenin

This protein has one known function to be an intranuclear RNAse (Shapiro et al., 1986; Palmer et al., 1986) that can aid RNA synthesis, at least from what reported in endothelial cells (Xu et al., 2002). Angiogenin, as its name suggests is involved angiogenesis but it is also expressed in the central nervous system, including motoneurons. Seven missense mutations were identified in 15 familial ALS patients and in 11 sporadic ALS patients (Greenway et al., 2006). Most mutations are located in the catalytic core of the protein and one of them in the nuclear localization signal, which suggest these mutations lead to a loss-of-function, resulting in ALS. However angiogenin mutations have only been found in Scottish and Irish populations but no other populations in the world, therefore ALS cases due to angiogenin mutations seem to be very rare and restricted to certain populations and thus contribute very little to hereditary cases.

CNTF

Ciliary Neurotrophic Factor is a neurotrophin that activates survival signals in neurons. Decreased levels of CNTF have been observed in ALS patients in cortico-spinal tract neurons (Ono et al., 1999), while a knock-out animal model for CNTF develops a progressive motoneuron degeneration (Masu et al., 1993). Noteworthy, CNTF has shown to be neuroprotective in transgenic Huntington Disease models where polyglutammine rich huntingtin aggregates (Emerich et al., 1997a, 1997b).

VEGF

Vascular Endothelial Growth Factor is a 41kD growth factor which for a long time was believed to be involved strictly in angiogenesis but recently it has been reported that VEGF itself can act as a survival growth factor on several neuronal populations, including motoneurons (Oosthuyse et al., 2001; Van Den Bosch et al., 2004) It has been reported that VEGF activates a survival cascade where IP3-Kinase and Akt are involved (Li et al., 2003). Interestingly, it was discovered by chance in a transgenic mouse model, when the hypoxia responsive element (HRE) was removed from the VEGF promoter, as part of a study on VEGF regulation and hypoxia, that the animals developed an ALS-like phenotype (Oosthuyse et al., 2001). The animals suffered from motoneuronal degeneration, muscular atrophy and neurofibrillary tangle accumulation. Screening of the VEGF gene promoter and the HRE element found no link between HRE variants and disease in humans (Gros-Louis et al., 2003) but two haplotypes in other regions of the promoter showed an increased risk of developing ALS in some populations (Lambrechts et al., 2003; Fernández-Santiago R., 2006).

Proteasome complex and ubiquitin

Ubiquitin is an 8kD protein that is bound to lysine residues on proteins that need to be degraded. The tagged proteins are then transported into the proteasome complex and they are degraded. In cell culture experiments, when the proteasome is inhibited, protein aggregates can be observed in the cells (Wyttenbach et al., 2000). Moreover, selective motoneuron death in organotypic cultures has been reported when the proteasome system is inhibited (Tsuji et al., 2005). It is hypothesized that protein aggregates could choke the proteasome machinery contributing to the apoptotic signal in the cell. Increased expression levels of the proteasome and ubiquitin have been reported in ALS patients when compared to controls (Mendonca et al., 2006).

Chaperones

Heat-shock proteins or chaperones are proteins that are responsible for the correct folding of proteins once they have been synthesized. When chaperones act on a neo-synthesized protein, the protein is forced to acquire its correct 3-D conformation. In cellular models of neurodegenerative diseases where protein aggregation is known (such as spinal bulbar muscular atrophy, spinal cerebellar ataxia, Alexander's disease and Huntington's disease), protein aggregates that show heat-shock protein immunoreactivity have been reported (Wyttenbach et al., 2000; Der Perng et al., 2006; Mitsui et al. 2002) suggesting that these proteins either attempt to correct the abnormal

folding of aggregated proteins or that chaperones get trapped and are sequestered from other important pathways in the cell. As it is known, some heat-shock proteins seem to posses an anti-apoptotic function as well (Brar et al., 1999; Wagstaff et al., 1999). In a transgenic ALS mouse model, treatment with arimoclomol increased survival by some days. Arimoclomol induces the phosphorylation-mediated activation of the HSP inducing factor HSF-1, thereby leading to increased levels of Hsp70 and Hsp90 in spinal cords (Kieran et al., 2004).

SMN and NAIP

Survival Motoneuron (SMN) is a 34kD protein and belongs to the family of Gemins, a set of proteins involved in RNA splicing. SMN is actually present as two copies on chromosome 5 (SMN1, SMN2), however the only functional protein seems to be SMN1. SMN2 undergoes one exon skipping because of one nucleotide difference (Lorson et al., 1999; Monani et al., 1999). SMN1 mutations are responsible for another motoneuron disease called Spinal Muscular Atrophy (SMA) which affects lower motoneurons at childhood (Lefebvre et al., 1995; Rodrigues et al., 1995). In another association study, SMN1 copy number has been proposed as a risk factor for sporadic ALS (Veldink et al., 2005; Corcia P., 2006).

Neuronal-apoptosis inhibitor protein (NAIP) is a protein that belongs to a family of antiapoptotic proteins. Mutations in this gene have been associated with SMA cases (Roy et al., 1995). In a study on an ALS transgenic mice, a modifier locus was mapped very close to the NAIP gene, suggesting that this gene or region could be involved in the penetrance of other causative genes of ALS (Kunst et al., 2000). Despite these findings, no modifier loci or genes have been found in humans, in fact, up to date, mutations in NAIP are considered to be SMA specific (Parboosingh et al., 1999).

Available ALS disease models

The first ALS transgenic model was developed by Gurney et al. in 1994. This model carries 8-9 tandem copies of the human SOD1 gene (cDNA) with a G93A mutation (haploid genome). These transgenic mice develop a progressive muscular atrophy and die around 4-5 months of age. Nowadays, several transgenic models based on mutant SOD1 have been developed including transgenic rats (Nagai et al., 2001) that also express mutant human SOD1 and also develop similar symptoms. It is important to state that there are SOD1 models that carry a low copy number and a high copy number of the human SOD1 gene in tandem and that the number of gene copies modifies disease onset and progression times before the animals die.

Mutation	Transgene copy number (diploid)	Onset (days)	Days before death (duration)	Reference
hG37R	42	120	ND	Wong et al., 1995
hG85R	148	240	7-14	Brujin et al., 1997
hD90A heterozygous	1	130	600	Brannstrom et al., 2000
hDA90A homozygous	2	ND	ND	Brannstrom et al., 2000
hG93A	25	90	< 60	Dal Canto et al., 1997
hG93A	18	130	< 70	Gurney et al., 1994, Dal Canto et al., 1997
hG93A	10	290	> 110	Dal Canto et al., 1997
hG93R	ND	230	10-13	Friedlander et al., 1997
hI113T	ND	330	slow	Kikugawa et al., 2000
mG86R	2	100	3	Ripps et al., 1995

h = human gene m= mouse gene ND= not documented

In addition to the transgenic SOD1 model, more models have been developed to study motoneuronal degeneration.

- Wst (wasted). This model has a 15.8kB deletion in the gene that codes for eEF1A-2 (translation elongation factor 1A-2). The deletion removes the promoter and the first exon in the gene which block its expression (Chambers et al., 1998). EF1A2 protein is only expressed in differentiated cells from the central nervous system. Homozygous wst^{-}/wst^{-} mice die about four weeks after birth and develop motoneuronal degeneration and abnormal accumulation of perikaryal phosphorylated neurofilaments (NF-H) (Lutsep et al., 1998).
- Nmd (neuromuscular degeneration). This model is based on an automosal recessive mutation in the gene that codes for Ighmbp2 (mu 2 immunoglobulin – binding protein). This protein works as a transcription activator and as a DNA helicase as well (Cox et al., 1998). Nmd mice develop a progressive paralysis and die about 4 weeks after birth. Because this gene is ubiquitously expressed, it is not understood why only motoneurons are affected in this model.
- As stated earlier, deletion of the HRE from VEGF causes motoneuronal degeneration in mice (Oosthuyse et al., 2001), so this can also be considered as a motoneuron disease model.

The transgenic mouse SOD1 model has been so far the most widely used. The first symptom transgenic mutant animals develop is slight twitching of the anterior limbs. Also, when mutant animals are held by the tail, they are not able to stretch their hindlimbs as a normal reflex when

compared to control littermates (Fig. 8)

In the late phase of the disease, the hindlimbs paralyze as well as the anterior limbs (Fig.9). Death usually occurs by asphyxia due to diaphragm paralysis.

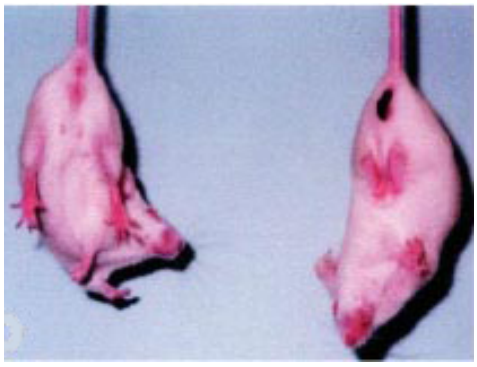


Fig. 8. Notice how the G93A animal is not able to extend its legs when lifted by the tai (right)l. Non-transgenic and wild-type animals on the other hand are able to extend their legs (left)

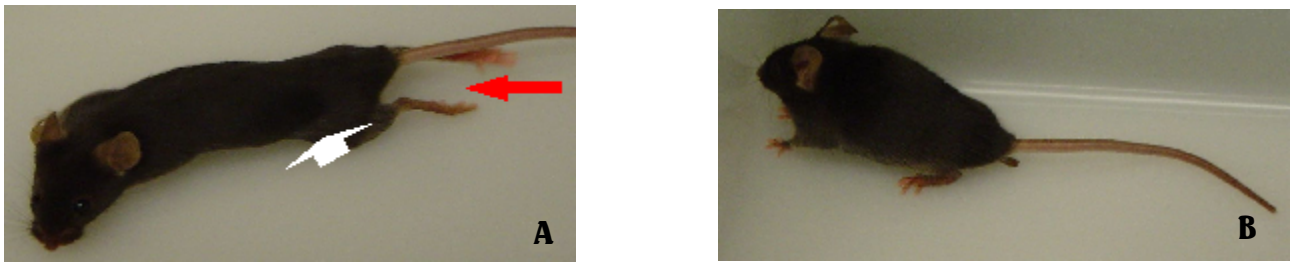


Fig. 9. Phenotype of *SOD1*^{G93A} mice (A) at the end stage of the disease, compared to a non-transgenic (B) littermate. Notice splayed hind limbs (red arrow) and pronounced muscle wastage (white arrow) in transgenic animal.

Transgenic SOD1 models have been further studied and manipulated in order to understand the mechanisms of disease in ALS. The different kind of experiments developed with murine SOD1 models can be divided as shown below:

1. Experiments in which transgenic SOD1 mice were treated with different compounds to test for disease onset/progression changes. Eg: antioxidants (vitamin E, acetyl-cysteine), creatine, minocycline, COX2 inhibitors, caspase inhibitors, AMPA antagonists.
2. Experiments in which double transgenic mice were developed from transgenic SOD1 mice to test for the effect of another overexpressed gene in SOD1 mice. Eg: calcium binding proteins (parvalbumin), neurofilament subunits, growth factors such as HGF, bcl2, ICE D^{-}.
3. Experiments in which gene therapy/delivery was applied to transgenic SOD1 mice to test for disease onset/progression changes. Eg: BDNF , GDNF (Wang et al., 2002), IGF-1 (Kaspar et al., 2003), cardiotrophin-1 (Bordet et al., 2001), bcl-2 (Azzouz et al., 2000).

Experiment	SOD1 mutation	Survival, no treatment (days)	Survival with treatment (days)	References
1%-2% Creatine administration	G93A	143 ± 2	157 ± 3; 169 ± 5	Klivenyi et al., 1999
Ferric porphyrin administration	G93A	129 ± 2	136 ± 2	Wu et al., 2003
zVAD-fmk (wide spectrum caspase inhibitor) administration	G93A	103 ± 3	124 ± 7	Li et al., 2000
NBQX (AMPA antagonist)	G93A	129.9 ± 3.6	143.3 ± 2.8	Van Damme et al.., 2003
Minocyclin administration	G93A	126 ± 3	139 ± 2	Zhu et al., 2002
Nimesulide administration	G93A	108 ±	120 ±	Pompl et al., 2003
NF-H and NF-L overexpression	G37R	285 ± 84	474 ± 195	Kong et al., 2000
HGF overexpression	G93A	147 ± 2	175 ± 6	Sun et al., 2002
Parvalbumin overexpression	G93A	132 ± 2	146 ± 5	Beers et al., 2001
Interleukin converting enzyme (dominant negative) expression	G93R	249 ± 4	270 ± 7	Friedlander et al., 1997
Bcl-2 overexpression	G93A	240 ± 2	275 ± 4	Kostic et al., 1997

Understanding ALS pathogenesis

Even though SOD1 has been proved as one of the genetic caused of ALS, the disease mechanisms are still not well understood. Several mechanisms have been proposed to try to explain ALS pathogenesis: Oxidative stress, Protein aggregation, Axonal transport disruption, Excitotoxicity, Mitochondrial dysfunction and Inflammatory response.

Oxidative stress

In the beginning, researchers thought that SOD1 mutations led to disease because of loss of function (Deng et al., 1993). This implied that the free radical scavenging function of SOD1 was lost and therefore free radicals would damage the cell and lead to degeneration. Despite these first suspicions, it was later proved that several mutant types of SOD1 retain catalytic activity (Borchelt et al., 1994). Surprisingly, a knock-out mouse model where the endogenous SOD1 gene had been removed, had a normal lifespan and developed normally (Reaume et al., 1996). Also, when wild-type hSOD1 was expressed in transgenic mice that expressed mutant hSOD1 as well, the disease was exacerbated instead of slowed down (Jaarsma et al., 2000), suggesting that the concomitant presence of normal catalytic activity had no effect on disease course. At this point, it was thought that mutant SOD1 developed a toxic gain of function. As explained earlier, SOD1 is responsible for

the conversion of superoxide ions to peroxide and then peroxide is converted into water an oxygen by catalase. The oxidative stress hypothesis suggests that the catalytic core in SOD1 develops an aberrant chemistry and could even perform the reverse chemical reaction by producing more free radicals such as peroxynitrite (Beckman et al., 1993) that can harm the cell (Hall et al., 1998). Otherwise, mutant residues could bind less tightly the zinc atom and it could be lost. Since the zinc atom could be structurally needed for correct copper atom orientation, this could also contribute to abnormal catalytic activity of the copper atom. Copper Chaperone for SOD1 (CCS) is a chaperone that loads copper into the catalytic core of SOD1 (Culotta et al., 1997). Subramaniam et al. (2002) demonstrated that CCS null transgenic mutant hSOD1 mice, which express a catalytically-inactive mutant enzyme (no copper is present in the enzyme), still develop ALS. This suggests that oxidative stress is not the main cause of ALS, since copper is responsible for the catalytic reactions and is not anymore loaded into SOD1. Some scientists however, proposed that copper could still be loaded through alternative pathways because some activity was still observed in SOD1 from CCS null mice, which indeed cannot be excluded (Beckman et al., 2002). But it could also be possible that when SOD1 is extracted from mouse tissue, copper from the tissues binds to SOD1 and therefore creates an artifact. However, transgenic mice that express QUAD hSOD1 (hSOD1 in which the four copper-binding histidines are mutated) and therefore express copper-free hSOD1, still develop ALS (Wang et al., 2003).

Nonetheless, antioxidant therapy with N-acetyl-L-cysteine (Andreassen et al., 2000) and ferric porphyrin (Wu et al., 2003), proved effective to slightly increase survival of affected mice but had no effect on disease onset at all. It is therefore suspected that oxidative stress on its own is not the cause of the disease but could possibly contribute at some extent.

Protein aggregation

The term aggregate in ALS has actually a dual meaning:

- It refers to ubiquitin reactive protein inclusions, neurofibrillary tangles and hyaline inclusions, as described earlier.
- It refers to SOD-1 immunoreactive detergent-insoluble species that can be detected by biochemical methods from affected tissues (Wang et al., 2002, 2003).

As we stated before, protein aggregates have been found in both sporadic ALS and familial ALS cases, including SOD1 induced fALS in human patients and transgenic mice (Bruijn et al., 1997, 1998; Gurney et al., 1994; Watanabe et al., 2001; Wong et al., 1995; Shibata et.al., 1996). These observations led scientists to speculate that mutant SOD1 could directly form protein aggregates in

the cells or trigger their formation. In fact, cell culture experiments have shown that mutant SOD1 tends to form aggregates (Johnston et al., 2000; Wang et al., 2003; ref). Metal-free (holo) SOD1 has been shown to form amyloid-like filaments when grown in crystals (Elam et al., 2003). . Experiments on non-neuronal cells where the proteasome system was inhibited showed protein aggregate accumulation inside the cells (Wyttenbach et al., 2000; Turner et al., 2004). Also apoptosis induction in neuronal cells was observed after proteasome inhibition (Rideout et.al., 2002). In fact one of the proposed disease mechanisms is that these protein aggregates sequester essential proteins for cell survival such as heat shock proteins (Watanabe et al., 2001), choke the proteasome system and/or disrupt the cytoskeletal architecture. This would lead to incorrect neurofilaments assembly and fibrillary tangle formation. The latter would block axonal transport and trophic factor transport to the cell body and ultimately lead to cell death. To support this view, a presymptomatic deficit of slow axonal transport has been reported in transgenic mutant hSOD1 mice (Williamson and Cleveland, 1999, Zhang et al., 1997).

Another set of main characters in protein aggregation are chaperones. Chaperones or heat-shock proteins (HSPs) are responsible for the correct folding of proteins to render them fully functional. During stress conditions, HSPs are activated (Morandi et al., 1989; Cox et al., 1993) to correct protein structure of unfolded proteins (Chai et al., 1999). HSPs such as hsp25 and hsp70 have been proposed to interact with SOD1 and mutant SOD1 could sequester them (Okado-Matsumoto and Fridovich, 2002). Since some HSPs also have an antiapoptotic role this could contribute to the apoptotic cascade in the cell.

However some experiments have shown that elevated wild-type hSOD1 overexpression causes some motoneuronal degeneration in transgenic mice (Jaarsma et al., 2000). Still, these mice appear healthy all throughout their lives and no wild-type hSOD1 aggregates have been reported. Care should be taken because the overexpression level of hSOD1 in transgenic animals is far higher than physiological levels. It could be possible that if wild-type hSOD1 was expressed at physiological levels, probably no pathologic signs would develop in mice. Perhaps only the mutant protein is able to form aggregates. It has been reported that some mutant proteins (A4V) are less stable (Hough et al., 2004) and only form aggregates in mutant SOD1 mice when wild-type hSOD1 is overexpressed as well (Deng et al., 2006). This could stabilize the mutant protein by forming a heterodimer. Having said so, other reports show that the degradation of SOD1 itself and its aggregates is proteasome mediated (Basso et al., 2006; Niwa et al., 2002; Urushitani et al., 2004; Urushitani et al., 2002), because these SOD1 aggregates are heavily ubiquitinated and a specific ubiquitin ligase, dorfin, that acts on mutant but not wild-type SOD1 has been discovered (Niwa et

al., 2002). While proteasome malfunction has been implicated in motor neuron death, it is not yet established whether it is a cause or consequence of aggregate formation. Contradictory results have been reported. In mutant hSOD1 G93A mice which accumulate mutant protein to high levels, proteasome activity is downregulated in the lumbar spinal cord well before the development of symptoms (Kabashi et al., 2004), while in a different line of mice with lower accumulated levels of the same mutant, increased proteasome activity in the spinal cord has been reported at symptomatic stages (Puttaparthi and Elliott, 2005).

On the other hand, in spinal cord extracts of presymptomatic ALS mice, an overall decrease of chaperone activity has been reported which persists throughout disease course, and multiple recombinant SOD1 mutants inhibit chaperone function in vitro (Bruening et al., 1999; Tummala et al., 2005). SOD1 induced protein aggregates could also provoke organelle dysfunction by translocating inside or blocking their functions (see mitochondrial damage below).

A proposed mechanism of protein aggregate effect is summarized in Fig. 10.

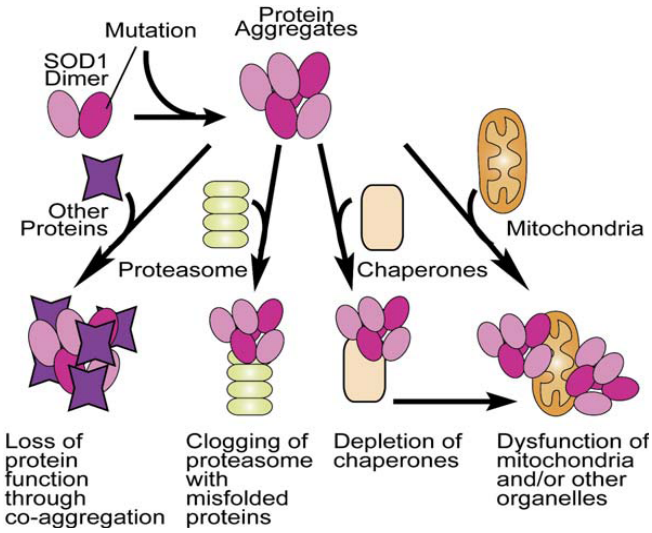


Fig. 10. Proposed protein-aggregation mechanism in ALS

It is interesting to point out that protein aggregates have also been reported in astrocytes and oligodendrocytes in ALS mouse models (Stieber et al., 2000). The direct implication of these aggregates in these two cell populations is still not understood. After having presented all this evidence, a clear idea of the role of protein aggregates in ALS pathogenesis is not known. Whether these aggregates are harmful or not, cause or consequence of the disease, still needs to be determined.

Axonal transport

Another characteristic of ALS is the reduced activity of axonal transport, described both in ALS

patients and ALS mice (Sasaki et al., 1996, 2005). The transport of molecules and organelles is a fundamental cellular process that is particularly important for the development, function and survival of neurons. This process is dictated by the highly polarized anatomy of neurons: axonal proteins are synthesized in the cell body and must be transported in an anterograde manner along the axons and dendrites to reach synapses, whereas substances such as peripherally located trophic factors must be transported centrally from the synaptic regions by retrograde transport. The molecular motors for anterograde and retrograde transport are kinesin and the dynein–dynactin complex, respectively (Fig. 11). Transport is conventionally regarded as either slow or fast. Presumably, functional and efficient axonal transport is particularly important for motor neurons, which are among the largest and longest cells in the body.

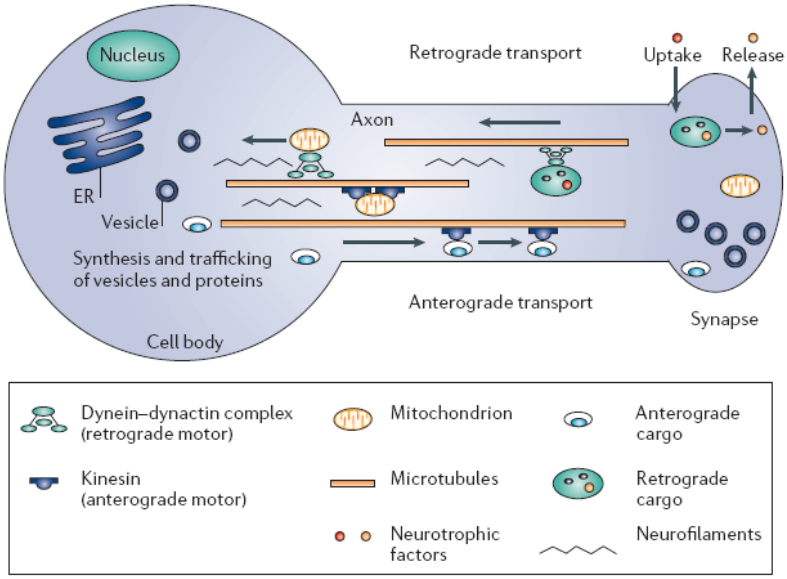


Fig. 11. Axonal transport schematic representation in motoneurons

Several findings indicate that defects in axonal transport might contribute to the demise of motor neurons in ALS. First, both slow and fast anterograde transport are slowed in transgenic G93A-SOD1 and G37R ALS mice prior to disease onset (Zhang et al., 1997; Borchelt et al., 1998; Williamson et al., 1999). Second, retrograde transport is also disrupted in ALS mice (Murakami et al., 2001). Third, although the molecular basis for this slowing is not fully elaborated, several authors suggest that aggregations of neurofilaments in the proximal axons (spheroids) might physically compromise the transport apparatus (Sasaki et al., 1996), at least for anterograde traffic. Neurofilaments have also been incriminated as modulators of axonal transport because they regulate axonal caliber (Rao et al., 2003). Diminution of retrograde transport in ALS mice has been attributed to the mislocalization and disruption of dynein function (Ligon et al., 2005).

As noted above, mutations in the $p150^{Glued}$ subunit of dynactin elicit an unusual form of human

lower motor neuron disease. Finally, in mice, disruption of the dynein–dynactin complex by the forced expression of dynamitin, a sub unit of dynactin, also produces a late onset motoneuron disease (LaMonte et al., 2002). Interestingly, when specific dynein mutations were introduced in transgenic mice expressing G93A mutant SOD1, an amelioration was observed (Kieran et al., 2005; Teuchert et al., 2006). This could derive from the fact that a toxic function performed by mutant SOD1 needs dynein-mediated transport along microtubules either in the axon or the cell body. All this observations suggest that axonal transport disruption is an important factor in ALS pathogenesis.

Excitotoxicity

Glutamate is one of the neurotransmitters used by neurons in the central nervous system. During glutamatergic neurotransmission, glutamate released from the presynaptic neuron activates ionotropic glutamate receptors present on the postsynaptic neuron. Activation of these glutamate receptors results in the influx of Na+ and Ca2+ ions into the cell, leading to depolarization and ultimately to the generation of an action potential. Once glutamate has been released for transmission, it is removed from the neuronal synapse by a glial glutamate transporter (GLT-1/EAAT2) expressed on the plasma membrane of astrocytes (Fig. 12).

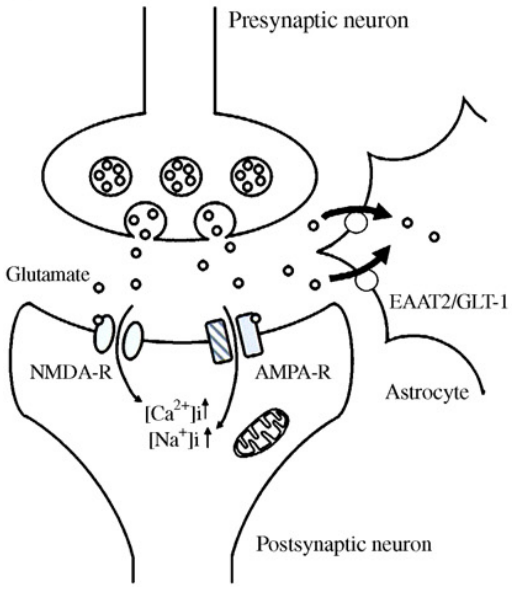


Fig 12. Schematic representation of glutamate release and uptake at a neuronal synapse

Excitotoxicity is the neuronal degeneration caused by over-stimulation of the glutamate receptors (Olney et al., 1978). At first, the NMDA receptor was considered to be uniquely responsible for

excitotoxicity (Choi et al., 1998). More recently, it became apparent that the activation of AMPA receptors is at least as important (Prehn et al., 1995). The predominant mediator of neuronal injury is Ca2+ influx through NMDA receptors, Ca2+-permeable AMPA receptors or voltage-gated Ca2+ channels (Kim et al., 1987; Carriedo et al., 1996; Van Den Bosch et al., 2000). Excessive influx of Ca2+ ions can result in the activation of several enzymes, such as lipases, phospholipases, caspases (Takadera et al., 1997), endonucleases, protein phosphatases, protein kinase C, xanthine oxidase and NO synthase. As it is well known, caspases induce programmed cell death or apoptosis. This can happen if glutamate is not removed from the synaptic cleft.

Two types of excitotoxicity can be defined: classical and slow excitotoxicity (Doble, 1999). Classical excitotoxicity refers to neuronal degeneration that occurs after an increase of the extracellular glutamate concentration, while slow excitotoxicity is the death of a weakened postsynaptic neuron in the presence of normal synaptic glutamate levels. Acute elevations of glutamate are thought to induce neuronal damage in conditions such as stroke, status epilepticus and neurotrauma. More chronic and milder elevations of glutamate are believed to underly excitotoxicity in neurodegenerative diseases.

Increased CSF glutamate levels have been reported in ALS patients when compared to controls (Rothstein et al., 1990; Plaitakis et al., 1988; Shaw et al., 1995). In addition, from human post-mortem tissue and transgenic models that express mutant SOD1, decreased levels of EAAT2 have been reported on astrocyte membranes as the disease progresses (Fray et al., 1998; Maragakis et al., 2004; Rothstein et al., 1995). It has also been shown that mutant SOD1 interacts with EAAT2 transcript splicing and therefore the resulting proteins are aberrant or non-functional (Lin et al., 1998). Moreover, AMPA GluR2 subunit editing is reduced in the spinal cord motoneurons from sALS patients when compared to controls (Kawahara et al., 2004; Takuma et al., 1999), but no AMPA editing difference was observed in G93A and H46R rat models (Kawahara et al., 2006). This AMPA GluR2 editing is important because the editing is responsible for reducing the permeability of the closed/inactive channel to calcium ions. The editing process changes the codon 586 from CAG into CGG, changing the aminoacide from a neutral glycine into a positively-charged arginine residue. Under normal conditions, the editing process is very efficient. When no correct editing is achieved, the permeability of the closed/inactive channel to calcium ions increases.

As stated earlier some motoneuronal populations (Haenggeli et al., 2002) are not affected in ALS. It has been shown that ocular, trochlear, abducens and Onuf's nucleus motoneurons express high amounts of calcium binding proteins such as calbindin and parvalbumin (Alexianu et al., 1994; Celio et al., 1990; Ince et al. 1993; Reiner et al., 1995) whereas ALS vulnerable motoneurons

express lower amounts of calcium binding proteins (Laslo et al., 2000). A high level of calcium binding proteins could definitely function as a calcium buffering system and thus protect from excitotoxic effects. Following these findings, double transgenic mice were developed. Transgenic mutant hSOD1 mice were bred with transgenic mice that overexpress parvalbumin, a calcium binding protein. The findings showed that double transgenic animals had a slightly increased survival when compared to control littermates (Beers et al., 2001).

Riluzole, the only drug approved for ALS therapy, has been shown to block glutamate release (Doble et al., 1996). This could explain why some slight effect on the disease is observed. Ceftriaxone, a B-lactam antibiotic, increases EAAT2 activity and extends survival in G93A mice (Rothstein et al., 2005). All these evidences suggest that excitotoxicity contributes to ALS.

Mitochondrial damage

Ultrastructural analysis shows that mitochondria start swelling and degenerating at very early stages in asymptomatic mutant animals, but only in those animals that express dismutase active (G37R, G93A) mutant proteins (Higgins et al., 2003; Kong and Xu 1998; Wong et al. 1995). Surprisingly, also mice that express wild-type hSOD1 develop vacuolated mitochondria but do not develop any disease (Jaarsma et al., 2000, 2001). It has been reported that in both mouse and patient samples, mutant SOD1 was purified from mitochondria-enriched fractions but only from affected tissues (Bergemalm et al., 2006; Deng et al., 2006; Liu et al., 2004).. This suggests that somehow SOD1 is able to translocate into these organelles. In fact, it has been proved by electron microscopy that SOD1 localizes in both isolated mitochondria (Liu et al., 2004) and in situ motoneuronal mitochondria (Higgins et al. 2002; Sasaki et al., 2007). SOD1 mutants that provoke disease at the lowest accumulated leves (G85R, G127X) are those that show the highest mitochondrial association (Liu et al, 2004). Another interesting observation is that endogenous wild-type SOD1 has not been found as mitochondrially associated and despite the mutant proteins are mitochondrially associated in the spinal cord, they have not been found in the liver (Liu et al., 2004). It is not yet understood how SOD1 affects mitochondrial function but proteome differences between mutant and non-mutant mitochondria have been reported (Fukada et al., 2004; Kirby et al., 2005; Lukas et al., 2006). Assuming that mutant SOD1 is preferentially imported into or deposited onto mitochondria in affected tissues, some mechanisms have been proposed to explain its effects (Fig. 13):

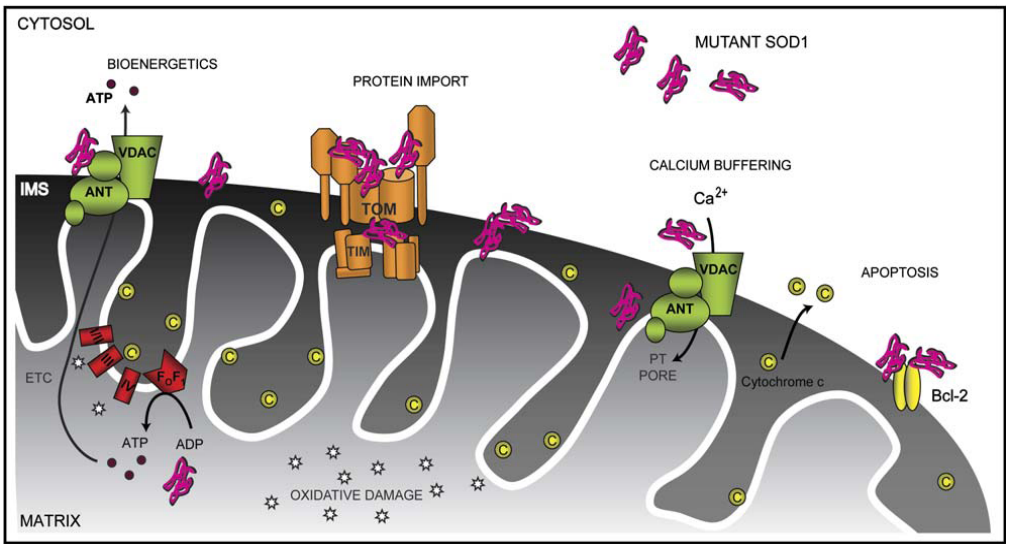


Fig. 13. Proposed mechanism for mitochondrial damage induced by mutant SOD1

1. Electron transport chain: mutant SOD1 may interfere with the elements of the electron transport chain, thereby disrupting ATP-generating oxidative phosphorylation.
2. Calcium homeostais: mutant SOD1 may also disrupt mechanisms by which mitochondria buffer cytosolic calcium levels.
3. Apoptotic machinery: mutant SOD1 aggregates may interfere with components of mitochondrial-dependent apoptotic machinery, such as Bcl-2, thereby triggering premature activation of an apoptotic cascade including cytochrome c release into the cytosol.
4. Mutant SOD1 may indirectly affect similar pathways linked to mitochondria by physically blocking the protein import machines, TOM and TIM.
5. Oxidative damage incurred by various mitochondrial proteins may also contribute to overall mitochondrial dysfunction.

All together, these mechanisms (or a combination) could disturb cellular homeostasis (within glial and/or motor neurons), ultimately triggering motor neuron death.

Further evidence that supports mitochondrial involvement in ALS, shows transgenic mutant mice treated with creatin, showed an increased survival when compared to untreated littermates (Klivenyi et al., 1999). Creatin contributes to increasing the ATP pool in the cells by donating a readily available phosphate group in order to regenerate ATP from ADP. In another experiment, transgenic mice were treated with minocyclin, a third generation tetracycline. Minocycline was shown to inhibit cytochrome c release from mitochondria which also reduced caspase 3 and 7 activation. Treated transgenic mutant mice indeed showed a prolonged survival when compared to untreated littermates (Zhu et al., 2002).

Inflammatory response

It has been hypothesized that external factors like pathogens could contribute to motoneuron degeneration. This observation comes from findings that show that enteroviral RNA sequences were found in ALS post-mortem tissue samples (Berger et al., 2000). This suggests that a viral infection could be a contributing factor in ALS either directly by killing motoneurons or indirectly by triggering an immune reaction that leads to degeneration or autoimmunity. However, the involvement of enteroviruses in ALS has long been discussed since contradictory results have been obtained in different reports (Nix et al., 2004; Cermelli et al., 2003). Nonetheless, a viral contribution cannot be completely outruled since the Sindbis virus induced motoneuron disease model needs to be considered as well (Darman et al., 2004).

Another interesting aspect involving the immune system in ALS pathogenesis is that several auto-antibodies have been found in the serum and CSF of ALS patients, such as anti-L-type Ca^{2+} channel (Kimura et al., 1994) expressed by motoneurons, anti-GM1 ganglioside (Li et al., 1991 and anti-acetylcholinesterase (Conradi et al., 1994) as well as anti-NFL and anti-a-actinin antibodies (Niebroj-Dobosz et al., 2006). These autoantibodies by binding to L-type calcium channels could constitutively activate them, leading to increased calcium influx and possible excitotoxicity (Smith et al., 1994). In addition, when cultured motoneurons were treated with CSF from affected patients, the cultured motoneurons degenerated and died (Demestre et al., 2005), therefore, some soluble factor in the CSF appears to be toxic to cells.

We must remember that motoneurons are not located in an isolated environment. In fact, motoneurons are surrounded by other kinds of cells such as microglia, astrocytes and Schwann cells. It is well established that microglial cells belong to a hematopoietic lineage and that these cells are immune system cells in the central nervous system. Astrocytes as stated before, are responsible for glutamate uptake and they also aid motoneuronal survival by secreting trophic factors. Schwann cells are responsible for myelin ensheathing of motoneurons fundamental for speeding up impulse transmission.

The reason we need to consider motoneurons as non isolated cells is because there is a neuroinflammatory interaction of microglia, neurons, and astrocytes. In the healthy CNS microglia present a ramified morphology (Fig. 14) providing immune surveillance in the neuronal environment (Raivich et al., 2005). In response to primary stimuli from neurons and astrocytes (initiating mechanism unknown in ALS), microglia become primed (Fig. 14) causing pro-inflammatory cytokine production and growth factor expression dowregulation (Perry et al., 2004). In response to secondary stimuli microglia become fully activated amoeboid cells with full

phagocytic potential (Fig. 14). These cells produce increased quantities of pro-inflammatory cytokines, reactive nitrating intermediates (RNI), reactive oxygenating intermediates (ROI), and glutamate. Astrocytes, on the other hand, decrease their growth factor contribution and release glutamate and inflammatory mediators. Neurons residing in the inflammatory microenvironment degenerate via apoptotic mechanisms (Fig 14).

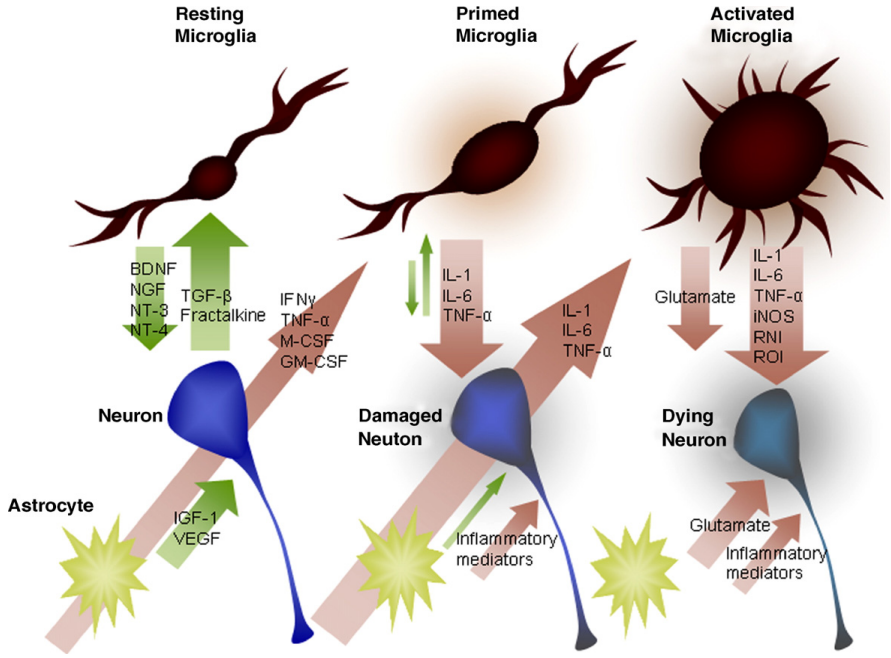


Fig 14. Proposed interaction between microglia, astrocytes and motoneurons in ALS

It has recently been shown that mutant SOD1 when secreted by affected motoneurons is a strong activator of microglia (Urushutani et al., 2006) Microglial activation has been reported before motoneuron loss and disease progression in ALS (Henkel et al., 2006). Other inflammation markers have been reported: COX-2 upregulation in the spinal cord of ALS patients has been reported (Yasojima et al., 2001; Yiangou et al., 2006). It is known that COX-2 is a marker of inflammation and is only induced under pathological circumstances. Another player, the chemokine fractalkine, has been identified as a chemoattractant in signaling the microglial response by injured motor neurons (Zujovic et al., 2000; Harrison et al., 1998). It has been reported to promote microglial proliferation in vitro (Hatori et al., 2002) and its receptor is expressed on both neurons and microglia (Meucci et al., 1998). In cell culture exporiments, fractalkine suppressed the production of nitric oxide (NO), IL-6 and TNF-α by activated microglia and suppressed neuronal cell death induced by microglia activated with LPS and interferon-gamma (IFN-γ), in a dose-dependent manner (Mizuno et al., 2003). Fractalkine is a chemokine that seems to have a role as an intrinsic inhibitor against microglia-induced neurotoxicity. Interestingly, when the fractalkine receptor is

ablated in ALS mice, the disease is exacerbated (Re and Przedborski, 2006), therefore fractalkine seems to have a role in ALS pathogenesis as well.

In addition, increased dendritic-cell populations have been found in the spinal cord of affected transgenic mice and also macrophage infiltrates have been revealed in the spinal cord of ALS patients (Henkel et al, 2006) suggesting an inflammatory response that goes actually beyond the CNS players of inflammation such as microglia. Taken together, all these reports show that inflammation is an important aspect of ALS.

Apoptotic mechanisms in ALS

Apoptosis markers can be observed in terminal stage ALS patients and animal models (Guegan et al., 2003; Pasinelli et al., 1998, 2000; Vukosavic et al., 1999). Cultured neuronal cells either injected or transfected with mutant SOD1 cDNA die by apoptosis (Durham et al., 1997; Pasinelli et al., 1998). Gradual decrease in intra-mitochondrial cytochrome-c levels has been associated with disease progression (Bacman et al., 2006). Bcl-2, an important antiapoptotic protein, has been reported to be bound and sequestered by mutant sod1 (Pasinelli et al., 2004). Activation of caspase 1 in G85R and G37R mice months before caspase-3 activation and disease onset has been reported (Pasinelli et al., 1998, 2000). Cytochrome-c translocation into the cytosol, and its interaction with APAF1 (apoptotic peptidase activating factor 1) and dATP binding form an oligomeric apoptosome. The apoptosome is then able to cleave and activate procaspase-9 . Caspase-9 activation is followed by caspase-1 activation before caspase-3 and caspase-7 activation. Once the pro-apoptotic signal has been activated in motoneurons, secondary events amplify this process such as microglial and T-cell activation with the release of inflammatory cytokines and factors such as interleukin- 1β, TNF-α and COX-2 (Alexianu et al., 2001; Robertson et al., 2001; Almer et al., 2001). TNF-α has been shown to activate caspase-8 in the late stage of ALS in transgenic mice and cultured embryonic motoneurons have been shown to be sensitive to a Fas-induced apoptosis (Raoul et al., 2002, 2006). This evidence suggests that these inflamatory cytokines could mediate a motoneuron-specific apoptotic pathway in ALS.

From the previously presented evidence, we can conclude that ALS pathogenesis is very complex and may involve all of the proposed mechanisms (Fig. 15).

One of the first events in ALS pathogenesis seems to be the disruption of the neuromuscular junction due to motoneuronal terminal retraction (Jokic et al., 2006). Also in the early phase of the disease, SOD1 mediated aggregates could disrupt neurofilament organization and mitochondrial

function. Moreover, secreted SOD1 (Urushitani et al., 2006) would trigger microglial premature activation. In the symptomatic stage of the disease, activated microglia and astrocytes would produce toxic and inflammatory factors harmful for the affected motoneuron. It has been reported that activated astrocytes produce NGF (Pehar et al., 2004) and that degenerating motoneurons in ALS express the $p75^{NTR}$ receptor (Lowry et al., 2001). This receptor is not normally expressed in adult motoneurons. An apoptotic mechanism mediated by the binding of NGF to the $p75^{NTR}$ receptor has been reported (Pehar et al., 2004). However, it has also been reported that motoneurons produce FGF-1 that potently activates astrocytes to produce and secrete NGF (Cassina et al., 2005). Still in the symptomatic stage, astrocytes would present lower levels of EAAT2 transporter (Fray et al., 1998; Maragakis et al., 2004; Rothstein et al., 1995) and aberrant EAAT2 products (Lin et al., 1998), contributing to excitotoxicity. Impaired axonal transport would also reduce trophic factor transport and organelle transport in the affected motoneuron.

Overloaded proteasomes would choke and protein aggregates would sequester other important proteins in the cell such as HSPs. Damaged mitochondria would not perform oxidative phosphorylation correctly, oxidative stress would increase and calcium buffering by mitochondria would be impaired, because of SOD1 induced dysfunction. All these events would lead to mitochondrial cytochrome-c release and caspase activation triggering motoneuronal apoptosis and ultimately to phagocytosis. Complete muscle atrophy would develop and glial scarring would be formed where the affected motoneuron used to be.

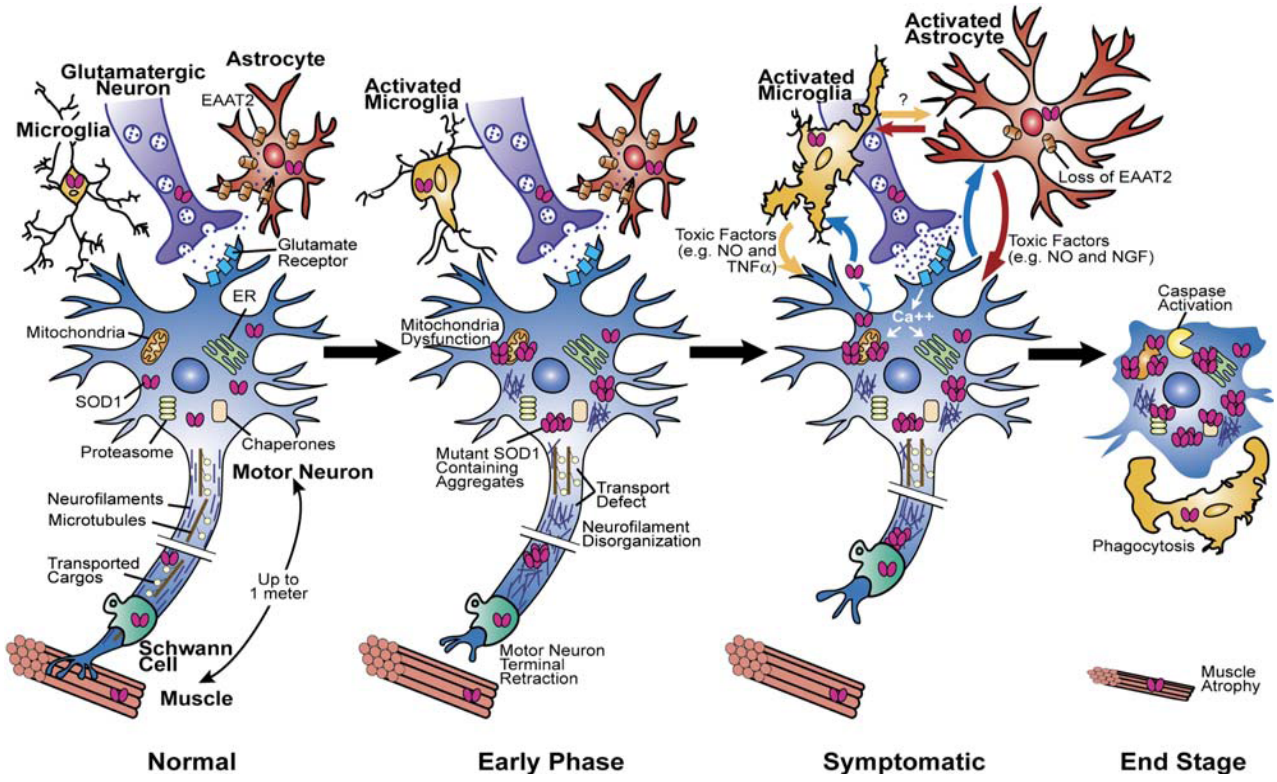


Fig. 15. Proposed ALS pathogenesis overview mechanism

Sometimes neighbors matter

Even though SOD1-induced degeneration is clearly motoneuron-specific, a contribution from surrounding cells is obvious. We must remember that SOD1 is ubiquitously expressed, therefore an important question was raised: in order to develop ALS, must mutant SOD1 be expressed ubiquitously or is it enough to express it only in motoneurons to develop disease? When mutant SOD1 was expressed selectively either in motoneurons or in astrocytes, mice did not develop motoneuron disease (Gong et al., 2000; Lino et al., 2002), suggesting that SOD1 toxicity requires mutant damage not just within motoneurons but also to non-neuronal cells. This was also demonstrated by Clement et al. (2003) who showed that in chimeric mice that are mixtures of normal and SOD1 mutant-expressing cells, toxicity to motoneurons is shown to require damage from mutant SOD1 acting within non-neuronal cells. Normal motoneurons surrounded by mutant SOD1-expressing non-neuronal cells developed aspects of ALS pathology. Conversely, mutant motoneurons could escape degeneration and death if surrounded by a relatively small minority of normal non-neuronal cells. Therefore, mutant SOD1 induced ALS can be defined as non-cell autonomous or as a neighbourhood disease, because only ubiquitous expression of the mutant protein can cause the disease.

On the other hand, let us consider ALS induced by dynactin mutations. Dynactin is a neuron specific protein and is not expressed by microglia and astrocytes. In this case, the mutant protein is expressed only by the affected cells. This suggets that dynactin induced ALS (Puls et al., 2003) is cell autonomous, meaning that some types of ALS can be cell autonomous but other types of ALS imply the contribution of different cells in order to develop disease.

Therapy and clinical trials

As stated before, the only drug so far approved for human use is riluzole. Riluzole shows to inhibit glutamate release and modestly extends survival of ALS patients by 2 months (Doble et al., 1996). Unfortunately, up to date, no clinical trial has been successful on humans. Many compounds that showed improvement in ALS animal models such as creatine (Klivenyi et al., 1999), proved ineffective in humans (Groeneveld et al., 2003). Growth factors such as IGF-1 (Borasio et al., 1998; Lai et al., 1997), BDNF (The BDNF Study Group, 1999) and CNTF (ALS CNTF Treatment Study Group, 1996) showed little or no benefit. However, some ALS clinical trials should be redesigned. Some of the substances were administered systemically whereas a localized administration could show some benefit and reduce side-effects. Also some of the administered substances were not

able to pass through the blood brain barrier (BBB). The BBB could be skipped (Fig. 18) by directly infusing the desired growth factor into the brain (intracerebroventricular) or spinal cord (intrathecal). In fact, continuous intracerebroventricular infusion of IGF-1 showed slight benefit in ALS patients (Nagano et al., 2005).

A possible target for clinical trials in humans could be RNA silencing of mutant SOD1. In order to do this, RNAi could be applied locally either by intracerebroventricular or intrathecal injection In fact, SOD1 transgenic mutant mice that were administered mutSOD1 RNAi using modified lentiviral vectors directly in the spinal cord (Raoul et al., 2005) showed a prolonged survival (Fig. 16).

A less invasive strategy is intramuscular injection of modified viral vectors that carry the mutSOD1 RNAi. These are then anterogradly transported to the motoneuronal cell body (Ralph et al., 2005; Miller et al., 2005). These mice showed an increased survival as well. Growth factors can also be delivered by using modified viral vectors. Survival in mutant G93A SOD1 expressing mice treated with viral vectors carrying IGF-1 (Kaspar et al., 2003) and VEGF (Azzouz et al., 2004) coding sequences, was increased after intramuscular injection (Fig. 18) , therefore it could be worth applying the same to humans.

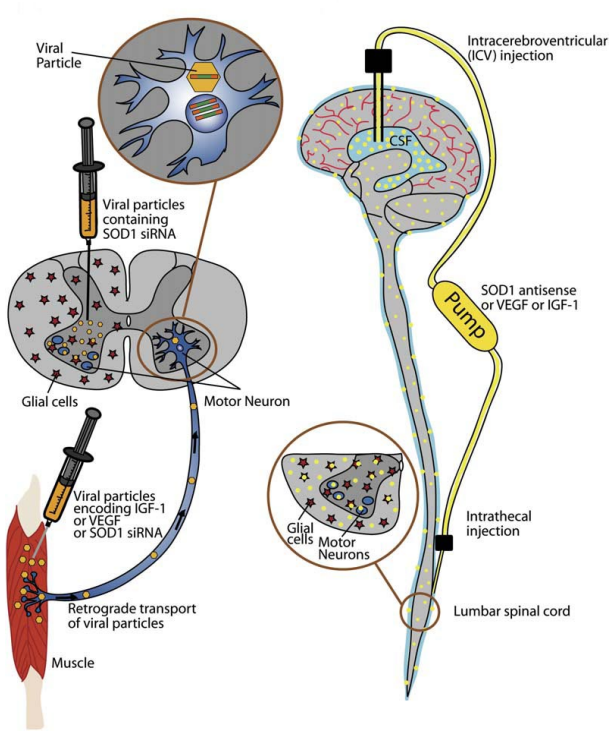


Fig 16. Proposed ALS therapies and delivery methods

Cell therapy in ALS

One of the most promising therapies in ALS is stem cell therapy. Stem cells are able to differentiate into several cell types (Fig. 19). Especially embryonic stem cells (ESCs) have been induced to differentiate into spinal motoneurons (Harper et al., 2004) by the combined treatment with retinoic acid which induces a horizontal neuralization and sonic hedgehog which induces a ventral orientation in these cells. ESC-derived motoneurons have also been show to grow axons (Harper et al., 2004) and generate neuromuscular junctions in periphernal nerve injury rat models (Craff et al., 2007). In another report, ESCs showed to implant and grow processes in the injured animals and contributed to movement recovery, although the extent of recovery cannot be determined (Desphande et al., 2006). Adult stem cells have also drawn attention because it has been reported that they can also differentiate into several cell lineages, including neural lineage progenitors (Kim et al., 2006). Because of ethical issues, adult stem cells would be easier to use. Despite all these encouraging reports, ALS stem cell therapy still needs further development before it can lead to successful results in humans. In order to achieve successful ALS stem cell therapy, differentiated motoneurons must be able to grow axons along the spinal cord and synapse lower motoneurons (this is the case for affected upper motoneurons). For affected motoneurons, the implanted motoneurons must be able to grow axonal processes that can be as long as one meter (lower limb muscles) and also develop neuromuscular junctions with innervated muscles.

Of course stell cell therapy must not only focus on motoneuronal degeneration, since as it has been shown and discussed earlier, microglia and astrocytes also play a fundamental role in cell-autonomous ALS. Whether oligodendrocytes are affected in ALS is not known yet.

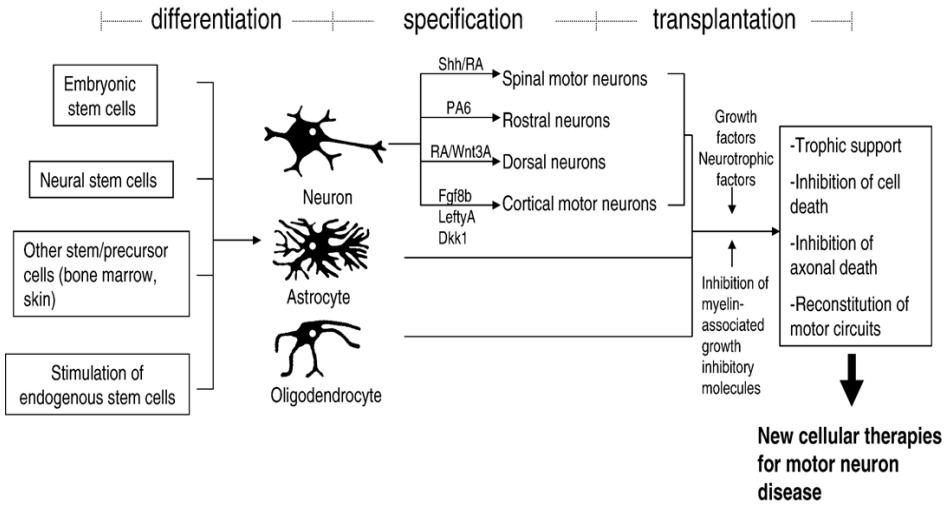


Fig. 17. Stem cell potential to differentiate into different cell types

Why neurochemistry studies?

Most ALS studies on animal models have focused on disease pathogenesis and have aimed at finding strategies to increase survival and slow disease onset and progression. However, few reports have dealt with topics such as behavior, learning and memory in ALS mice. These aspects during disease course are also interesting to study because cognitive deficits and various degrees of dementia have been documented in ALS patients (Kew et al., 1993; Kato et al., 1994; Massman et al., 1996; Hanagasi et al., 2002), and no attention has been so far paid to possible alterations of brain systems related to learning and memory. We therefore decided to study cholinergic systems in ALS transgenic mice during disease course.

On the other hand, long-term dietary supplementation with creatine has been proposed to be neuroprotective in both acute and chronic neurodegenerative diseases. Neuroprotective effects of creatine have been demonstrated in some animal models of neurotoxicity/excitotoxicity but not in others (Malcon et al., 2000; Matthews et al., 1998, 1999), in traumatic brain and spinal cord injury (Hausmann et al., 2002; Sullivan et al., 2000) and in ischemia (Zhu et al., 2004). Furthermore, attenuation of disease symptoms and increased lifespan through dietary creatine supplementation has been described in animal models of human neurodegenerative diseases (Ellis et al., 2004). Surprisingly, reports on dietary creatine supplementation in animal models of neurodegenerative injuries and diseases have not dealt, so far, with the derangement of neurochemical parameters related to degenerating neurons and with their rescue through the dietary manipulation. This information is very relevant, because specific alterations of neurochemical parameters are one of the most reliable markers to evaluate the extent of neural damage. Furthermore, this approach may be potentially able to put in evidence differential responses to insults and protection of different neuronal populations present in the same brain region.

Finally, polyamines are a family of several polycationic compounds found in virtually all the living organisms, from simple prokariotic to eukariotic cells. In mammals, they are produced in a highly regulated biosynthetic pathway in which the key enzyme, ornithine decarboxylase (ODC), converts the immediate precursor ornithine into the diamino compound putrescine that, in turn, gives rise to higher order derivatives, including spermidine and spermine, the two physiologically most important polyamines. The widespread distribution together with the highly regulated levels of cell polyamines are thought to reflect a prominent physiological role of these compounds. In recent years, many studies have demonstrated the involvement of polyamines in various cellular processes such as regulation of gene expression, cell proliferation and differentiation and tissue

regeneration (Pegg and McCann, 1982; Banan et al., 1998; Thomas and Thomas, 2001; Childs et al., 2003;). In parallel with interest raised by the physiological roles of polyamines, a growing attention has been paid to the possible pathological involvement of these compounds. For instance, based on their ability to promote cell proliferation, polyamines are suspected to be involved in tumor growth (Gerner and Meyskens, 2004; Gugliucci, 2004). In the central nervous system (CNS), polyamines, that have been localized to both neurons and glial cells (Bernstein and Muller, 1999), show characteristic profiles of regional and developmental abundance.

For instance, ODC activity and putrescine levels are found at high levels during perinatal phases of neurogenesis and nerve cell differentiation (Slotkin and Bartolome, 1986). On the other hand, in the mature brain the ODC/polyamine system is regulated at very low levels, but the ODC activity and the parallel synthesis of polyamines can be rapidly induced by various traumatic insults and in neuropathological conditions (Paschen, 1992). Increase of polyamine levels has been described in focal cerebral ischemia in humans (Els et al., 2001) and in various animal models of transient and permanent brain ischemia (Baskaya et al., 1997; Adibhatla et al., 2002; Babu et al., 2003). Alterations of ODC/polyamines system have been also reported in post-mortem investigations on patients affected by Alzheimer's and Parkinson's diseases (Morrison and Kish, 1995; Morrison et al., 1998; Gomes-Trolin et al., 2002). In spite of relatively abundant data on ODC activation and polyamines accumulation in various neuropathological states, not much is known about cellular and molecular mechanisms affected by these changes. Since glutamate excitotoxicity is from a long time considered a major determinant of neurodegenerative processes, an excessive positive modulatory effect of polyamines on NMDA receptor has been suggested to be involved in polyamine neurotoxicity (Contestabile et al., 1994; Sparapani et al., 1997; Williams, 1997). Moreover, abnormally elevated polyamine levels can induce activation of enzymes involved in the apoptotic cascade of neuronal death (Poulin et al., 1995; Schipper et al., 2000). By contrast, neuroprotective roles of natural polyamines have also been reported by several investigators (Ferchmin et al., 2000; Harada and Sugimoto, 1997; Clarkson et al., 2004). In view of these controversial results, it seems important to explore systematically the temporo-spatial profiles of ODC/polyamine alterations in multiple animal models of neuropathological disorders in order to unmask coincidental and common features. Data about changes of ODC/polyamines system in rat CNS during aging have been reported in a previous paper and a marked increase in ODC activity and putrescine and spermidine accumulation in the spinal cord but not in brain areas of aged rats were reported (Virgili et al., 2001). This suggested that the spinal cord can be a sensitive target for neurodegeneration and a region where derangement of ODC/polyamine system is correlated with

impairment of motor function (Virgili et al., 2001). On this basis, it was deemed interesting to study alterations of this system in a pathological condition primarily involving degeneration of spinal cord motor neurons, such as amyotrophic lateral sclerosis (ALS). The only available report on this disease, revealed no significant alteration in polyamine levels in post-mortem studies in the spinal cord of patients deceased by ALS (Ekegren et al., 2004). Therefore we decided to perform a study on a mice model of ALS.

Why proteomics?

There are several compelling reasons to use proteomics and approach biological questions from the protein perspective (Banks et al., 2000; Lopez and Melov, 2002; Petricoin et al., 2002; Schulenborg et al., 2006). The term proteome describes the ensemble of proteins expressed by a cell, tissue or the whole organism, and unlike the temporally constant genome, it is dependent on intra- and extra-cellular parameters, dynamics and variables. Proteins are the functional output of the cell and therefore they are expected to provide the most relevant information in the context of the biological system and specific networks they are involved in. Their expression and function can be modulated in many ways, from transcription to post-translation (e.g. alternative splicing of the pre-mRNA and post-translational modifications), leading to multiple protein products from a single gene that can not be predicted from analysis of nucleic acids alone. These modifications may affect the catalytic activity, association, stability, half-life or localisation of a specific protein in a disease state and thus their detection would be important to understand underlying disease mechanisms.

The central tool for displaying the proteome is two-dimensional gel electrophoresis (O'Farrell, 1975). In 2D electrophoresis, proteins in a sample are first separated according to their isolectric point: isoelectric focusing (IEF), this is the first dimension. In the second dimension, poteins are separated according to their molecular weight.

Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino- and carboxyl-termini. The isoelectric point (pI) is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI. The presence of a pH gradient is critical to the IEF technique. In a pH gradient and under the

influence of an electric field, a protein will move to the position in the gradient where its net charge is zero. A protein with a net positive charge will migrate toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a net negative charge will migrate toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This is the focusing effect of IEF, which concentrates proteins at their pIs and allows proteins to be separated on the basis of very small charge differences.

When comparing the proteome of healthy versus diseased tissue, distinct protein alterations can be detected. Several post-translational modifications that modify molecular weight of a protein to a minimal extent (such as phosphorylation) can be detected with 2D electrophoresis, because the protein's pI changes. Differentially expressed proteins can be identified by mass spectrometry. Mass spectrometry is an analytical tool used for measuring the molecular mass of a sample. For large samples such as biomolecules, molecular masses can be measured to within an accuracy of 0.01% of the total molecular mass of the sample i.e. within a 4 Daltons (Da) or atomic mass units (amu) error for a sample of 40,000 Da. This is sufficient to allow minor mass changes to be detected, e.g. the substitution of one amino acid for another, or a post-translational modification. In matrix assisted laser desoprtion ionization (MALDI) sample molecules are bombarded with a laser and become ionized. The type of a mass spectrometer most widely used with MALDI is the TOF (time-of-flight mass spectrometer), mainly due to its large mass range. The time-of-flight analyser separates ions according to their mass(m)-to-charge(z) (m/z) ratios by measuring the time it takes for ions to travel through a field free region known as the flight, or drift, tube. The heavier ions are slower than the lighter ones. Peptide mass fingerprinting is used with a MALDI-ToF mass spectrometer. In this case the unknown protein of interest is cleaved into peptides by a protease such as Trypsin. The collection of peptides resulting from this cleavage comprise a unique identifier of the unknown protein. The absolute masses of the (still unknown) peptides are accurately measured with a mass spectrometer such as MALDI-TOF

Differentially expressed proteins then have to be validated and characterized in depth to elucidate their function and affected pathways.

To date numerous proteomic studies have been performed in the field of neurodegeneration. In Parkinson’s disease (PD), a 2D gel based differential proteome study reported mitochondrial dysfunction and oxidative damage in ventral midbrain of parkin deficient mice. Parkin is an E3 ubiquitin ligase, known to be impaired by mutations in an early onset form of familial PD. The

study resulted in the detection of 13 downregulated proteins in the parkin null mice, eight of which seemed to be linked to mitochondrial respiration or detoxification of byproducts of mitochondrial respiration (Palacino et al., 2004). In another proteomic study, Basso et al. (2004) analysed protein extracts from human post-mortem substantia nigra of PD patients and identified several differentially expressed proteins, including neurofilaments, peroxiredoxin II and complexes III and IV of the respiratory chain. Calmodulin, cytochrome c and cytochrome c oxidase were among the proteins that were differentially expressed in dopamine depleted brain regions of brain tissue sections from a rat model of PD (Pierson et al., 2004). In Alzheimer's disease, several proteomic studies have been also published over the last years. Cottrell et al. (2005) analysed 21 proteins in human AD brain, which co-precipitated with the main component of neuritic plaques amyloid precursor protein (APP), by 2D gel electrophoresis and identified them by mass spectrometry. In addition to six known interactors of APP, they identified 15 new interacting partners including proteins involved in axonal transport (dynein, myosin, dynamin), cytoskeletal and structural proteins (actin, tubulin, GFAP, spectrin, neurofilaments), as well as chaperone proteins (αB-crystallin, HSP90, HSC71, cyclophilin A). Oxidative modification of proteins, due to oxidative stress and free radical formation, is an early event of neurodegenerative diseases and can be detected using oxyblot prior to their separation by 2D-PAGE. In this case the carbonyl group, introduced to lysine, arginine, proline or threonine residues by oxidative processes, can be derivatized to 2,4-dinitrophenylhydrazone using 2,4-dinitrophenylhydrazine and eventually detected using a specific antibody. This method has been used extensively by the group of Butterfield who reported several differentially oxidized proteins in the hippocampus but not in the cerebellum of AD patients compared to matched controls (Castegna et al., 2002; Sultana et al., 2006). Excessively oxidized proteins included α-enolase, triose phosphate isomerase (TPI), dihydropyrimidinase-related protein-2 (DRP-2), creatine kinase and HSC71. Proteomic technologies have also been used to screen body fluids, such as cerebrospinal fluid (CSF), serum and plasma, with the aim to identify specific disease biomarkers for early diagnosis. Two reviews by Davidsson and Sjogren (2005, 2006) summarize results from proteomic studies of differential protein patterns in neurodegenerative diseases with focus on AD.

In the field of ALS, a few proteomic studies have also been designed in order to identify new potential marker proteins involved in the pathogenesis of motor neuron degeneration. As described earlier, a recent CSF proteomic study reported the identification of three protein species (a 4.8-kDa VGF peptide, cystatin C and a 6.7-kDa cationic protein species) that were significantly lower in concentration in the CSF from patients with ALS compared to normal controls as well as

patients with purely motor peripheral neuropathy (Pasinetti et al., 2006). The combination of the three protein species correctly identified patients with ALS with 95% accuracy, 91% sensitivity and 97% specificity from the controls, during the early timepoint of the disease. Decreased cystatin C was identified in the CSF of ALS patients, in another study, along with transthyretin and carboxy-terminal fragment of neuroendocrine protein 7B2 (Ranganathan et al. 2005). In addition, Ekegren et al. (2006) identified several differentially expressed proteins in post-mortem spinal cords from ALS patients compared to control subjects, including GFAP, haemoglobin α and β chain, myelin basic protein, thioredoxin, α-enolase and choline acetyltransferase. Using a motor neuron-like cell line, Fukada et al. (2004) identified 45 mitochondrial proteins that are altered in the presence of the G93A-SOD1 mutation. Nine and 36 protein spots displayed elevated and suppressed abundance respectively in $SOD1^{G93A}$-expressing cells. Half of the differentially expressed proteins were found to be involved in various metabolic pathways, mainly the mitochondrial respiratory chain, while the remaining included proteins involved in membrane transport, antioxidant proteins, and heat shock molecular chaperones among others. A proteomic analysis of protein expression in the $SOD1^{G93A}$ mouse model was also undertaken to identify differences in protein expression in the spinal cords of end-stage mice (Lukas et al., 2006). Protein profiling was done on soluble and particulate fractions of spinal cord extracts, using 2D liquid chromatography coupled to tandem mass spectrometry, and revealed that changes in mitochondria-associated proteins were particularly prevalent, suggesting that part of the "gain of function" of mutant SOD1 is to target specific mitochondrial proteins. Recently, in another project, Massignan et al. (2007) performed a proteomic analysis of spinal cord extracts from asymptomatic (9 week-old) wild-type and mutant SOD1 expressing transgenic mice. The analysis was performed by using 2D gel electrophoresis. The group found that 10 proteins were significantly over-represented and 5 proteins were under-represented in SOD1 mutant mice when compared to wild-tpye animals. The identified proteins belonged to several biological processes such as mitochondrial metabolism, glycolitic metabolism and protein degradation among others.

We thought that 2D-PAGE could be a useful technique in separating hundreds of proteins present in insoluble protein fractions extracted from spinal cords of $SOD1^{G93A}$ expressing mice with a high resolution, helping identifying the components of this fraction. The present proteomics project is the continuation of a proteomic analysis perfomed on insoluble protein fractions from end-stage (16 week-old) animals (unpublished).

CHAPTER 2: MATERIAL AND METHODS

Animals

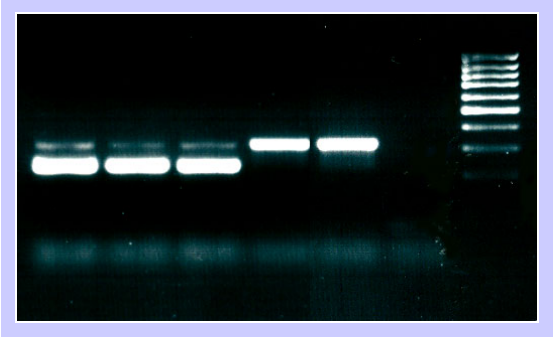
Transgenic mice overexpressing a mutated form of human SOD1 gene, an animal model for familial ALS, were used. The mutation consists of a glycine 93 to alanine (G93A) substitution and is located in exon 4 of the gene (Gurney et al., 1994). Mice carrying the mutated gene, designated B6SJL-TgN ($SOD1^{G93A}$) 1GUR and mice overexpressing the wild-type SOD1 human gene, designated B6SJL-TgN ($SOD1^{G93A}$) 2GUR), were obtained from Jackson laboratories. The colony was established and maintained by crossbreeding male transgenic mice with B6EiC3Sn females. To assess the genotypes of transgenic offspring, DNA was extracted from the tail of 30-40 day-old mice and amplified by using a PCR assay with specific primers for wild-type mouse IL-2 (Horak, Ivan 1991) :

forward 5'- CTA ggC CAC AgA ATT gAA AgA TCT -3'
reverse 5'- gTA ggT ggA AAT TCT AgC ATC ATC C -3'

and for exon 4 of human SOD1 (Brown, Robert Jr 1993 exon 4 set b) :

forward 5'- CAT CAg CCC TAA TCC ATC TgA -3',
reverse 5'- CgC gAC TAA CAA TCA AAg TgA -3'

as shown by the image below:



Transgenic animal DNA amplification results in two bands corresponding to IL-2 as an internal control (upper band 324bp) and exon 4 of human SOD1 (lower band 236bp). Lanes 1-3 transgenic animals, 4-5 non-transgenic animals, 6 molecular weight ladder

The experiments were designed to compare results from three groups of mice: G93A carrying a

high copy number of copies of the human mutated gene, wild-type transgenic mice carrying an equivalent number of copies of the human wild-type gene and non transgenic littermates.
Male Wistar rats form Harlan Italy were also used where specified.

Neurochemistry studies

Forebrain cholinergic system alterations in SOD1 mutant transgenic mice

Initial neurochemical experiments considered males and females as separate groups but, after data analysis, we found no significant differences related to sex and thus data from both groups were pooled. In survival experiments, only females were included in the experimental groups to avoid sex-related variability, as it is known from the literature that mean lifespan of G93A females exceeds by few days that of males. Experiments of choline supplementation were performed by treating pregnant females with 35 mM choline chloride (Sigma, St. Louis) added to the drinking water (sweetened with 0.5% saccharine) from gestational day 11 to birth. The daily average choline intake was 7 mmol choline/(kg day), including choline added to the water and choline present in the standard food diet, which amounted to 1.5 mmol choline/(kg day). In addition, groups of choline-treated or untreated progeny of G93A mice were supplemented with 25 mM acetyl carnitine hydrochloride (ALCAR, Sigma, St. Louis), starting from 40 days of age and continuing until death. Average intake was 3.5 mmol/(kg day). Experiments were conducted according to the Italian law on the use of animals for experimental purposes, which conforms to European Community rules and were authorized by a local bioethical committee. Veterinary surveillance on animal health and comfort was ensured by a University Veterinary committee.

Neurochemical assays

At 55, 110 and 125 days of age, animals were killed by decapitation and various brain samples were dissected, immediately frozen on dry ice and stored at -80°C until used. On the day of experiment, collected tissue was homogenized in ice-cold solution containing 50 mM Tris–HCl, pH 7.4, and 0.5% Triton-X 100. The homogenate was used to determine protein content and to perform enzyme activity assays. Choline acetyltransferase (ChAT) and glutamate decarboxylase (GAD) activity were determined according to established radiochemical methods (Fonnum, 1975; Fonnum et al., 1977). Acetylcholinesterase (AchE) and glutamine synthetase (GS) activity were quantified

using colorimetric methods (Ellman et al., 1961; Patel et al., 1983). All chemicals were purchased from Sigma (St. Louis, MO), except for radiochemical substrates originated from Perkin-Elmer ([acetyl-1-^{14}C]-labeled coenzyme A; Boston, USA) and from GE Healthcare ([^{14}C]-labeled glutamate; Buckinghamshire, UK), respectively.

Assay for nucleosomal DNA fragmentation

Oligonucleosomal DNA fragmentation, a characteristic feature of apoptotic cell death, was determined by binding of a monoclonal antibody to internucleosomal proteins using a commercially available kit (Cell Death Detection ELISA Plus, Roche, Indianapolis, IN). Lumbar spinal cord and cortex from 110-day-old G93A mice and non-transgenic littermates were homogenized in the provided lysis buffer and centrifuged. The supernatant was used to quantify the index of apoptosis following the manufacturer's instructions.

Immunohistochemical procedures

Mice were anesthetized and perfused with 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. Cervical and lumbar spinal cord segments and brains were dissected and postfixed overnight in the same fixative. After washing, samples were immersed in 15% sucrose for 24 h and sectioned at 40 mm thickness with a freezing microtome.

For immunohistochemistry, all washing and incubation steps were performed on a flatbed shaker at room temperature, unless specified. Sections were first washed with PBS 0.1% Triton X-100 for 15 minutes. Following this step, endogenous peroxidases were blocked by incubating the sections for 30 minutes in 0.3% (v/v) H_2O_2 diluted in methanol. The sections were then washed 3 times for 10 minutes with PBS 0.1% (v/v) Triton X-100. Aspecific binding sites were blocked by incubating the sections with PBS-T 0.1% Triton X-100 plus BSA 2% (w/v, Sigma) and 1.5% (v/v) normal rabbit serum (Sigma). Primary antibody incubation (1:100 dilution) was performed at 4°C overnight in PBS 0.1% Triton plus BSA 1% and normal rabbit serum. A goat anti-ChAT polyclonal antibody (Chemicon) was used to label ChAT reactive cells. The next days the sections were washed 3 times for 5 minutes and then incubated for 1 hour with a biotinylated mouse anti-goat (Sigma) secondary antibody (1:500 dilution) in PBS 0.1% Triton X-100 plus BSA 1% and 1.5% normal rabbit serum (v/v). After this incubation, the sections were washed 3 times for 5 minutes in PBS. The sections were then incubated for 1 hour with ABC reagent (Vectastain). ABC reagent was prepared at least 30 minutes before the incubation as specified by the manufacturer. Following this incubation, the

sections were washed 3 times for 5 minutes in PBS and aftwerwards with 50mM pH 7.6 Tris-HCl. Immunoreactivity was revealed through DAB staining following the manufacturer's protocol (Vectastain). DAB staining was blocked by washing the sections with double-distilled water when the required staining was achieved.

Sections were mounted on gelatin-coated glass slides and left to dry overnight. The following day the mounted sections were dehydrated by incubating the glass slides for at least 1 minute in 95% ethanol, then 100% ethanol, a second incubation in 100% ethanol and a last incubation in xylene. Sections were coverslipped in permount mounting medium and left to dry.

Statistics

Experimental data were subjected to statistical analysis by using Student's t-test or, when appropriate, analysis of variance (one-way ANOVA) followed by post hoc comparison through Bonferroni's test.

Differential neuroprotecion by creatine supplementation

Male Wistar rats form Harlan Italy were divided into two groups at 40 days of age. The first group was fed ad libitum with standard diet. The second group received the same diet supplemented with 2% creatine. Rats underwent surgical operation at 70 days of age and were then allowed to survive for additional 30 days, continuing to be fed with the normal or the supplemented diet during all these periods.

For surgery, rats were anesthetized with ether, fixed to a David Kopf stereotaxic instrument and holes were drilled in the skull at the appropriate stereotaxic coordinates. Two different models of excitotoxic neurodegeneration were adopted to test possible neuroprotection from creatine supplementation. In the first model, rats received unilateral injections of the neurotoxin, ibotenic acid (IBO, 7 Ag/1 Al saline), into the left striatum at the following coordinates (Paxinos et al., 1982): 1 mm in front of bregma, 3 mm lateral, 5 mm below the dura. This treatment results in widespread degeneration of striatal neurons and in particular of the cholinergic and GABAergic neuronal population present in this brain region (Ciani et al., 2001). In the second model, IBO (5 Ag/1 Al saline) was unilaterally injected in the area of the nucleus basalis magnocellularis, where cholinergic neurons providing most of the cholinergic input to the neocortex are localized (Mesulam et al., 1983; Woolf et al., 1991), at the following coordinates: 1 mm behind bregma, 2.7 mm lateral, 7.8 mm below the dura. The excitotoxic lesion of these neurons results in fall of cholinergic parameters in cortical areas as a consequence of the degeneration of cholinergic

terminals (Casamenti et al., 1998; Contestabile et al., 2004). Injections were performed slowly (about 3 min followed by 2 min during which the needle was left in place), using a 10 Al Hamilton syringe operated by a micrometric device. In both cases, the contralateral (right) side of the brain received an equivalent injection of saline and served as control for the excitotoxic lesion. After 30 days of recovery, rats were killed by decapitation, the brains were rapidly removed and sliced with a Sorvall tissue chopper. Samples of the striata (for striatum-injected animals) obtained from two consecutive slices comprised between levels bregma 1.2 and bregma 0.2 (Paxinos et al., 1982), or the fronto-parietal cortex (for nucleus basalis magnocellularis-injected animals) obtained from three slices comprised between levels bregma 1.2 and bregma 1.3 (Paxinos et al., 1982), from the two brain sides were separately microdissected under the stereomicroscope, frozen in dry ice and stored at -80°C until assayed. This procedure, which has been used for many years in our lab (Ciani et al., 2001; Contestabile et al., 2004; Crochemore et al., 2005), allows reproducible dissections of equivalent samples from different animals without compromising reliability of the neurochemical determinations indicated below. Samples were homogenized in 0.32 M sucrose, added with 0.5% Triton X-100 (final concentration) and the whole homogenate was used to assay the activity of the cholinergic marker, choline acetyltransferase (ChAT) (Fonnum et al., 1975) and of the GABAergic marker, glutamate decarboxylase (GAD) (Fonnum et al., 1977), as well as sample protein content (Lowry et al., 1951). ChAT activity was assayed by incubating aliquots of the homogenates containing known amount of protein, for 10–30 min at 37°C in the presence of choline (8 mM) and ^{14} CacetylCoA (0.2 mM, NEN, specific activity 51.6 mCi/mmol) and adding eserine (0.1 mM) to block acetylcholinesterase. The labeled acetylcholine formed through the enzymatic reaction was extracted by Kalignost (0.5% in acetonitrile), brought to the organic phase of a scintillation cocktail (Instafluor, Packard), counted and expressed as Amol formed/unit protein weight. GAD activity was assayed by incubating aliquots of the same samples for 1 h at 37°C in the presence of ^{14}C-glutamate (21.6 mM, NEN, specific activity 45 mCi/mmol), trapping the CO_2 evolved by the enzymatic reaction with hyamine hydroxyde, counting and expressing the activity as Amol of CO_2 formed/unit protein weight. We knew from previous experiments that glial proliferation consequent to the lesion does not alter the protein content of the affected regions (Virgili et al., 1992).

Starting from 40 days of age, wild type and G93A transgenic mice were divided in groups, composed of approximately the same number of males and females, which received standard diet or diet supplemented with 2% creatine. When 110 days old, an age at which disease symptoms are manifested in G93A mice and cholinergic deficit is evident not only in the degenerating segments

of the spinal cord, but also in some forebrain regions (Crochemore et al., 2005), wild type mice and part of the G93A ones were killed by decapitation.

Samples from the lumbar spinal cord, the hippocampus, the olfactory cortex and the striatum (Franklin et al., 1997) were rapidly collected in parallel by two experienced operators and stored in the deep freezer for subsequent determination of ChAT activity.

The remaining G93A mice were used to assess survival and were therefore maintained under the different diets until euthanized when they reached the final stage of the disease, characterized by complete limb paralysis and inability to move and feed.

Statistical analysis was carried on through Student's t test or analysis of variance (one way ANOVA) followed by post-hoc Bonferroni's test. The number of animals used for each experiment is indicated in figure legends. Experiments were performed in accordance with the Italian and European Community law on the use of animals for experimental purposes and were approved by the University of Bologna bioethical committee. Throughout the study, animals were kept under veterinary surveillance for their comfort and health.

ODC/polyamine system alterations in ALS-G93A mice

For the study of the polyamine system in ALS G93A mice, animals from each group, taken at pre-symptomatic (55 days of age) and at symptomatic (125 days of age) stages, were killed by decapitation and the selected regions from brain and spinal cord were immediately dissected, frozen in dry ice and stored at°80°C until used.

ODC activity

To extract the enzymatic protein, tissue from the different samples was homogenized in ice-cold 50 mMTris–HCl (pH 7.5), containing 0.1 mMEDTA, 5 mM dithiothreitol and 0.04 mM pyridoxal-5-phosphate, by a motor-driven homogenizer equipped with a Teflon pestle (1200 rpm, 12 strokes). The homogenate was centrifuged at 18,000 g for 20 min and aliquots of the supernatants were transferred to small tubes where they were assayed essentially according to a previous described procedure (Baudry et al., 1986). Briefly, samples were incubated (for 1 h at 37 8C) with a reaction mixture containing [^{14}C]ornithine as a substrate (NEN, specific activity 40–60 mCi/mmol, final concentration 0.05 mM). At the end of the decarboxylation reaction, the [^{14}C] CO_2 released was trapped using hyamine hydroxide and measured by liquid scintillation spectrometry. Enzyme activity was expressed as CO_2 released/unit of protein content in the supernatant (Lowry et al.,

1951).

Determination of polyamines

The determination of the three polyamines, putrescine, spermidine and spermine, was performed by HPLC after pre-column derivatization with dansyl chloride. The tissue samples from the various CNS regions were homogenized in 5–10 volumes of 0.3N $HClO_4$ (1000 rpm, 14 strokes) and the homogenate was centrifuged at 14,000 g for 15 min. The pellet was dissolved in 0.5N NaOH and subsequently utilized for protein content determination by the method of Lowry et al. (1951). To obtain the dansylation of polyamines, 300 ml of a standard mixture (containing 1.25 nmoles of putrescine and 10 nmoles of spermidine and spermine) and 300 ml of the supernatant from every sample were added to 20 ml of 100 mM 1,8-diaminooctane, used as an internal standard, 20 ml of 3N KOH and 180 ml of 1.5 M Na_2CO_3 (pH 10.5). After adding 0.8 ml of a dansyl chloride solution (8 mg dissolved in 1 ml acetone), the reaction mixture was allowed to stay in the dark overnight. At the end of the incubation, 200 ml of 0.3N KOH were added and the dansylated polyamines were extracted in 1 ml of diethyl ether under vigorous shaking conditions. The samples were then gently centrifuged and 900 ml of the diethyl ether phase were transferred, dried under vacuum and resuspended in 400 ml of methanol. Twenty ml of this sample were injected into a twin pumps HPLC apparatus equipped with a reverse phase C18 column (4.6 mm 150 mm, 5 mm ODS) and with a fluorimetric detector set at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Polyamines were eluted at a flow rate of 1.2 ml/min with a mobile phase resulting by mixing two solutions: eluent A (50% water, 30% acetonitrile and 20% methanol) and eluent B (60% acetonitrile and 40% methanol). The elution was performed by a linear gradient changing from 50 to 100% B in 16 min.

Statistical analysis

To assess the significance of the differences between the experimental groups all results were subjected to one way analysis of variance (ANOVA) followed by Bonferroni post test only if overall $p < 0.05$.

Proteomics studies

Insoluble protein fraction extraction

For the proteomic study of insoluble protein complexes, male animals from each group were taken at an early pre-symptomatic (6 weeks of age) and an early symptomatic (11 weeks of age). Males were taken to exclude possible gender variability.

The animals were anesthetized with 70mg/kg of pentobarbitone and perfused with PBS containing 4U/ml of heparin at a constant flow of 25ml/min using a pump in order to wash out blood that could otherwise interfere with the 2D PAGE analysis. The spinal cords were then quickly dissected and snap frozen in liquind nitrogen and then stored at 80°C until needed.

6 spinal cords from 6-week and 6 spinal cords from 11-week old transgenic mutant animals and control littermates were used for bidimensional electrophoresis. The insoluble protein fraction was extracted using a modified Wang protocol (Wang et al., 2002) in order to make it compatible with subsequent bidimensional electrophoresis. The spinal cords were weighed and all buffers were added in a 1:10 proportion according to tissue weight. Briefly, the spinal cords were firstly homogenized in TEN-1 (100mM Tris pH 8.0, 25mM EDTA pH 8.0, 400mM NaCl) and TEN-2 (100mM Tris pH 8.0 , 25 mM EDTA pH 8.0, 400mM NaCl, 1% NP-40, 2X sigma protease inhibitor cocktail) buffers and then sonicated with a probe sonicator (70W; TEKMAR, Cincinnati, OH, USA) in ice at 40% intensity for 3 cycles of 30 seconds. An aliquot of this first fraction was stored for further western blotting analyses and stored at -80°C. The samples were centrifuged in an Optima (Beckman) preparative ultracentrifuge at 100,000xg, 4°C for 10 minutes. The supernatant (S1) was then stored and 1:10 volumes of TEN-3 (100mM Tris pH 8.0, 1% NP-40) were added to the pellet (P1) and then the samples were sonicated in ice at 40% intensity for 3 cycles of 30 seconds in ice and centrifuged at 100,000xg, 4°C for 10 min. The supernatant (S2) was stored and 1:10 volumes of TEN-4 (100mM Tris pH 8.0, 1% NP-40, 0.25% SDS, 0.5% sodium deoxycholate) buffer were added to the pellet (P2) and the samples were sonicated (no ice) at 40% intensity for 3 cycles of 30 seconds. The samples were then centrifuged at 100,000xg, 4°C for 10 min. The supernatant was stored (S3) and the remaining pellet was washed again with another 1:10 volumes of TEN-4 buffer and centrifuged at 100,000xg, 4°C for 10 minutes. The supernatant was completely removed and the remaining pellet (P3) was solubilzed using 2D-PAGE rehydration buffer (8M urea, 0.5% CHAPS) and sonicated at 40% intensity for 3 cycles of 10 seconds to prevent overheating of the samples that could lead to protein carbamylation and create artifacts. The samples were left to

solubilize in a spinning wheel overnight and the next day spun at 13,000 rpm for 10 min to remove any insoluble material.

Protein content assay

A Non-Interfering Protein Assay (NIPA, G-Biosciences St. Louis MO, USA) was used to assay all samples. The manufacturer's protocol was used for all samples.

2D-electrophoresis

Strip rehydration

50ug of P3 fraction were made up to 350ul with rehydration buffer and DTT, pharmalytes and bromophenol blue were added (8M urea, 0.5% (w/v) CHAPS, 0.8% (v/v) pH 3-10 GE Healthcare Pharmalytes, 18mM DTT, 0.002% (w/v) bromophenol blue (Sigma). The samples were loaded into a rehydration strip tray and 18cm pH3-10 non-linear IPG strips (GE Healthcare) were loaded facing the samples and let to rehydrate for at least 10 hours (overnight).

First dimension

The next day, the first dimension was run using a Multiphor II (GE Healthcare) at 50uA per strip using the following steps:

Step	Voltage	Time (minutes)
1	100 V	60
2	150 V	60
3	300 V	60
4	600 V	30
5	1200 V	30
6	1800 V	30
7	2400 V	30
8	3000 V	30
9	3500V	up to 80kV Total

The first two steps were run at a low voltage in order to eliminate ions from salts that could otherwise interfere with the focusing step. The electrode wicks were changed after the two one-

hour initial steps and by the end of the day. Once the first dimension was finished, the strips were stored at -80°C until needed for the second dimension.

Strip equilibration

Before the second dimension, the strips needed to be equilibrated. The equilibration was performed by incubating the strips in equilibration buffer (6M urea, 75mM Tris-HCl ph 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue) first in 1% DTT for 15 minutes and then in 2.5% iodoacetamide for 15 minutes. Following the equilibration, the strips were briefly washed in Laemli SDS electrophoresis buffer (25mM Tris base, 192mM glycine, 0.1% SDS).

Second dimension

During the second dimension, proteins migrate along the strip and once they reach their isolectric point, that is the pH where they are not anymore charged, they stop migrating. The equilibrated strips were loaded onto 12% acrylamide gels by gently pushing them down against the top part of the gel making sure not to trap any air bubbles between the gel and the IPG strip. 2ul of RPN800 rainbow protein marker (GE Healthcare) were loaded onto filter paper and loaded next to the IPG strips. The IPG strips were overlaid with agarose sealing solution (0.5% (w/v) agarose, 0.002% (w/v) bromophenol blue in Laemli SDS electrophoresis buffer) in order to keep them on top of the gels all throughout the second dimension. The second dimension was performed in an Ettan Dalt six (GE Healthcare) tank using the following protocol keeping the buffer tank temperature constant at 20°C all throughout the second dimension:

30 min at 2W per gel

5-6 h at 15 W gel until the blue dye front reached the bottom of the gel.

Silver staining

All solutions were prepared as described in the table below. After the second dimension was completed, the gels were taken out from the glass plates and places in glass trays with enough fixing solution and stored overnight at 4°C.

The next day, the fixing solution was removed and enough sensitizing solution was added to the gels and incubated on a shaker for 30 minutes at RT. Glutaraldehyde was added just before the sensitizing solution was added to the gels. The gels were then washed 3 times for 5 minutes with

double-distilled water and then the gels were incubated with the silver solution for 20 minutes at RT. Formaldehyde was added fresh to the silver solution prior to incubating with the gels. Following this incubation, the gels were quickly washed 2 times for 1 minute with double distilled water and then the developing solution was added to the gels. Formaldehyde was added fresh to the developing solution before the gels were incubated. The best incubation time according to spot number and background intensity was determined empirically. All gels were incubated for 7 minutes in order to increase reproducibility of the spots visualized on the gels and to avoid high background. In order to stop the silver staining reaction, the developing solution was removed and the gels were incubated with stop solution for at least 10 minutes. The gels were then washed 3 times for 5 minutes with double distilled water and stored in sealed plastic bags with double distilled water at 4°C until required for image scanning for computer analysis.

Silver stain fixing solution 40% (v/v) ethanol, 10% (v/v) acetic acid	**Final concentration**
Ethanol, absolute	40%
Acetic acid, glacial	10%
Sensitizing solution 30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate, 0.125% (v/v) glutaraldehyde	**Final concentration**
Ethanol, absolute	30%
Sodium acetate, anhydrous (FW 82.03)	6.8%
Sodium thiosulphate, pentahydrate (FW 248.18)	0.2%
25% glutaraldehyde	0.125%
Silver solution 0.25% (w/v) silver nitrate, 0.015% (v/v) formaldehyde	**Final concentration**
Silver nitrate (FW 169.87)	0.25%
37% formaldehyde	0.015%
Developing solution 2.5% (w/v) sodium carbonate, 0.0074% (v/v) formaldehyde	**Final concentration**
Sodium carbonate, anhydrous (FW 105.99)	2.5%
37% formaldehyde	0.0074%
Stop solution 1.5% (w/v) Na_2EDTA	**Final concentration**
Na_2EDTA (ethylenediaminetetraacetic acid, disodium salt) (FW 372.24)	1.5%

Computer analysis

Phoretix 2D evolution v2005 (now Progenesys PG600, Non-linear dynamics) was used for computer analysis following these guideline steps:

1. Spot detection. All intensity signals above the gel background are considered to be spots by the program. Care should be taken as some detected spots can actually be false positives due to impurities or gel handling. Therefore detected spots should also be checked one by one by the user.
2. Gel matching. The detected spots are then matched between all the gels. Manual matching of spot patterns that can be recognized by eye can help by setting some spot matches as seeds that can further aid the program to finish automatic matching.
3. Gel warping. Once gel matching has been done, gel images can be warped or overlapped on each other to increase positive matches. Manual warping by introducing some manual anchors according to visible spot patterns can aid automatic warping. After warping, matching should be performed once again and this process is repeated until the percentage of positive matches is high enough to proceed with analysis. At least 80% of positive spot matching was used for this project.
4. Background subtraction. The background value from each gel is subtracted from each spot value.
5. Spot normalization. In order to be able to compare similar spots from different gels, the spot intensity values need to be normalized by creating an absolute ratio when compared with the total spot intensity from each gel.
6. The normalized spot intensity values can then be used for statistical analysis. For this project, 6 gels for each disease stage and animal type were used (6+6+6 animals 6 weeks, 6+6+6 animals 11 weeks).
7. Image production. 2D images can be directly created by selecting a specific area with the cursor from the visualized image and then copied onto the clipboard. 3D images can be created using the same specific selected area and then using the 3D visualization option. Protein spot peaks can be reduced or increased with this function and the images can be rotated as well.

Statistical analysis of 2D gel derived data

Comparative quantitative analysis is performed by resolving the ratio for a single protein spot between experimental groups. That is, the mean normalized volume of a spot in the Experimental group is divided by that for the same spot in the Control group. This enables the detection of spots of increased or decreased expression in the Experimental group. Comparisons were made between

all experimental groups, i.e. G93A vs hSOD, G93A vs non-transgenic and hSOD vs non-transgenic. Firstly, an F-Test was performed, to determine whether the variances in the experimental and control groups were significantly different, using a two-tailed probability calculation, where p was set at 0.05. F-Test could not obviously return any value for spots that were present in less than two gels. The F-Test result was then used in a 'nested' Student's T-Test. Based on the F-Test result two versions of the Student's T-Test were performed (assuming either equal or non-equal variance) for the means of each spot, and a statistically significant result was reported after setting the P value at 0.05. A list of spots with at least a 2-fold change in normalized expression between G93A and hSOD as well as G93A and non-transgenic was produced.

Preparative gels

For preparative gels 400ug of protein were loaded per 18cm pH 3-10NL IPG strip (GE Healthcare). The first dimension protocol was similar to the analytical gel protocol but required more time to achieve 80kV. The second dimension protocol was also similar to the analytical gel protocol and required more time as well because of increased protein load. The silver staining protocol was performed the same way as for analytical gels except that for the sensitizing step where glutaraldehyde was omitted and the silver staining step where formaldehyde was omitted. Formaldehyde was kept in the developing solution instead.

Mass spectrometry

Spots of interest were excised using the One Touch Plus (Web Scientific; Crewe, UK) spot picking pipettes from MS compatible preparatory gels (see 2.7.3). Two sizes of pipettes and tips were used to remove either 1.5mm or 3.0mm diameter spots from 2D gels. A new tip was used for every spot, eliminating cross contamination from one sample to the next. Spot excision was performed in clean environment and eppendorf tubes were rinsed with ddH_2O in order to minimise contaminating proteins such as keratin entering the protein identification workflow at this stage.

In-gel trypsinolysis was performed using an Investigator Progest (Genomic Solutions, Huntington, UK) robotic digestion system (Westbrook *et al.*, 2001). Tandem electrospray mass spectra were recorded using Q-Tof hybrid quadrupole/orthogonal acceleration time-of-flight spectrometer (Waters, Manchester, UK) interfaced to a Waters CapLC capillary chromatograph. Samples were dissolved in 0.1% aqueous formic acid, injected into a Pepmap C18 column (300 μm x 0.5 cm; LC

Packings, Amsterdam, The Netherlands), and eluted into the electrospray with an acetonitrile/0.1% formic acid gradient (5-70% acetonitrile over 20 min).

Data-dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3, or 4 over a survey mass range of 540-1200. Known trypsin autolysis products and keratin-derived precursor ions were automatically excluded. Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in SwissProt/TREMBL, using ProteinLynx Global Server (Wait *et al.*, 2002). One missed cleavage per peptide was allowed, and the fragment ion tolerance was set to 100 ppm. Carbamidomethylation of cysteine was assumed, but other potential modifications were not considered in the first pass search. All matching spectra were reviewed manually, and in cases in which the score reported by ProteinLynx Global Server was less than 100, additional searches were performed against the NCBI non-redundant data base using MASCOT, which utilises a robust probabilistic scoring algorithm (Perkins *et al.*, 1999). Mass spectrometry was performed by Prof. Wait's group at the Kennedy Institute of Reumatology, Imperial College London.

Western blotting

10ug of protein extract per lane were loaded and separated in 10% acrylamide gels using Laemli SDS running buffer [25mM Tris base, 192mM glycine, 0.1% (w/v) SDS] in Mini-Protean 3 cells (Bio-Rad) initially at 1W per gel until the samples entered the separation gel and then the power was set to 2W per gel until the blue dye front reached the bottom of the gel. 5ul of rainbow marker (GE healthcare, RPN800) were loaded per gel. The proteins from the gel were then transferred onto nitrocellulose membranes (Hybond-ECL, GE Healthcare) in Mini trans-blot 3 cells (Bio-Rad) using transfer buffer (50mM Tris, 200mM glycine) at a constant current of 400mA for 2 hours. Large format gels destined for western blotting were run as described before in the second dimension section and were subjected to a semi-dry transfer at a constant current of 400mA for 2 hours in a Multiphor II unit (GE Healthcare). Following the transfer, Ponceaus-S staining [0.1% (w/v) Ponceaus-S, 10% (v/v) acetic acid] was performed in order to check for transfer efficiency. The membranes were then first washed in ddH20, then in TBS (2mM Tris pH 7.6, 1.37 M NaCl) for 5 minutes and blocked with 5% (w/v) milk (BioRad) and TBS-Tween 20 0.1% (v/v) (Sigma). After blocking, the primary antibodies were diluted in 3% milk TBS-T 0.1% and the membranes were incubated overnight at 4°C. The antibody used was sheep anti-SOD1 (1:3000, Calbiochem) and

then the secondary antibody was diluted in milk 3% TBS-T 0.1% and the membranes were incubated for 1 hour at RT. The secondary antibody used was donkey anti-goat (1:3000, Chemicon). Following the secondary antibody incubation, the membranes were washed 3 times for 5 minutes with TBS-T 0.1% and then 1 time for 5 minutes in TBS. In the dark room, the membranes were incubated with ECL detection reagents (Chemicon), wrapped with Saran wrap and X-ray film (GE Healthcare) was exposed to the membranes for the required times.

CHAPTER 3: RESULTS

Forebrain cholinergic system alterations in SOD1 mutant mice

Brain regional neurochemistry at different stages of disease progression assays were based on samples taken from G93A mice, wild-type transgenic mice and non-transgenic littermates at a pre-symptomatic (55 days of age), a symptomatic (110 days of age) and an advanced symptomatic (125 days of age) stages. In agreement with reports from the literature, motor behavior of G93A mice was normal at 55 days, while posterior limb motility was clearly affected at 110 days. Severe paralysis of the posterior limbs and incipient paralysis of the anterior ones was evident at 125 days. Mean lifespan of G93A mice (136◎5 days) was evaluated in a group of female animals, euthanized at the terminal stage of the disease, which is characterized by complete limb paralysis and inability to eat and drink. Brain cholinergic systems were initially studied in various brain areas of the three groups of mice at 55 and 125 days of age by measuring ChAT catalytic activity. At 125 days of age, ChAT activity was significantly decreased in the hippocampus (22, 18%), the olfactory–entorhinal cortex (18, 20%) and the fronto-parietal cortex (13,9%) of G93A mice compared to wild-type transgenic mice and non-transgenic littermates, respectively (Fig. 1A). These differences were clearly related to the disease progression, as they were not present at the pre-symptomatic stage (Fig. 1A). In other brain regions, such as the striatum and the cerebellum, no significant alterations of ChAT activity were measured (Fig. 1B). Neurochemical alterations of the forebrain cholinergic systems were already present at an earlier symptomatic stage (110 days of age) and showed a trend towards aggravation with age. This is demonstrated in Fig. 1C, where the percent decrease of ChAT activity in the various brain regions of G93A mice is plotted against the value of wild-type transgenic mice at the different ages examined. In the same brain regions, no significant alterations of the cholinergic–cholinoceptive marker, AchE, were measured (Table 1). Furthermore, the GABAergic system appeared completely unaffected in the hippocampus and the olfactory–entorhinal cortex regions showing the largest decrease of cholinergic activity (Table 1). Forebrain regions, in which cholinergic neurons projecting to the cortex and the hippocampus are localized, were examined by ChAT immunocytochemistry at 125 days of age. Compared to non-transgenic littermates, in G93A mice cholinergic neurons were less abundant in the medial septum–ventral diagonal band (Fig. 2A and B) and appeared to some extent reduced in number also in the nucleus basalissubstantia innominata area (Fig. 2C and D). The striatum, possessing a well-characterized population of cholinergic interneurons, did not show any regressive alterations

of ChAT immunoreactive neurons (Fig. 2E and F).

Neurochemical correlates of motor neuron degeneration in the spinal cord and brainstem

Assays of cholinergic activity at the main sites of motor neuron degeneration, the lumbar and the cervical spinal cord as well as the brain stem, revealed decreases in ChAT catalytic activity in G93A mice at a symptomatic stage (125 days of age), while no changes were observed at 55 days of age (Fig. 3A). The reduction found in G93A mice reached 22–29% in the cervical spinal cord, 35–39% in the lumbar spinal cord and 17–15% in the brain stem when compared to wild-type transgenic mice and non-transgenic littermates, respectively. In addition, AChE enzymatic activity was significantly decreased in both the spinal cord segments of 125-day-old G93A mice (Fig. 3B). A marker for GABAergic neurons (GAD) was unaffected in the spinal cord of G93A mice, as compared to wild-type transgenic animals. When compared to non-transgenic littermates, both types of transgenic mice showed a significant decrease of GAD enzymatic activity at the cervical spinal cord level (Fig. 3C). Immunocytochemistry of cervical and lumbar spinal cord segments at 125 days of age showed that most large ChAT immunoreactive motor neurons present in the ventral horn of non-transgenic littermates, had disappeared in G93A animals (Fig. 4). At the neurochemical level, a significant decrease of ChAT activity was already detectable in the spinal cord and the brain stem of G93A mice at 110 days of age (Fig. 5). Other assays (AChE and GAD) carried on at 110 days of age, gave results similar to those reported above for the 125 days stage. In particular, AChE activity was already significantly decreased in both the cervical and the lumbar spinal cord of G93A mice while GAD activity showed no differences (data not shown). As these data indicated that a substantial loss of spinal motor neurons was already occurring in G93A mice at 110 days of age, we used this stage to obtain an evaluation of presumably apoptotic cell death, an aspect of human ALS and of mutated SOD1 animal models that has raised controversies in recent literature (reviewed in Guegan and Przedborski, 2003). By using a sensitive assay to detect the amount of nucleosomal DNA fragmented in an apoptotic like way in tissue homogenates (White and Barone, 2001;Monti and Contestabile, 2003), we found significantly higher levels of DNA fragmentation in the lumbar spinal cord of G93A mice compared to wild-type transgenic animals, while no significant differences were detected in homogenates from a cortical region taken from the same animals (Fig. 6).

Fetal choline supplementation and postnatal ALCAR treatment

In rats, fetal choline administration has been shown to favorably affect the development of the forebrain cholinergic system (Meck et al., 1989; Williams et al., 1998; Sandstrom et al., 2002; Meck et al., 2003).

Two groups of G93A mice and non-transgenic littermates supplemented with choline during gestation were used to assay, at 125 days of age, ChAT levels in spinal cord and some brain regions in order to verify whether the treatment was able to modify the adult levels of cholinergic activity and/or to counteract the cholinergic deficit found to be present in symptomatic G93A mice. As shown in Table 2, gestational choline supplementation did not result in any relevant effect on the decline of ChAT activity in the spinal cord and the brain regions assayed in G93A with respect to non-transgenic littermates (compare percent decreases with those derived from Fig. 1A). Interestingly, however, by comparing the absolute value of the catalytic activity of gestationally choline-supplemented mice (Table 2) with the respective values of their untreated counterparts (data from Figs. 1 and 3), it resulted that these values were significantly ($p < 0.001$, Student's t-test) higher in choline supplemented mice, basal ChAT activity resulting increased by 30–55% depending on the region considered. The catalytic activity of AChE was not affected by fetal choline supplementation (data not shown). Survival of gestationally choline supplemented G93A mice was not significantly different from that of untreated animals (mean lifespan was 136 5 days for G93A untreated mice compared to 138 3 days for choline supplemented animals; means S.E. for groups of five to seven female mice). In rats, long-term administration of ALCAR has been claimed to be neuroprotective by positively affecting the brain cholinergic systems (Piovesan et al., 1994; Taglialatela et al., 1994). G93A mice supplemented or not with choline during gestation and administered with ALCAR from postnatal day 40 until death did not show any improvement of their survival (mean lifespan was 130 ± 6 days for ALCAR supplemented mice and 139 ± 3 days for choline plus ALCAR supplemented mice; means S.E. for groups of five to seven female mice).

Glutamine synthetase activity

As gliosis has been repeatedly reported to parallel motor neuron loss in the spinal cord of ALS patients and SOD1 animal models, we also measured the activity of glutamate synthetase, an enzyme that is considered to be an astrocytic marker and that has been shown to increase in experimental models of neurodegeneration (Virgili et al., 1992). As shown by the data presented in Table 3, we did not find any significant alteration of glutamine synthetase activity in the spinal cord, as well as in some other brain regions, of G93A mice with respect to controls, at 125 days of age.

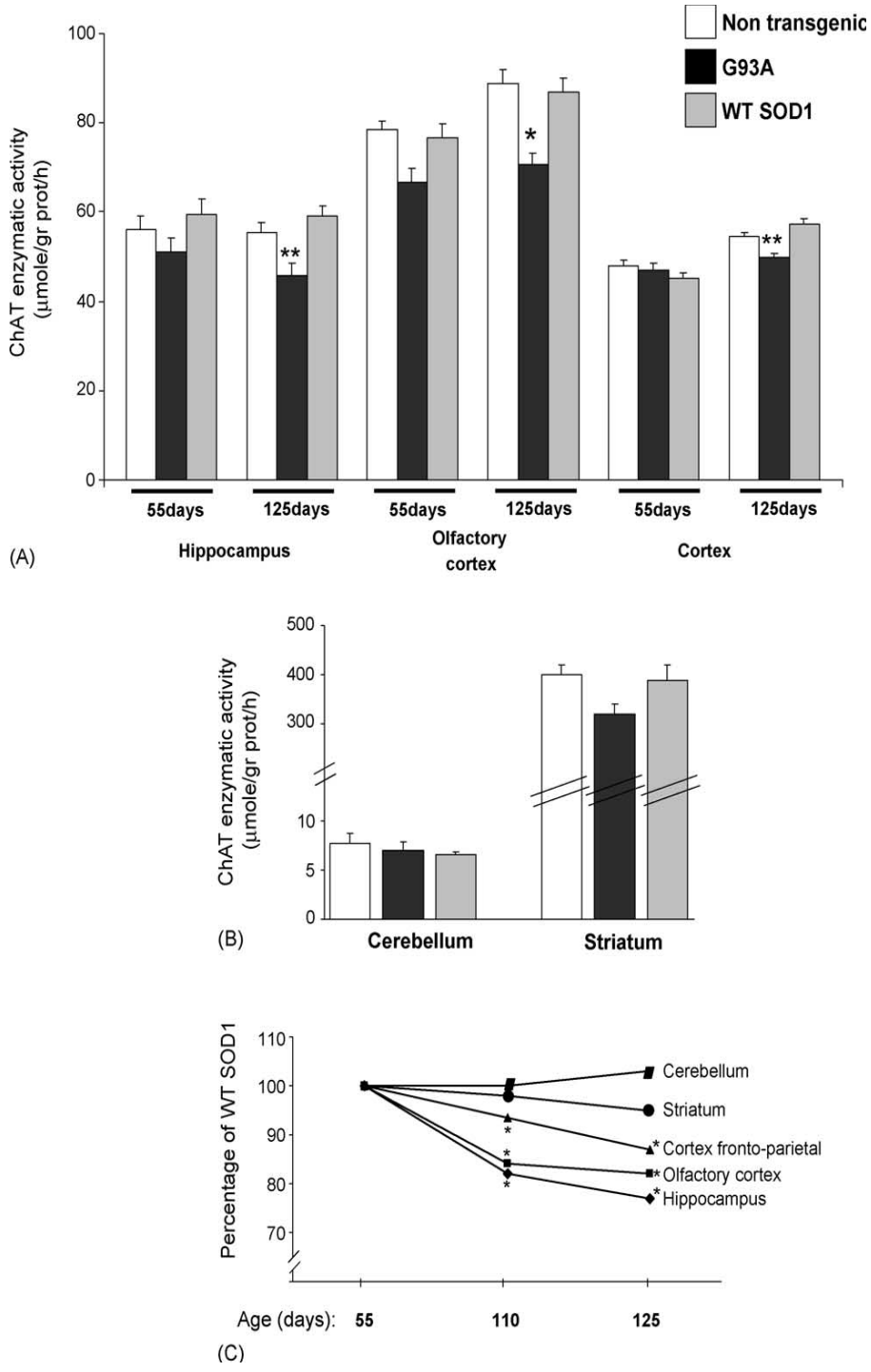


Fig. 1. ChATactivity in brain regions of G93A, wild-type transgenic mice and non-transgenic littermates at 55 and 125 days of age (A and B). Bars represent the mean ± S.E. of seven to nine animals. *$p < 0.05$, **$p < 0.01$ as compared to both wild-type transgenic mice and non-transgenic littermates, Bonferroni's test after ANOVA. (C) Composite graph showing the percent decrease of ChAT activity in brain regions of transgenic mice carrying the mutated SOD1 gene at different ages. Results are expressed as percent activity found in G93A mice with respect to wild-type transgenic mice of the same age. Each point is the mean of seven to nine animals; (*) denotes statistically significant differences of at least $p < 0.05$ (Student's t-test).

Table 1
AchE and GAD activity in various brain regions of the three groups of mice at 125 days of age

	Ache			GAD		
	Non transgenic	G93A	WT SOD1	Non transgenic	G93A	WT SOD1
Cerebellum	2.39±0.37	2.44±0.22	2.9±0.14	-	-	-
Striatum	45.4±1.2	38.2±2.9	42.2±2	-	-	-
Cortex fronto-parietal	5±0.12	4.5±0.15	4.8±0.17	-	-	-
Olfactory cortex	9±0.29	8.3±0.5	7.9±0.3	100±9	110±9	114±9
Hippocampus	7±0.17	6±0.17	6.7±0.56	56.1±3.1	56.1±2.7	53.1±1.4

Data are expressed as mmole/g prot/h for AChE and mmole/g prot/h for GAD and are the mean ± S.E. of seven to nine animals

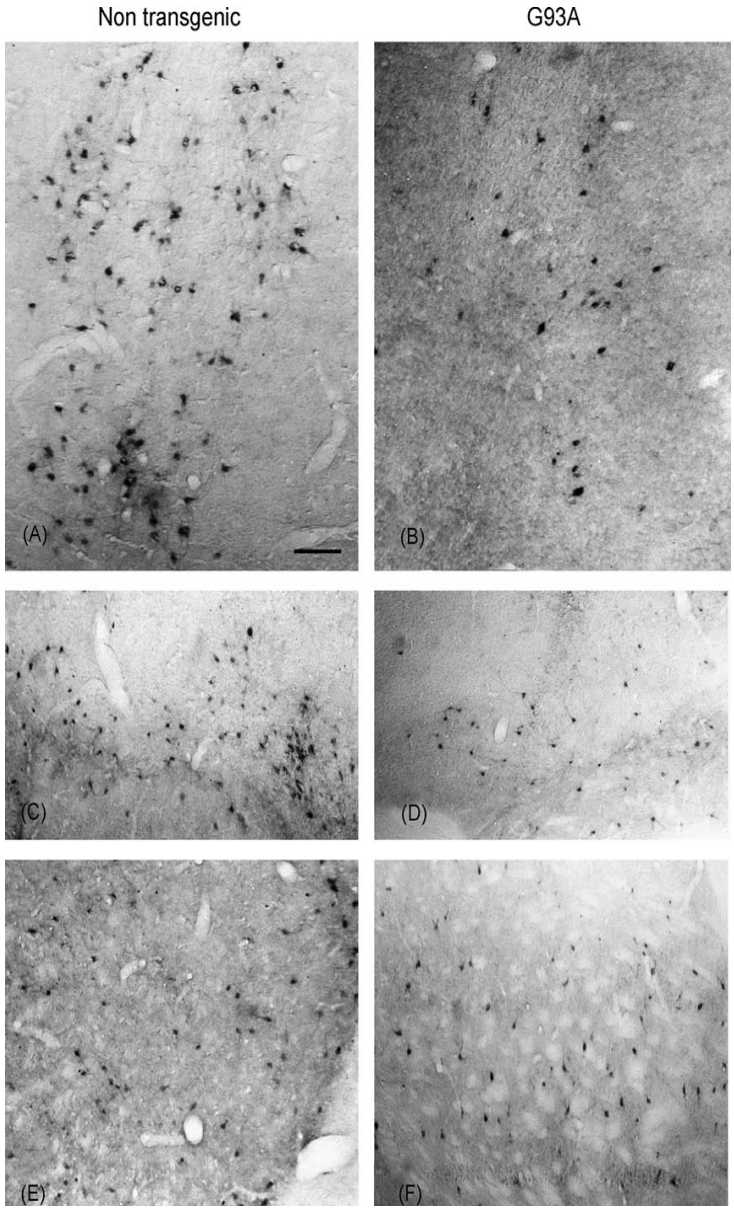


Fig. 2. Cholinergic neurons, revealed through ChAT immunohistochemistry, in the medial septum–ventral diagonal band (A and B), in the nucleus basalis magnocellularis (C and D) and in the striatum (E and F) of 125-day-old G93A mice and non-transgenic littermates. Note the decreased number of immunopositive neurons in the basal forebrain areas of G93A mice. Calibration bar: 150 mm.

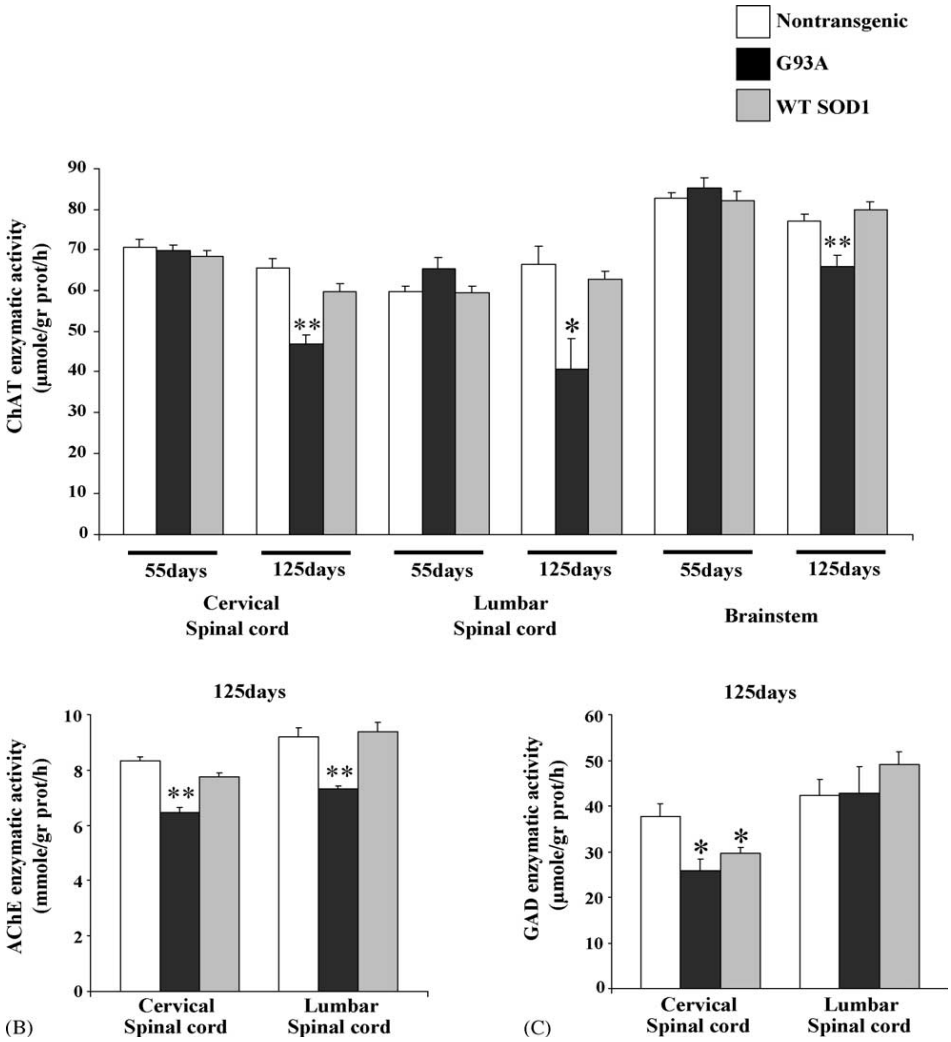


Fig. 3. Enzyme activities in the spinal cord and brainstem of the three groups of mice. Bars represent the mean ± S.E. of seven to nine animals. *$p < 0.05$, **$p < 0.01$ as compared to both wild-type transgenic mice and non-transgenic littermates, Bonferroni's test after ANOVA.

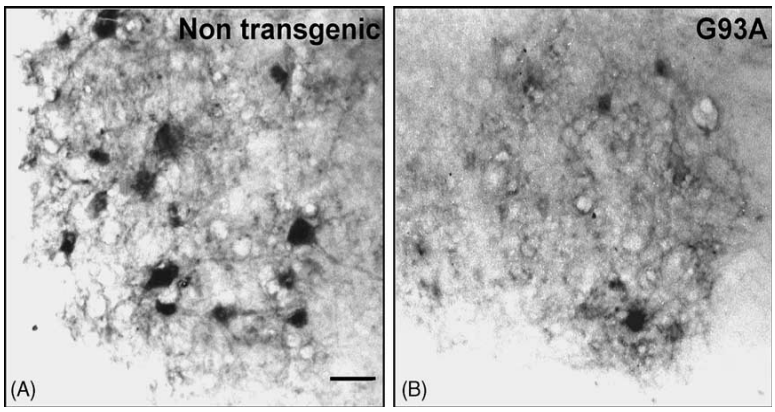


Fig. 4. Cholinergic motor neurons in the ventral horn of the spinal cord of G93A mice and non-transgenic littermates at 125 days of age. Note the dramatic decrease of the large cholinergic motor neurons in the G93A mice. Calibration bar: 50 mm.

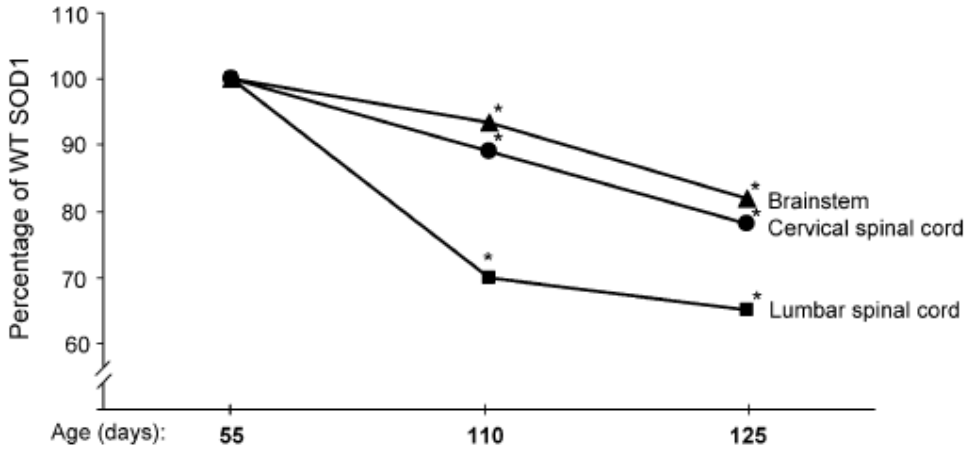


Fig. 5. Composite graph showing the percent decrease of ChATactivity in the spinal cord and brain stem of transgenic mice carrying the mutated SOD1 gene at different ages. Results are expressed as percent activity found in G93A mice with respect to wild-type transgenic mice of the same age. Each point is the mean of seven to nine animals. (*) Denotes statistically significant difference of at least $p < 0.05$ (Student's t-test).

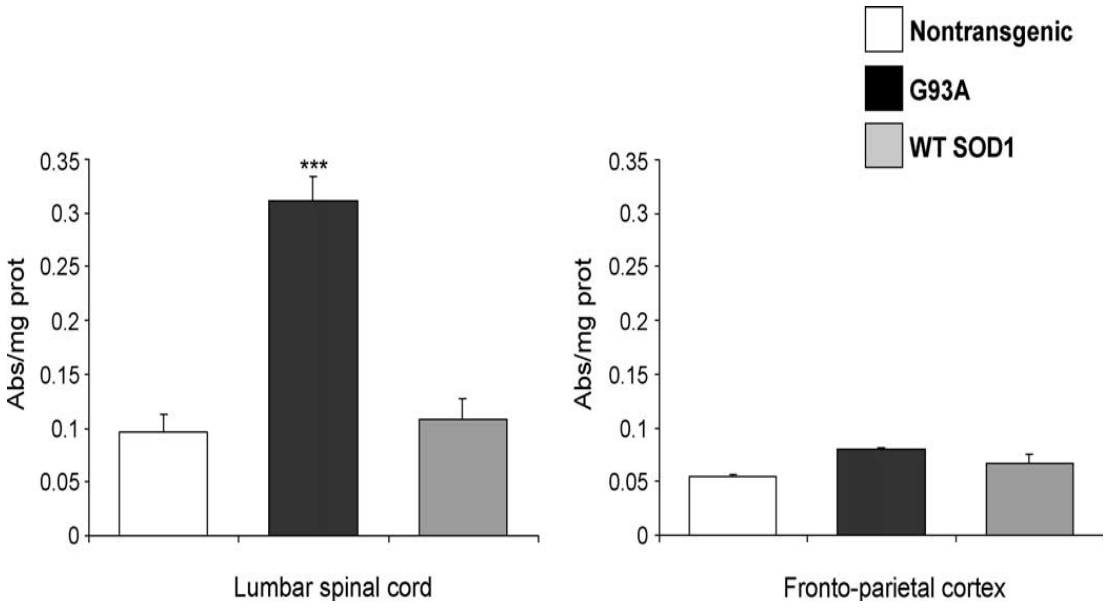


Fig. 6. Evaluation of nucleosomal DNA fragmentation through ELISA method in tissue extract from 110-day-old mice. Bars are the mean ± S.E. of three animals. ***$p < 0.001$ as compared to wild-type transgenic animals and non-transgenic littermates, Bonferroni's test after ANOVA.

Chat activity in choline-supplemented G93A and non-transgenic littermates at 125 days of age

	Non-transgenic (NT)	G93A	% ChAT decrease respective to NT (untreated)	% of ChAT decrease in G93A respective to NT (choline-supp)
Cervical spinal cord	65.5±2.3	46.7±2.5	29**	19**
Untreated	86.1±1.4 (131%)º	69.7±2.3		
Choline supplemented				
Lumbar spinal cord	66.4±4.5	40.7±7.4	39**	32**
Untreated	103±3 (155%)º	69.8±2.1 (171%)		
Choline supplemented				
Hippocampus				
Untreated	55.6±1.9	45.9±2.8	17.4**	16**
Choline supplemented	88.5±2.1	74.6±2.7		
Olfactory cortex				
Untreated	88.7±3.8	70.8±12.4	20**	22.5**
Choline supplemented	133±3 (150%)º	103±2		

Data are expressed as mmole/g prot/h and are the mean ± S.E. of six to nine animals.

º Denotes that the percent difference with respect to untreated animals (in brackets) is statistically significant ($p < 0.01$, Student's t-test).

Table 3

Glutamine synthetase in the spinal cord and brain regions

	125 days		
	Non-transgenic	G93A	WT SOD1
Cervical spinal cord	2.42±0.12	2.26±0.10	2.3±0.08
Lumbar spinal cord	1.71±0.09	1.55±0.13	1.8±0.10
Brainstem	1.92±0.04	1.71±0.05	2.07±0.15
Hippocampus	1.46±0.10	1.69±0.11	1.88±0.10
Olfactory cortex	2.46±0.16	2.22±0.15	2.05±0.10

Data are expressed as mmol/g prot/h and are the mean ± S.E. of seven to nine animals

Differential neuroprotecion by creatine supplementation

The dose of creatine supplementation used for present experiments was the same recently adopted for studies of neuroprotection in rats and mice, including G93A mice (Dedlu et. al., 2003; Klivenyi et al., 1999; Matthews et al., 1998; Matthews et al., 1999). Creatine is transported to body organs, including the brain, where it is assumed by cells through an active membrane transport system (Ipsiroglu et al., 2001). Long-term (4–8 week) oral administration of creatine, resulting in average intake similar to the one produced by our diet, increased by 15–30% total creatine and phosphocreatine concentration in brain of rats and mice [18]. Rats fed with normal or with creatine-enriched diet did not show any significant difference in daily food intake and body weight increase. Unilateral intrastriatal injection of ibotenic acid in rats resulted in large depletion of markers for cholinergic and GABAergic striatal neurons (Fig. 7), reflecting the widespread excitotoxic death of these neurons, as previously shown (Ciani et al., 2001). Long-term feeding with creatine-enriched diet was remarkably neuroprotective towards the excitotoxic lesion. In the IBO-injected striatum, the marker for cholinergic neurons, ChAT, was decreased by 63% as compared to the contralateral side in normally fed rats, while the decrease was only 32% in rats fed with creatine-enriched diet (Fig. 7A). Similarly, the marker for GABAergic neurons, GAD, was decreased by 68% in the IBO-injected striatum of normally fed rats, while it was significantly less reduced (49%) in rats fed with creatine enriched diet (Fig. 7B). Interestingly, while creatine supplementation did not affect, per se, the striatal ChAT level, the GABAergic marker was significantly increased (+35%) in the saline-injected striatum of rats fed with creatine-enriched diet (Fig. 7B). The levels of enzymatic activities measured in saline injected striata were not different from those present in sham-operated animals assayed in parallel (Figs. 7A, B). In a different model of excitotoxicity, IBO injection in the nucleus basalis magnocellularis (NBM) with degeneration of cholinergic basal forebrain neurons and consequent decrease of cholinergic innervation to the cortex (Casamenti et al., 1998; Contestabile et al., 2004), dietary creatine supplementation was not neuroprotective, as in both groups of rats a similar decrease of ChAT activity in the ipsilateral cortex was recorded (Fig. 8).

In G93A SOD1 mutant mice, the progression of the ALS-like disease can be monitored by the decrease of the cholinergic marker, ChAT, not only in the affected regions of the spinal cord where cholinergic motor neurons degenerate, but also in some forebrain areas receiving cholinergic innervation from basal forebrain cholinergic neurons (Crochemore et al., 2005). Creatine supplementation results in increased survival of G93A mice and in delay of some disease-related symptoms (Klivenyi et al., 1999; Zhang et al., 2003) and it may, therefore, favorably affect the loss

of the cholinergic marker. In the present experiment, 2% creatine supplementation starting at the age of 40 days resulted in a small, but significant, increase in the survival of transgenic mice (mean lifespan of normally fed G93A mice: 132.4 ± 2.6 days; mean lifespan of creatine-supplemented G93A mice: 142 ± 3 days; n = 7, $P < 0.05$, Student's t test). Some G93A mice were sacrificed at the age of 110 days, together with age-matched wild type littermates, as at this stage of progression the disease is already characterized by significant decrease of ChAT activity in the spinal cord, as well as in some forebrain areas (Crochemore et al., 2005). The levels of ChAT activity in wild type mice were not changed by the different dietary regimen in any of the regions examined and, accordingly, data from these animals were pooled together. In the lumbar spinal cord, ChAT activity was similarly decreased in G93A mice, either fed with normal or creatine-supplemented diet, compared to wild type animals (Fig. 9A). In the olfactory cortex and the hippocampus of the same mice, instead, the decrease in ChAT activity present in G93A mice at 110 days of age was completely counteracted by the long-term dietary supplementation with creatine (Figs. 9B, C). Interestingly, also in mice, creatine supplementation increased GAD activity in the striatum, but not in the hippocampus of both wild type and transgenic mice (Fig. 10).

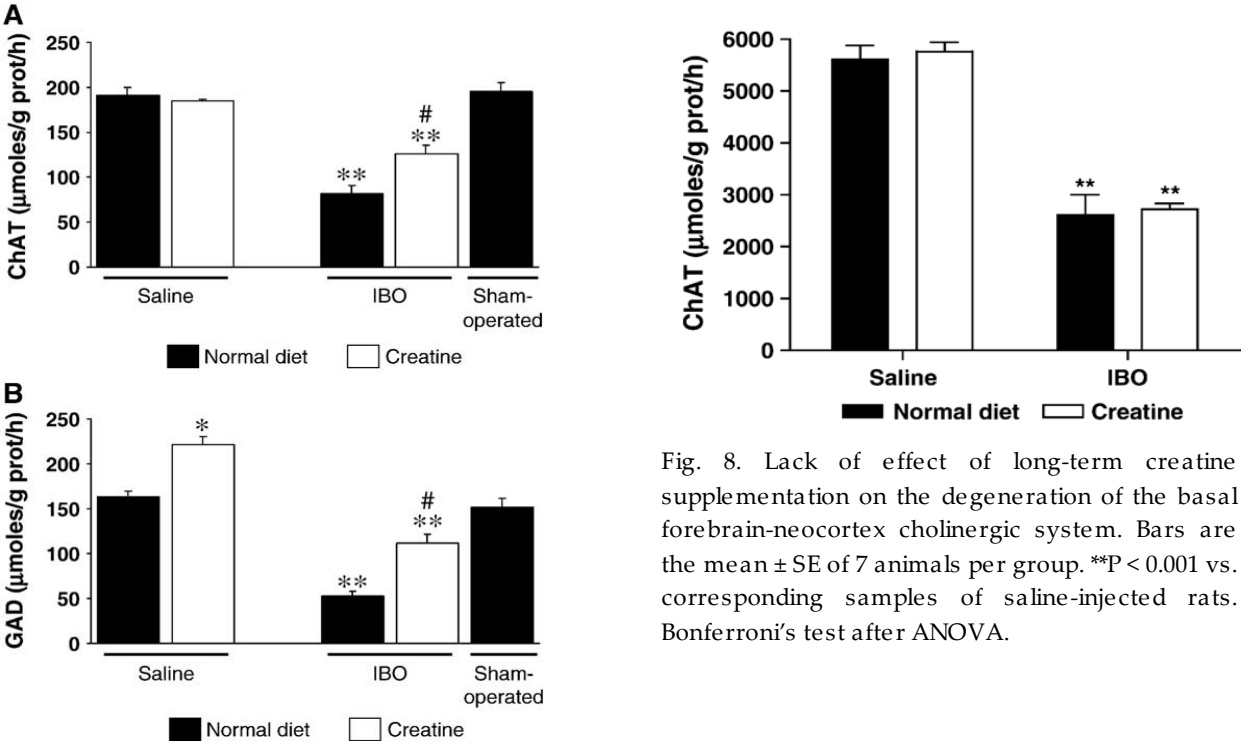


Fig. 8. Lack of effect of long-term creatine supplementation on the degeneration of the basal forebrain-neocortex cholinergic system. Bars are the mean ± SE of 7 animals per group. **P < 0.001 vs. corresponding samples of saline-injected rats. Bonferroni's test after ANOVA.

Fig. 7. Effect of long-term creatine supplementation on neuroprotection of striatal cholinergic and GABAergic neurons from ibotenic acid excitotoxic insult. Bars are the mean ± SE of 7 animals per group. (A) ChAT activity in the striatum of IBO-injected rats fed with normal- or creatine-supplemented diet, compared to the saline-injected striatum or to the striatum of shamoperated rats. (B) GAD activity assayed in the same samples. **P < 0.001 vs. respective saline-injected striata; #P < 0.01 vs. the striatum of normal diet-fed rats. Bonferroni's test after ANOVA.

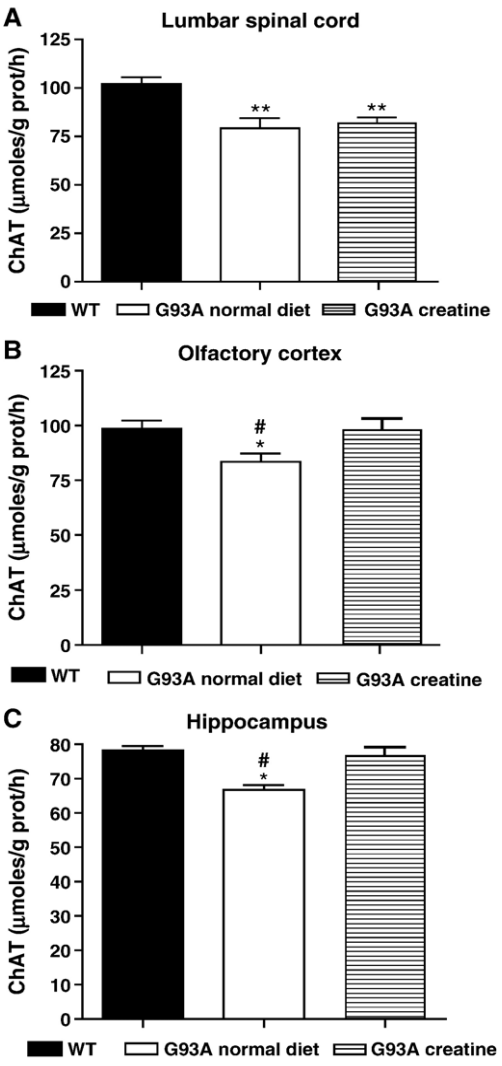


Fig. 9. Differential effect of long-term creatine supplementation on the cholinergic marker in the spinal cord and forebrain areas of G93A mice at 110 days of age. Bars are the mean ± SE of 7 (G93A mice) or 10 (wild type mice) animals per group (A) ChAT activity in the lumbar spinal cord of wild type and transgenic mice. (B) ChAT activity in the olfactory cortex of wild type and transgenic mice. (C) ChAT activity in the hippocampus of wild type and transgenic mice. *P < 0.05 vs. wild type mice; #P < 0.05 vs. creatine-supplemented G93A mice. Bonferroni's test after ANOVA.

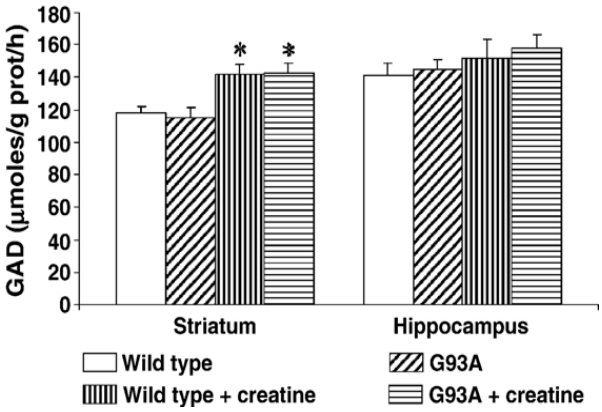


Fig. 10. Effect of long-term creatine supplementation on GAD activity in the striatum and hippocampus of wild type and G93A mice. Bars are the mean ± SE of 7 (G93A mice) or 5 (wild type mice) animals per group. *P < 0.05 vs. corresponding normally-fed animals. Bonferroni's test after ANOVA.

ODC/polyamine system alterations in ALS-G93A mice

ODC activity was measured in tissue extracts from cervical and lumbar tracts of the spinal cord, and brain stem (Fig. 11). In all cases no significant differences were found comparing G93A,wild-type transgenic and non transgenic mice at a presymptomatic stage (55-day-old mice). By contrast, at a symptomatic stage (125-day-old mice), the analysis revealed a marked increase of ODC activity in the spinal cord and brain stem of G93A mice, compared to both wild-type transgenic and non transgenic animals (Fig. 11). In particular, ODC activity was about three times higher in cervical spinal cord and about four times higher in lumbar spinal cord of G93A mice. A smaller, but still significant, increase was detected in brain stem, where ODC activity was higher by 150–200% in G93A mice compared to wild-type transgenic and non transgenic animals (Fig. 11). By contrast, the enzymatic activity did not show any significant change in the cortex and cerebellum of G93A mice,compared to wild-type transgenic and non transgenic animals, both at 55 and 125 days of age (Table 4).

Tissue polyamine levels

Figs. 12–14 show the amounts of the three main polyamines, putrescine, spermidine and spermine, measured in the cervical and lumbar spinal cord, as well as in the brain stem from 55-and 125-day-old G93A mice compared to wild-type transgenic mice and non transgenic littermates. In agreement with alterations detected in ODC enzymatic activity, no differences were detected in younger animals, while levels of putrescine were consistently higher in 125-day-old G93A mice, compared to wild-type transgenic and non transgenic animals. In the cervical spinal cord the putrescine level of G93A mice was six times and 4.5 times that of wild-type transgenic and non transgenic animals, respectively (Fig. 12). In the lumbar spinal cord, putrescine increased about three times with respect to both controls (Fig. 13) and a smaller, but still significant increase was also observed in the brain stem of G93A mice (Fig. 13). As shown in Figs. 12–14, levels of spermidine in both the cervical and lumbar spinal cord of G93A mice appeared higher, compared to the other two types of mice, but this difference was statistically significant only in the case of the cervical spinal cord. No significant differences were found in spermine levels in any of the regions examined, among the three experimental groups of animals. As shown in Table 5, no significant changes in the levels of any of the polyamines considered were detected among the three groups of experimental mice in the cortex and cerebellum,analyzed in 125-day-old animals.

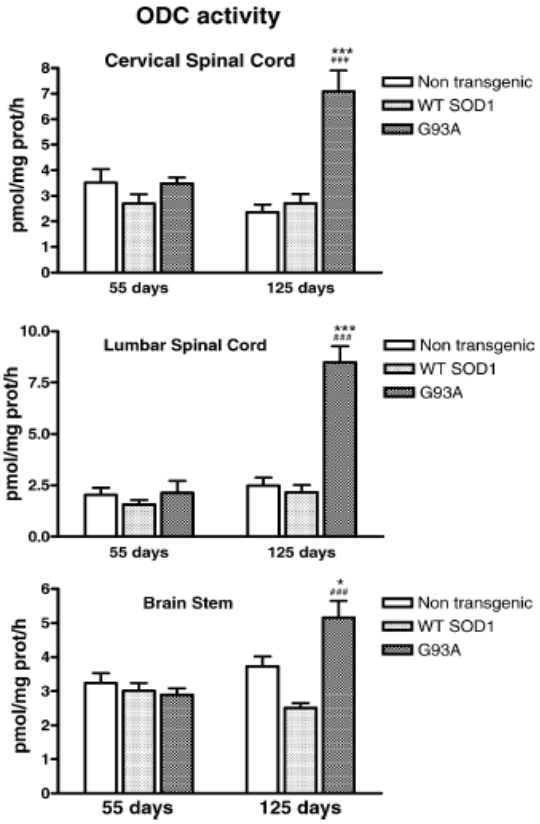


Fig. 11. ODC activity in spinal cord and brain stem of G93A, wild-type transgenic and non transgenic mice at 55 and 125 days of age. Bars are the mean ± S.E.M. of seven animals. *p < 0.05, ***p < 0.001 as compared to non transgenic mice; ###p < 0.001 as compared to wild-type transgenic mice, one way ANOVA with Bonferroni's test.

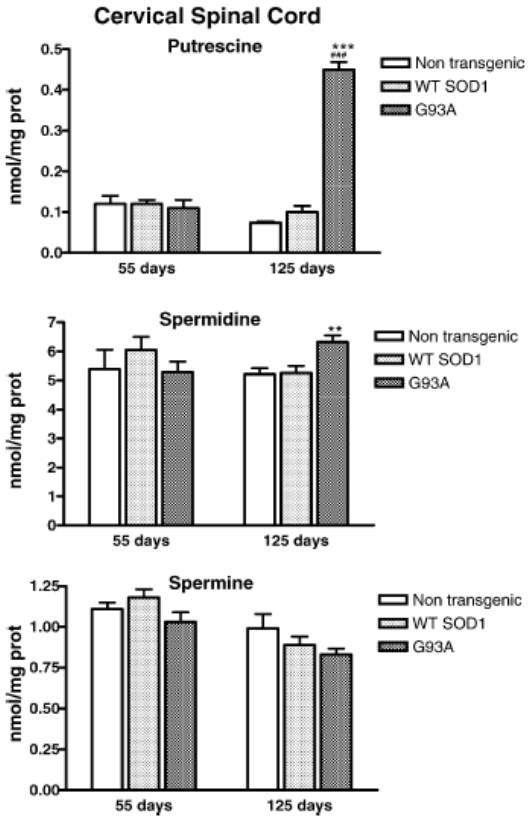


Fig. 12. Polyamine content in the cervical spinal cord of G93A, wild-type transgenic and non transgenic mice at 55 and 125 days of age. Bars are the mean ± S.E.M. of five to seven animals. **p < 0.01, ***p < 0.001 as compared to non transgenic mice; ###p < 0.001 as compared to wild-type transgenic mice, one way ANOVA with Bonferroni's test.

Table 4
ODC activity in cerebral cortex and cerebellum of G93A, wild-type transgenic and non transgenic mice at 55 and 125 days of age

Days	Fronto-parietal cortex			Days	Cerebellum		
	NTG	WT SOD1	G93A		NTG	WT SOD1	G93A
55	3.09±0.51	3.27±0.24	3.48±0.41	55	2.59±0.35	2.41±0.28	2.37±0.27
125	3.41±0.39	3.86±0.22	3.56±0.16	125	2.22±0.28	2.37±0.28	2.29±0.29

Data, expressed as pmoles/mg protein/h, are the mean ± S.E.M. of four to six animals.

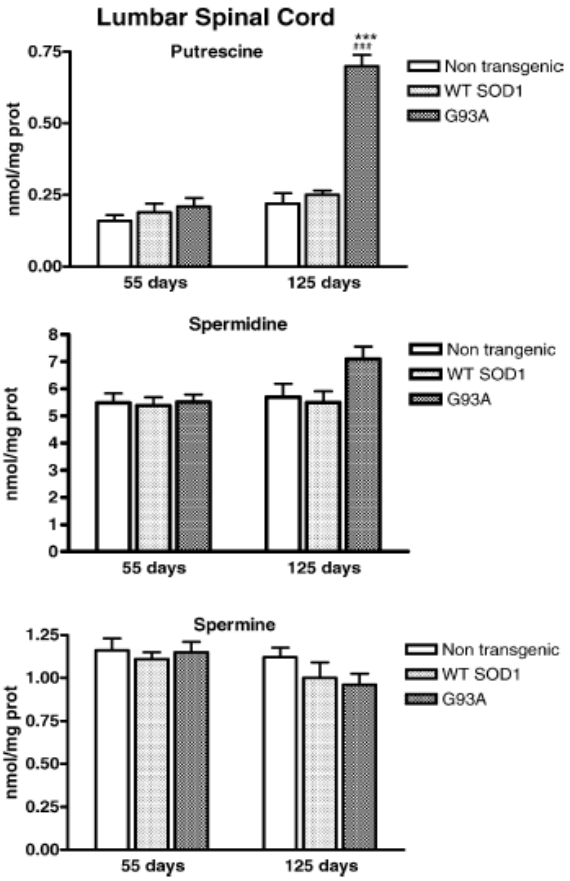


Fig. 13. Polyamine content in the lumbar spinal cord of G93A, wild-type transgenic and non transgenic mice at 55 and 125 days of age. Bars are the mean ± S.E.M. of five to seven animals. ***$p < 0.001$ as compared to non transgenic mice; ###$p < 0.001$ as compared to wild-type transgenic mice, one way ANOVA with Bonferroni's test.

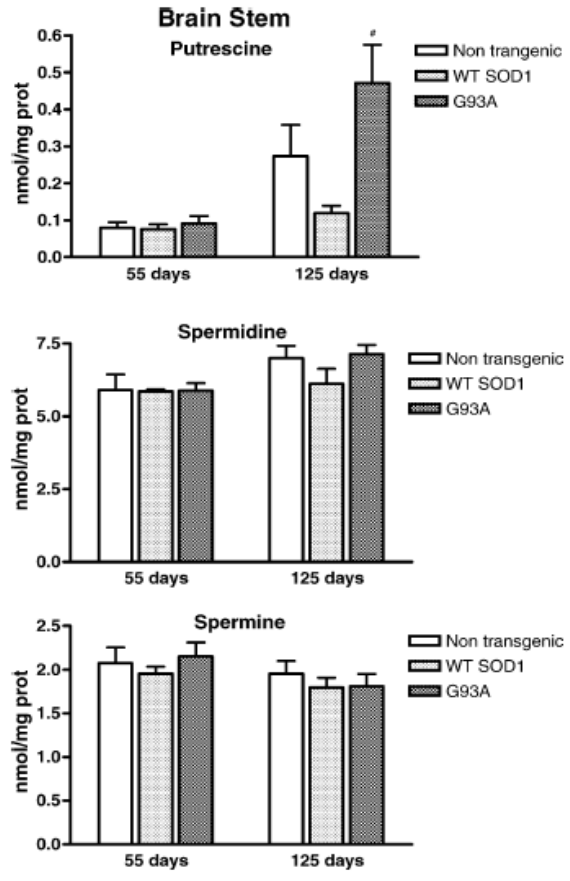


Fig. 14. Polyamine content in the brain stem of G93A, wild-type transgenic and non transgenic mice at 55 and 125 days of age. Bars are the mean ± S.E.M. of 5–10 animals. #$p < 0.05$ as compared to wild-type transgenic mice, one way ANOVA with Bonferroni's test.

Table 5
Polyamine levels in cerebral cortex and cerebellum of G93A, wild-type transgenic and non transgenic mice at 125 days of age

Days	Fronto-parietal cortex			Days	Cerebellum		
	NTG	WT SOD1	G93A		NTG	WT SOD1	G93A
Putrescine	0.042±0.007	0.022±0.003	0.049±0.008	Putrescine	0.116±0.043	0.107±0.032	0.263±0.06
Spermidine	2.389±0.097	2.069±0.104	2.304±0.129	Spermidine	6.197±0.285	5.650±0.503	6.160±0.66
Spermine	2.619±0.111	2.405±0.085	2.537±0.130	Spermine	5.154±0.181	4.910±0.426	4.820±0.46

Data, expressed as nmoles/mg protein, are the mean ± S.E.M. of six to seven animals.

Proteomics of insoluble protein complexes

P3 fractions from mutant animal spinal cords showed strong immunoreactivity for SOD1 as shown by western blotting. It has previously been reported (Basso et al. 2006) that Triton extracted protein insoluble fractions that show strong SOD1 immunoreactivity in mutant G93A animals are in part composed of polyubiquitinated SOD1. Notice that in 6 week-old animals there are no high molecular bands when compared to 11 week-old animals:

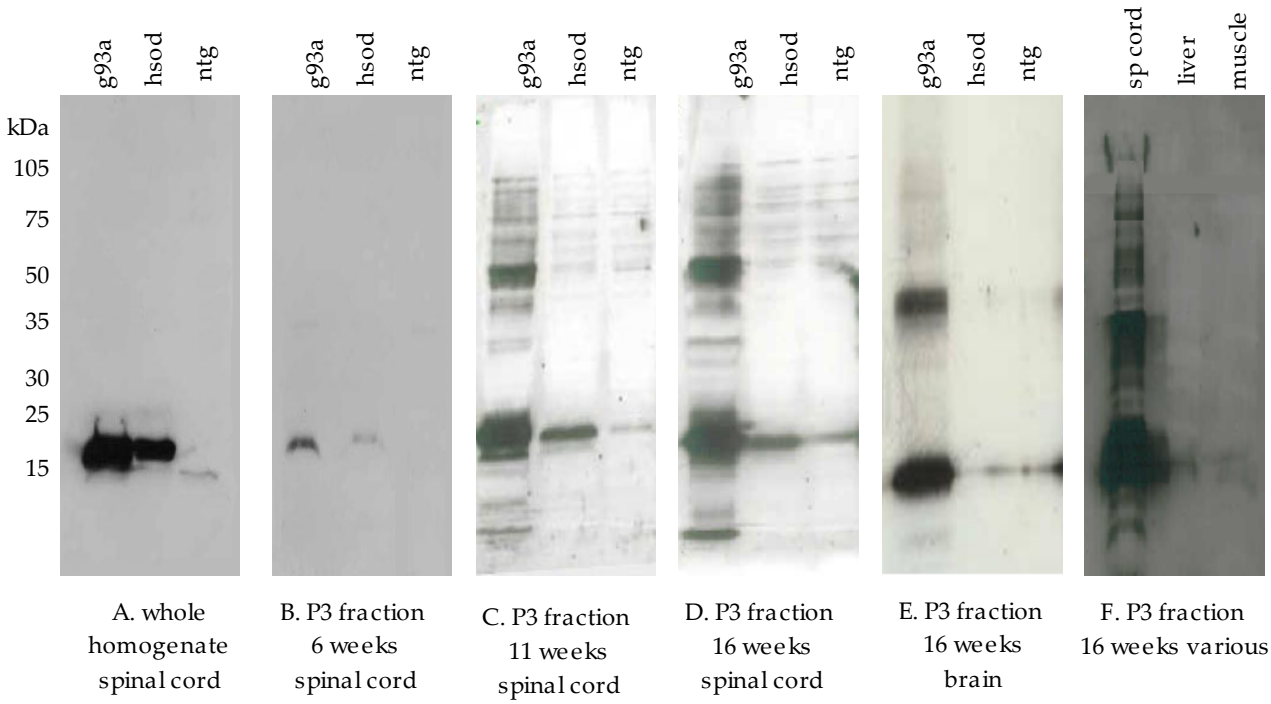


Fig 15. Spinal cord extracts and P3 fractions probed against a specific anti-SOD1 antibody

Interestingly, these high molecular weight bands were very strong in the spinal cord and brain when compared to other tissues/organs such as muscle and liver (Fig 15A-F). The high molecular bands could not be detected in the whole homogenate in any of the animals (Fig. 15A) neither in the P3 fraction from asymptomatic animals (Fig. 15B). However they could already be detected in symptomatic (Fig. 15C) and end-stage (Fig. 15D-F) animals. Because of this strong SOD1 immunoreactivity and high molecular bands in symptomatic animals, it was assumed that SOD1 mediated insoluble aggregates could be present as well in this insoluble fraction (P3).

About 700-800 protein spots were detected in analytical gels by 2D-PAGE and silver staining as shown below (Fig. 16), SOD1 is circled in red as a reference. The insoluble protein was also separated by 2D-PAGE, blotted onto nitrocellulose membrane and probed with a specific anti-SOD1 antibody. However, because the signal spots were strong and too close, the observed signal was actually observed as a blotch laying in the pH 4-7 range. Therfore we decided to further separate the insoluble fraction in a pH4-7 NL gradient and proceeded as before (Fig. 17). Several

protein spots immunoreactive for SOD1 were found in G93A animals when compared to wild-type animals.

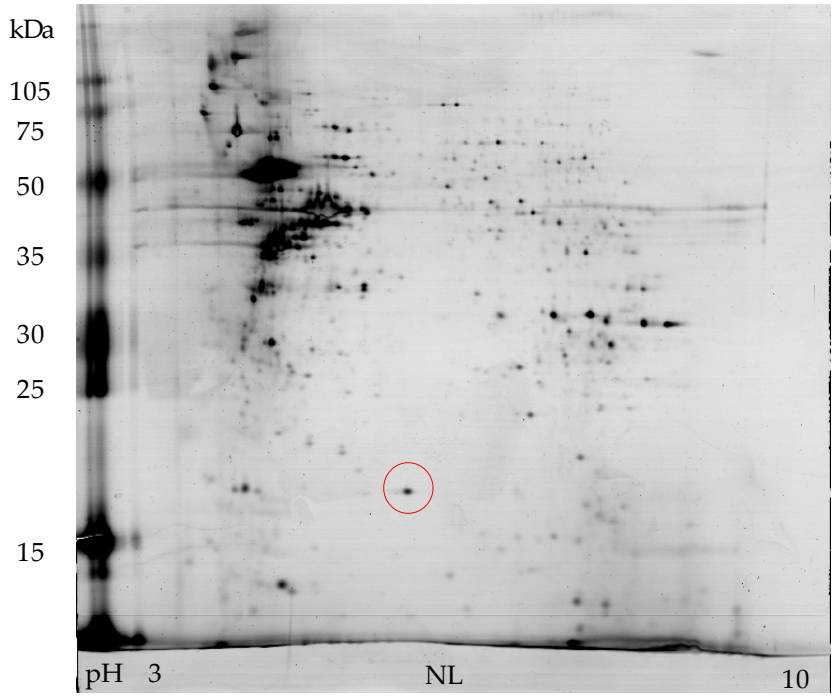


Fig. 16 A representative insoluble fraction analytical 2D gel. The gel was silver stained and then scanned. SOD1 is circled in red.

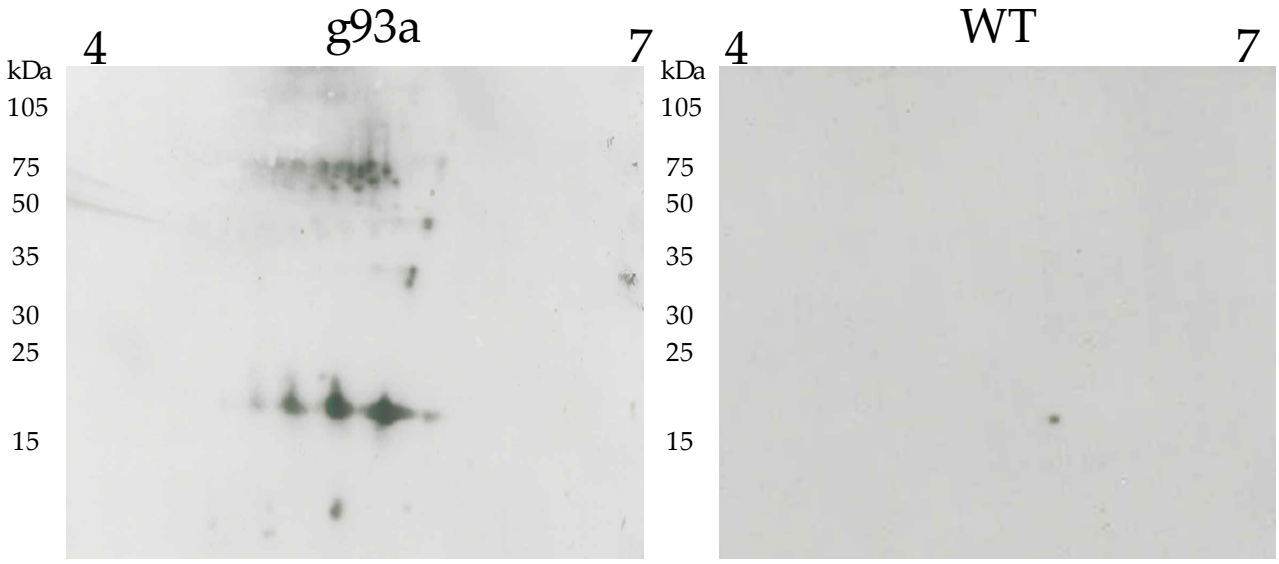


Fig. 17 2D-PAGE blotting of P3 fractions. The blotted P3 fractions were probed with a specific anti-SOD1 antibody. 50ug of protein were loaded per 18cm IPG strips

Common proteins in the insoluble fraction

A set of protein spots common to the three animal types was sequenced in order to have an idea of which proteins tend to be in the insoluble fraction. These proteins belonged mainly to metabolic pathways and cytoskeletal architecture as shown below:

	Some proteins present in the P3 fraction common to all animals			
Protein	Name	PI	Predicted MW kDa	Aproximate observed MW kDa
COX5A	Cytochrome c oxidase polypeptide Va	6.5	16	15
COX5B	COX5B_MOUSE Cytochrome c oxidase polypeptide Vv	8.8	13.7	15
CX6A1	Cytochrome c oxidase polypeptide VIa-liver	10.2	12.3	15
MBP	Myelin basic protein	9.9	27.1	15
TBB3	TBB3_MOUSE Tubulin beta-3	4.9	50.4	20
TBB4	TBB4_MOUSE Tubulin beta-4 chain (20kD fragment)	4.8	49.6	20
TBB5	TBB5_MOUSE Tubulin beta-5 chain	4.8	49.6	20
VATB2	Vacuolar ATP synthase subunit B,brain isoform	5.7	56.5	50
ENOG	Gamma-enolase	5.0	47.1	50
ATPB	ATP synthase beta chain	5.3	56.2	50
NFL	Neurofilament triplet L protein	4.6	61.5	60
Chain R COX	Chain R Bovine Heart Cytochrome C oxidase	5.0	12.4	10
MYL6	Myosin light polypeptide 6	4.6	16.9	15
ATPD	ATP synthase delta chain	5.0	17.6	15
hSOD1	Human Superoxide dismutase	5.7	15.9	18
ODPB	Pyruvate dehydrogenase E1 component beta subunit	6.4	38.9	35
β-TUBULIN	beta-tubulin	5.0	50.0	32
AINX	alpha-internexin	5.2	55.7	60
NUHM	NADH-ubiquinone oxidoreductase 24kDa subunit	7.0	27.3	25
VIME	vimentin	5.1	53.7	42
GFAP*	glial fibrillary acidic protein	5.36	49.9	variable

Successful spot sequences from MALDI-ToF

A total of 39 protein spots were identified as statistically significan differences. However only a total of 16 spots could be sequenced by MALDI-ToF. In the 6 weeks age stage, 29 different protein spots were found to be statistically significant. Protein spots detected only in G93A animals but neither in non-transgenic nor hSOD transgenic animals, were defined as ON changes. Upfold changes were defined as UP changes and downfold changes were defined as DOWN changes. The changes displayed were as follows: 12 ON changes (1 identity: TBB4), 7 UP changes (3 identities: all GFAP), 11 DOWN changes (7 identities: VATA1, VATB2, HSC70, GBB1, VDAC2x3). A total of

11 protein spots were sequenced in this stage. In the 11 week-age stage the changes were as follows: 6 ON changes (4 identities: CRMP2, GFAPx3), 3 UP changes (1 identity: GFAP), 1 DOWN change. A total of 5 protein spots were sequenced in this stage. A summary of the unsequenced protein spots is reported below:

6 weeks unsequenced protein spots

ON	kDa	UP	kDa	DOWN	kDa
3412	35	1532	35	1702	30
874	50	1593	30	1791	25
899	50	1693	30	1924	75
1695	30	2733	10		
1822	25				
1879	25				
1941	25				
2165	23				
2761	10				
2845	10				

11 weeks unsequenced protein spots

ON	kDa	UP	kDa	DOWN	kDa
2191	15	858	40	1341	25
777	40	890	40		
1085	32				
1103	30				

A summary of the successfully sequenced and identified protein spots is shown below:

				ratio	ratio			
Stage	ID	Name	Change	mut/wt	mut/ntg	PI	MW kDa	Peptide matches
6 weeks	TBB4	tubulin beta-4	ON			4.8	49.6	2
	VATA1	vacuolar ATPase catalytic subunit A	DOWN	13.8	6.3	5.5	68.2	5
	Q8CHX2/ HSC70	heat-shock protein 70/ATPase H+ transporting V1 subunit A, isoform 1	DOWN	3.9	2.2	5.5/5.4	68.3/70 .8	1/1
	VATB2	vacuolar ATPase subunit B2	DOWN	2.5	2.1	5.7	56.5	2
	GBB1	guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	DOWN	2.1	2.4	5.9	37.3	5
	VDAC2(1)	volage-dependent anion channel 2	DOWN	5	9.6	7.8*	31.7	3
	VDAC2(2)	volage-dependent anion channel 2	DOWN	4	4.2	7.8*	31.7	3
	GFAP	glial fibrillary acidic protein	varied	varied	varied	varied	varied	varied
11 weeks	CRMP2	collapsin response mediator protein-2	ON			6.3	62.2	4
	GFAP	glial fibrilary acidic protein	varied	varied	varied	varied	varied	varied

As reported in the table above, the only two proteins that could be detected in G93A gels but not in any of the control gels were TBB4 (six weeks) and CRMP2 (eleven weeks), so they were defined as

ON changes. All other statistically significant changes were reported as downfold changes. 2D gel images and representative 3D images were created for the successfully sequenced proteins comparing non-transgenic, transgenic mutant G93A hSOD1 and wild-type hSOD1 animals. The first 2D image shows the representative area in the gel where the spot of interest was present and another 2D image shows the specific protein spot. The 3D image is a 3D representation of the 2D image with the specific spots that gives a clearer idea of protein level differences. TBB4 at 6 weeks was recognized by Progenesys but was located in a very crowded area and it was not possible to create good 2D or 3D images. Only those protein spots that showed statistically significant differences in five out of six gels and only protein spots that significantly changed in mutant animals when compared to the two control animals were chosen.

VATB2 (6 weeks) showed an approximate two-time downfold (Fig. 17), VATA1 levels (6 weeks) showed an average downfold of 6 to 13 times when compared to controls (Fig. 18). Q8CHX2/HSC70 showed an average downfold between 2 and 4 times (Fig. 18). VDAC2 was present as three different spots, all with the same molecular weight but with different isoelectric points. The spots had an average downfold change between 2 and 9 times when compared to controls (Fig.19-20). GBB1 (6 weeks) showed an average downfold of 2 times (Fig.21). GFAP was found at 6 and 11 weeks as different spots with different molecular weight and PI, showing both up and downfold changes (not shown). CRMP2 (11 weeks) in G93A animals was present as a very faint spot very close to other protein spots (Fig. 22). The 3D image is different from the previous ones because Proteomeweaver analysis software (BioRad) was used to create this representation in another laboratory.

Two different proteins Q8CHX2/HSC70 were actually detected in one protein spot. Both proteins have a very similar molecular weight and isoelectric point.

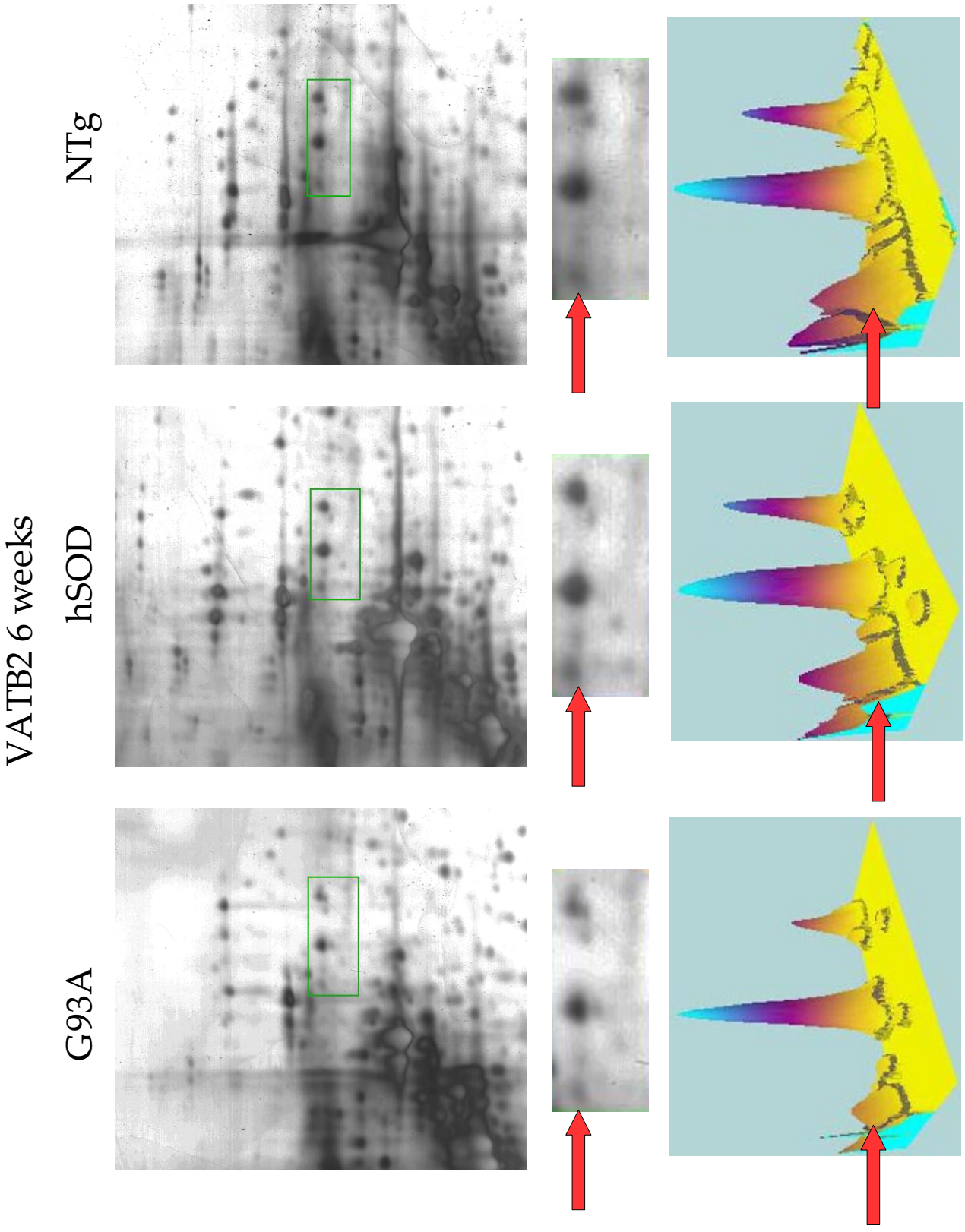


Fig. 17

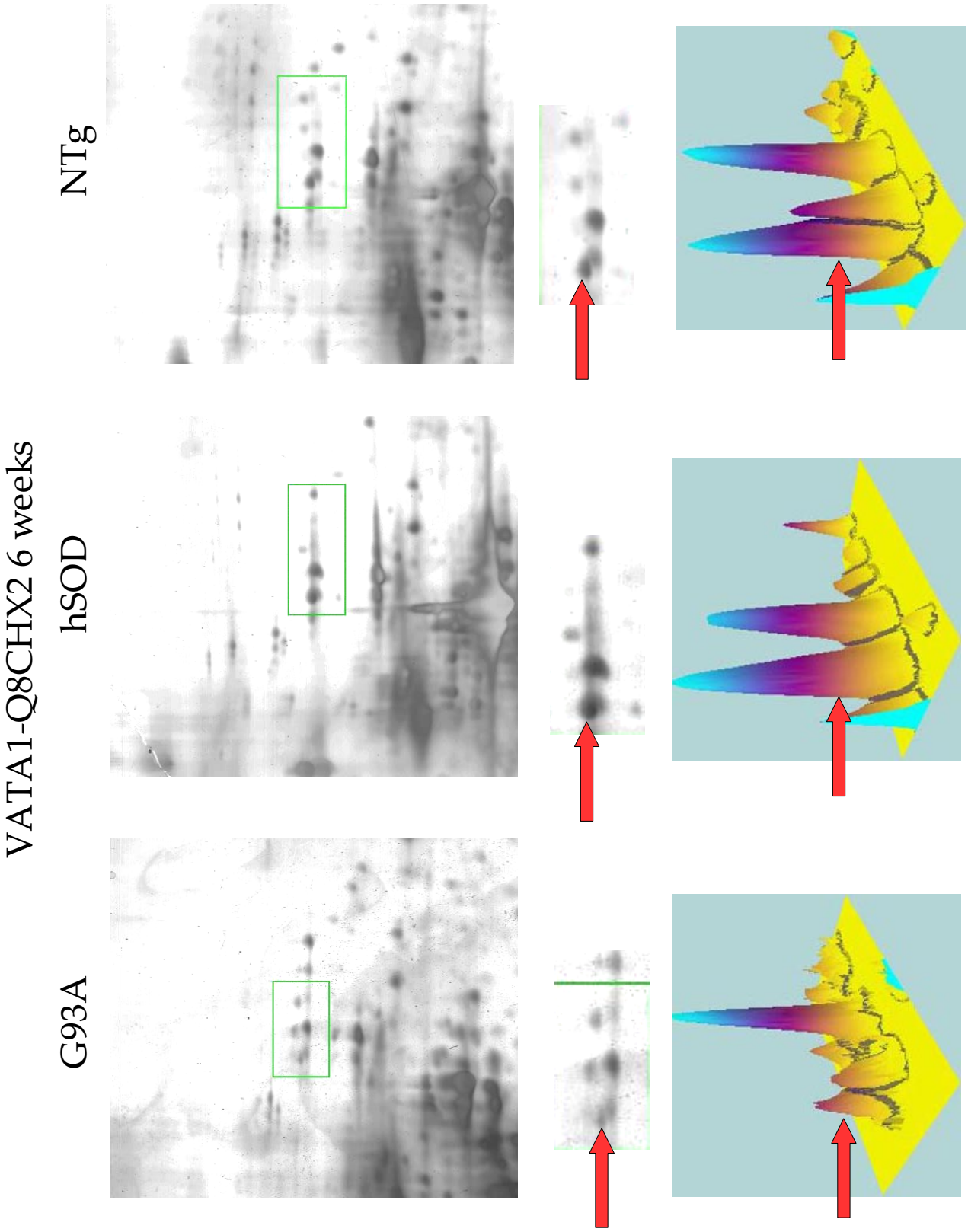


Fig. 18

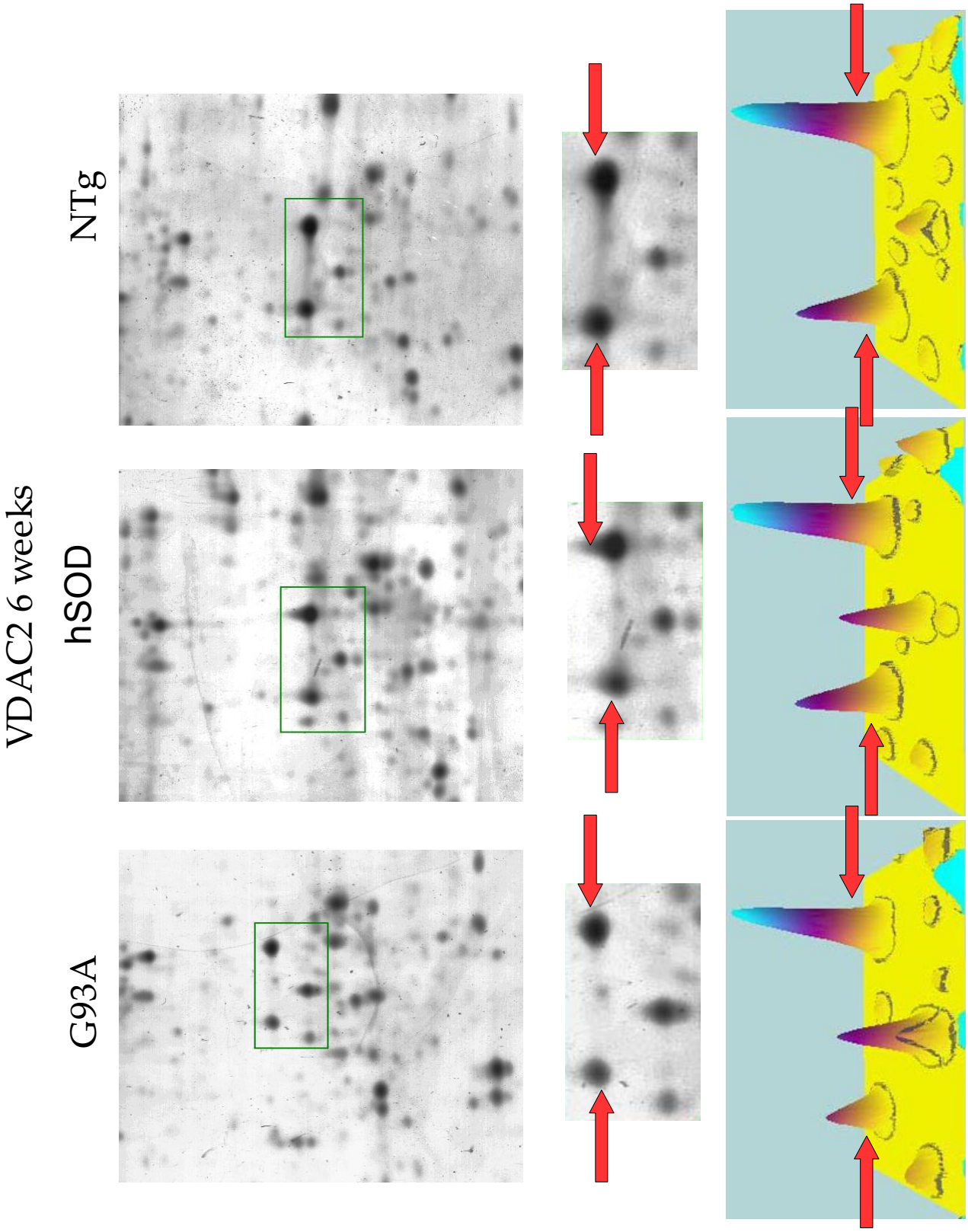


Fig. 19

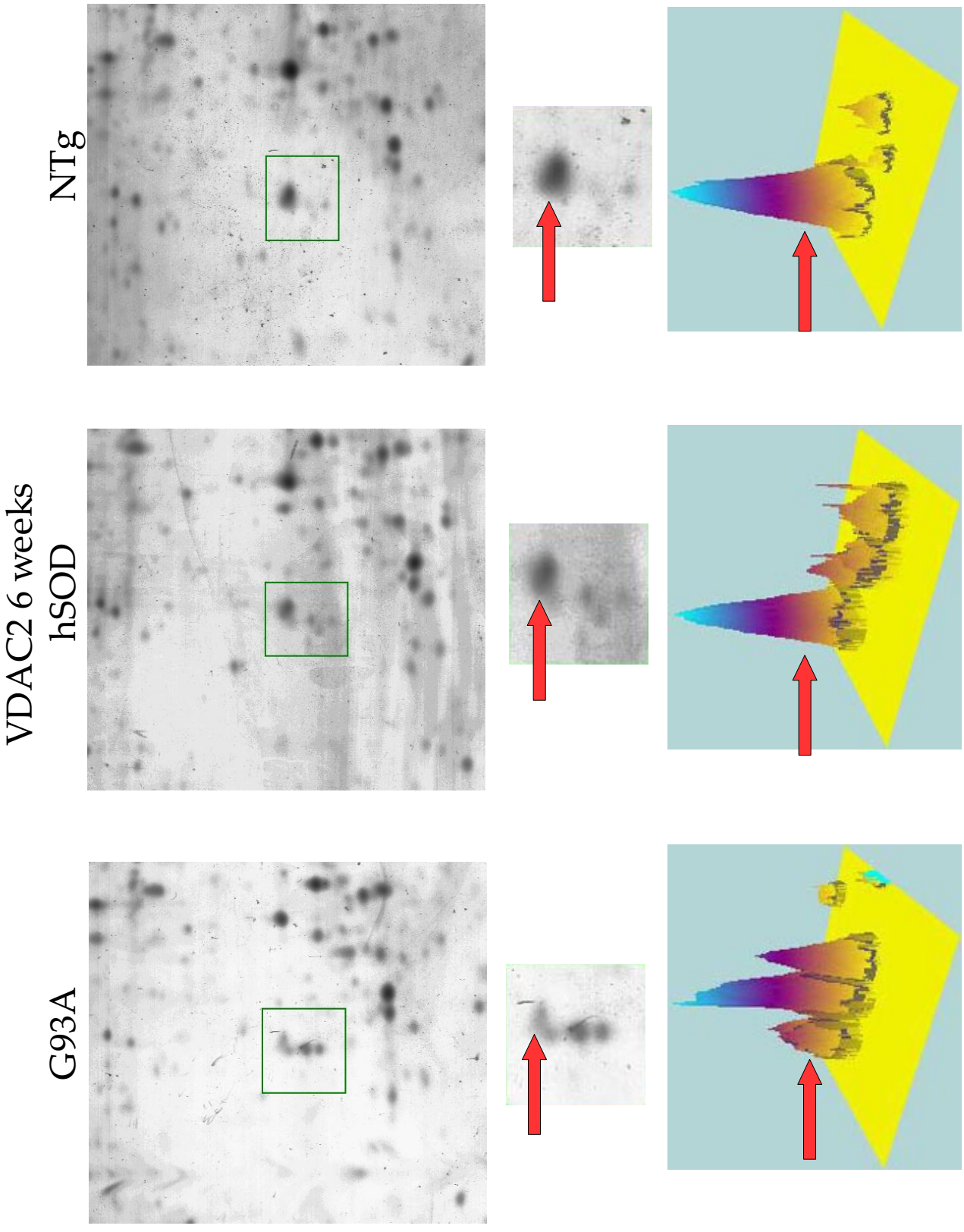


Fig 20

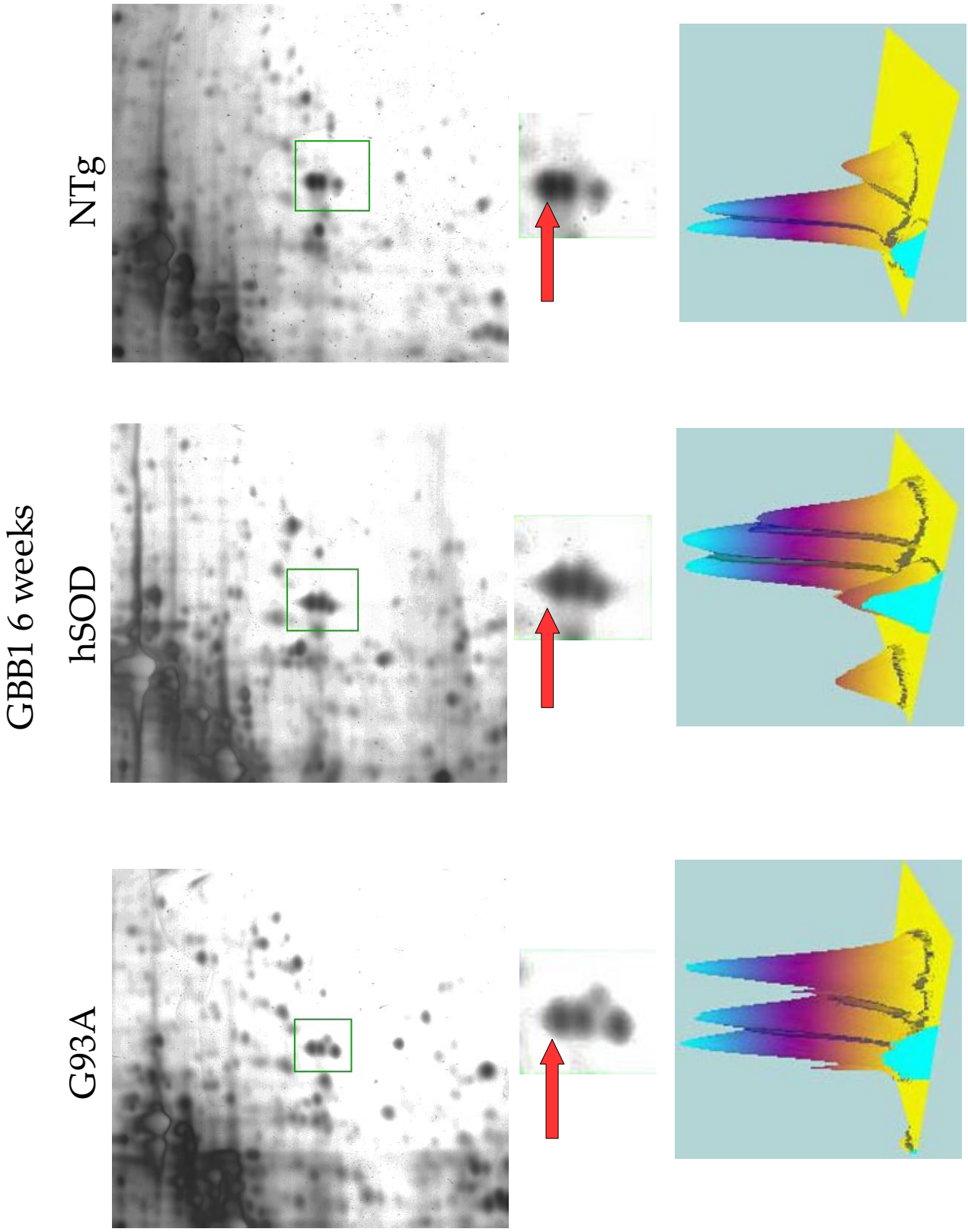


Fig 21

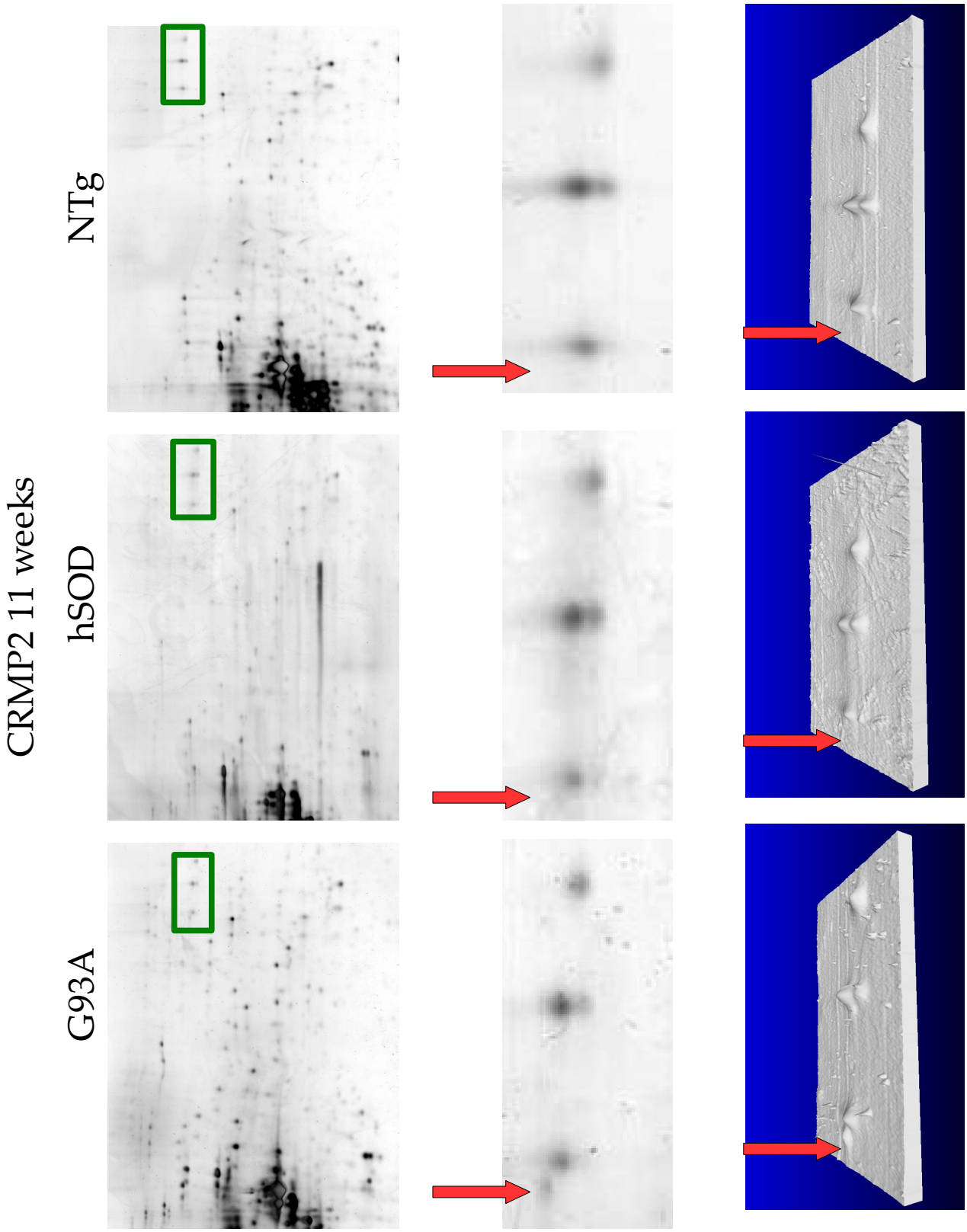


Fig 22

Overview of the identified proteins

VATB2 and VATA1

Vacuolar ATP synthase subunit B (VATB2) also known as Vacuolar ATP synthase subunit B, brain isoform is a 56kDa protein that belongs to the ATPase alpha/beta chains family. It is a non-catalytic subunit of the peripheral V1 complex of vacuolar ATPase (V-ATPase). Vacuolar ATP synthase catalytic subunit A (VATA1) also known as Vacuolar ATP synthase catalytic subunit A, ubiquitous isoform is a 68kD protein that belongs to the same family as VATB2 and it is the catalytic subunit of the peripheral V1 complex of vacuolar ATPase. ATPase is a heteromultimeric enzyme composed of a peripheral catalytic V1 complex (main components: subunits A, B, C, D, E, and F) attached to an integral membrane V0 proton pore complex (main component: the proteolipid protein). Vacuolar ATPase is a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles. V-ATPase dependent organelle acidification is necessary for such intracellular processes as protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation. VATB2 and VATA1 present an intracytoplasmic membrane and peripheral membrane location.

Q8CHX2

ATPase H^+ transporting V1 subunit A, isoform 1 (Q8CHX2) also known as ATPase, H+ transporting, lysosomal V1 subunit A is a 68kDa protein that belongs to the ATPase alpha/beta chains family. It may be involved in organelle acidification.

HSC70

Heat shock cognate 71kDa protein also known as heat shock 70kDa protein 8 is, as the name states a 71kDa protein that belongs to the heat shock protein 70 family. It is present as two isoforms. This protein binds to nascent polypeptides to facilitate correct folding. It also functions as an ATPase in the disassembly of clathrin-coated vesicles during transport of membrane components through the cell (De Luca-Flaherty et al., 1990). Its activity is suppressed by interacting with HSP105alpha (Yamagishi et al., 2004). It has a cytoplasmic localization though it translocates rapidly from the cytoplasm to the nuclei, and especially to the nucleoli, upon heat shock (Tsukahara et al., 2004). Hsc70 has been reported in protein inclusions from sporadic ALS cases (Watanabe et al., 2001).

VDAC2

Voltage-dependent anion-selective channel protein 2 is a 38kDa protein that belongs to the eukaryotic mitochondrial porin family. It is ubiquitously expressed although it is a low-abundance VDAC isoform. It forms a channel through the mitochondrial outer membrane which allows diffusion of small hydrophilic molecules and adopts an open conformation at low or zero membrane potential and a closed conformation at potentials above 30-40 mV. The open state has a weak anion selectivity whereas the closed state is cation-selective. VDAC2 is present as 6 different isoforms, all derived from alternative splicing. It has been shown to bind the apoptotic protein Bak (Cheng et al., 2003). Because Bak binds and inactivates the antiapoptotic protein bcl-2 (Enyedy et al., 2001), VDAC2 also has an antiapoptotic function.

GBB1

Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta 1 also known as transducin beta chain 1, is a 37kDa protein. Guanine nucleotide-binding proteins (G proteins) are involved as modulators or transducers in various transmembrane signaling systems. G proteins are composed of 3 subunits, alpha, beta and gamma.The beta and gamma chains are required for the GTPase activity, for replacement of GDP by GTP, and for G protein-effector interaction.

TBB4

Tubulin beta-4 chain, also known as tubulin 5 beta, is a 49kDa protein and belongs to the tubulin family. Tubulin is the major constituent of microtubules. It is a dimer of alpha and beta chains It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain. The highly acidic C-terminal region may bind cations such as calcium.

CRMP2

Collapsin-response mediator protein-2 also known as Dihydropyrimidinase-related protein 2 is a 62kDa protein that belongs to the dehydropyrimidinase family. It is ubiquitously expressed and has a cytoplasmic localization. It has been reported to bind to tubulin and promote microtubule assembly (Fukata 2002) and to transport the Sra-1/WAVE1 complex to axons in a kinesin-1-dependent manner, thereby regulating axon outgrowth and formation (Kawano et al., 2005). It has also been linked to Alzheimer's disease: a monoclonal antibody which strongly stains neurofibrillary tangles in Alzheimer disease brains, specifically labels CRMP2 when

phosphorylated on Ser-518, Ser-522 and Thr-509 (Gu et al., 2000). Aberrant expression of CRMP-2 has been documented in fetal Down-syndrome brains (Weitzdoerfer et al., 2001).

GFAP

GFAP is a 50kDa type-III intermediate filament protein. It is a cell-specific marker that during the development of the central nervous system, distinguishes astrocytes from other glial cells. Mutations in this gene cause Alexander disease, a rare disorder of astrocytes in the central nervous system (Der Perng et al., 2006). 3 isoforms have been so far reported. Isoform 3 (epsilon) interacts with the N-terminus of presenilin-1 (Nielsen et al., 2002), involving this protein in Alzheimer's disease as well. An additional transcript variant has been described, but its full length sequence has not been determined.

CHAPTER 4: DISCUSSION

Forebrain cholinergic alterations in SOD1 mutant mice

The present results reveal in an established animal model of human ALS that the cholinergic activity of cortical and hippocampal areas receiving their cholinergic innervations from the basal forebrain neurons significantly decreases at symptomatic stages of the disease. Accordingly, at the same stages these cholinergic neurons appear remarkably reduced in number. These alterations temporally parallel the degeneration of lower motor neurons. Gestational supplementation of choline, while resulting in long-term enhancement of cholinergic activity, was unable to increase the lifespan of transgenic mice or to counteract the cholinergic impairment in brain regions and spinal cord.

A previously unsuspected decrease of the cholinergic activity in some brain areas of G93A mice, namely the hippocampus, the olfactory–entorhinal cortex and, to a lower extent, the fronto-parietal cortex has been found in the present study. This effect is clearly linked to the disease progression, as it only appears at symptomatic stages, in parallel with motor neuron loss and ChAT decrease in the spinal cord. The effect on forebrain cholinergic neurons is somehow specific, as it is not accompanied by any change in the activity of an unrelated neurotransmitter system such as the GABAergic one, which is also unchanged in the affected segments of the spinal cord. The immunohistochemical observations establish a clear correlation between the decreased ChAT activity in cortical and hippocampal areas and the decrease in the number of basal forebrain neurons that provide most of the cholinergic innervations to these structures. Anterior cholinergic neurons of the medial septum-diagonal band, that mainly project to the hippocampus and olfactory areas (Mesulam et al., 1983; Woolf, 1991) are drastically decreased in number at symptomatic stages of the disease. More posterior and lateral basal forebrain cholinergic neurons of the nucleus basalis magnocellularis-substantia innominate, that are mainly responsible for the cholinergic innervation to neocortical areas (Mesulam et al., 1983; Woolf, 1991) appear also reduced in number. While the loss of cholinergic neurons was not directly measured by counting them, the decreased ChAT activity in their terminal

fields of innervations, the cortices and hippocampus, constitutes a reliable quantitative evaluation of the alterations of this system with the progression of the disease. Other cholinergic systems, for instance, the one constituted by striatal interneurons, were unaffected in symptomatic G93A mice both concerning the biochemical evaluation of ChATactivity and the number and distribution of ChAT-immunoreactive neurons. A previous study (Azzouz et al., 1999) reported an increased mRNA ChATexpression in the striatum of 130-day-old G93A mice, suggesting to the authors that it could reflect some form of compensation for the death of spinal motor neurons. The present observations do not support this conclusion, as they demonstrate the invariance of the striatal cholinergic system at symptomatic stages of G93A mice. There are severeal reports demonstrating that ALS patients develop with the progression of the disease cognitive deficits and forms of dementia (Kew et al., 1993; Kato et al., 1994; Massman et al., 1996; Hanagasi et al., 2002). It could be of interest to investigate whether in human patients these cognitive dysfunctions are accompanied by a decreased activity of the forebrain cholinergic system, whose alterations have been often found to be involved in cognitive deficits. The presence, at symptomatic stages of the disease, of a sizeable deficit in basal forebrain system providing cholinergic innervations to cortical and hippocampal areas, indicates that this system undergoes regressive changes in the mouse model of ALS and suggests that the same may also happen in the human disease. Post mortem studies in brain areas of ALS patients are needed in order to verify whether this is actually the case.

In the present study, an attempt was made to counteract the cholinergic deficits observed in symptomatic G93A mice through gestational supplementation of choline and/or long-term administration of ALCAR. Fetal supplementation of choline through maternal diet in the rat has been shown to favorably affect the development of the forebrain cholinergic system, possibly by increasing the level of NGF in the target regions of these neurons and to improve learning and memory (Meck et al., 1989; Williams et al., 1998; Sandstrom et al., 2002; Meck et al., 2003). ALCAR, a putative cholinergic substance, has been shown to improve memory performance in the rat after lesions and in aging condition (Piovesan et al., 1994; Taglialatela et al., 1994). In the present study, neither gestational choline supplementation, nor chronic adult ALCAR supplementation, nor both treatments

combined together, were able to significantly improve survival of G93A mice. However, gestational choline supplementation significantly increased the basal level of cholinergic activity in several brain and spinal cord areas, an observation that is made here for the first time in mice. The fact that this relevant increase of the basal cholinergic activity had no significant beneficial consequences on its disease-related late impairment or on survival of G93A mice, may be interpreted as an evidence that the cholinergic deficit is not the primary factor in the development of the disease.

However, the fact that the expression of the mutated SOD1 specifically results in regressive alterations of motor neurons and of the cholinergic neurons of the basal forebrain, that have in common the relatively large size and the high content of ChAT protein, may not be merely circumstantial. The decrease of ChAT catalytic activity in the cervical and lumbar segments of the spinal cord of affected mice, that is measured here for the first time in the present animal model, should theoretically match the decrease of motor neuron number previously reported at equivalent stages of the disease in the same animals. Based on cell count in Nissl stained sections of the lumbar spinal cord in G93A mice at late symptomatic stages (more than 120 days of age), a total estimate of around 50% decrease in motor neuron number has been reported, with large (>25 mm in cell soma size) neurons appearing decreased by more than 90% in number (Klivenyi et al., 1999; Guegan and Przedborski, 2003). At slightly earlier symptomatic stages (110–115 days of age), the estimate was around 40% decrease in cervical and 40–45% in lumbar spinal cord (Beers et al., 2001; Li et al., 2003). Similar estimates derive from studies on G93A mice exhibiting a slower disease progression, due to a reduced number of copies of the mutated gene, by comparing stages of the disease equivalent in terms of symptomatic features (Joosten et al., 2001; Sun et al., 2002; Copray et al., 2003). In evaluating the decrease of ChAT catalytic activity in the spinal cord of the affected mice, one should take into account the fact that the biochemical assay collectively measures the catalytic activity of motor neurons as well as of cholinergic interneurons of the spinal cord, that have not been reported to be affected by the disease. Furthermore, enzyme expression and activity may be differently regulated in healthy, atrophic and damaged neurons. Data from post mortem tissue from ALS patients suggest that some of the affected motor neurons,

exhibiting a definite atrophic state, may still increase the efficiency of acetylcholine synthesis through enhanced ChAT activity (Kato, 1989). This may contribute to the fact that loss of ChAT activity does not

result so sharp when assayed in post mortem patient spinal cord (Berger et al., 1992). Recent data suggest that motoneurons of symptomatic mice modify the expression of proteins that may in turn affect directly or indirectly the overall cholinergic activity (Copray et al., 2003). In the brain stem, the moderate decrease of ChAT activity measured in the present study likely reflects the fact that motor neurons of different nuclei are not equally affected in the disease and that anyway these cholinergic nuclei only represent a small fraction of the total brain stem volume (Reiner et al., 1995; Nimchinsky et al., 2000; Haenggeli and Kato, 2002).

Death of motoneurons in the spinal cord of ALS patients or mouse models has been considered to be essentially apoptotic and to belong to a typical programmed cell death mechanism by several authors, mainly on the basis of the signaling pathways found to be altered in the disease (Mu et al., 1996; Friedlander et al., 1997; Martin, 1999; Li et al., 2000; Pasinelli et al., 2000). However, studies on the morphological changes or in situ labeling with methods to detect DNA fragmentation have not been conclusive (Guegan and Przedborski, 2003). In human ALS, as well as in mouse models of the disease, some authors have reported evidence for nucleosomal DNA fragmentation in the spinal cord (Yoshiyama et al., 1994; Martin, 1999), while others have not (Migheli et al., 1994, 1999; He and Strong, 2000). The relatively long time course of the disease in human patients and in transgenic mice with low number of copies of the mutated SOD1 may render difficult to have a substantial number of apoptotic neurons in any single sample of the spinal cord (Guegan and Przedborski, 2003). In this respect, instead, mice with rapid progression of the disease may offer a more favorable model as the occurrence of some TUNEL-positive neurons can be demonstrated in the spinal cord of G93A mice at symptomatic stages of the disease (Kang et al., 2003).

Taking advantage of a sensitive Elisa method to detect fragmented DNA in brain homogenates, that had been previously demonstrated to be reliable for developmental programmed neuronal elimination as well as for age-related neuronal loss (White and

Barone, 2001; Monti and Contestabile, 2003), the results here show that a sizeable amount of DNA fragmentation can be detected in samples of mutated transgenic mice spinal cord at a stage characterized by substantial motor neuron loss and ChAT decrease. This experimental evidence supports the notion that motor neuron death in ALS models is, to a significant extent, a programmed cell death of apoptotic type.

Reactive astrogliosis in ALS patients has been described in the spinal cord and the cortex from post mortem samples (Nagy et al., 1994; Schiffer et al., 1996). In SOD1 transgenic mice, astrogliosis and microglial activation in the spinal cord of affected animals closely parallels the progressive motor neuron degeneration and is associated with up-regulation of neuroinflammatory markers (Levine et al., 1999; Xie et al., 2004). Being considered a marker of the metabolic state of astrocytes, glutamine synthetase is theoretically expected to be increased in cases of astrogliosis consequent to neuronal degeneration (Virgili et al., 1992).The previous results confirm, instead, that the absence of increased activity and expression of the enzyme, previously reported for the lower spinal cord of G93A mice (Fray et al., 2001), is also a feature of the cervical spinal cord as well as of the brain stem and other brain regions. The apparent dissociation between gliosis and metabolic activity of astrocytes, may be related to the complex interplay between neurons and astrocytes in glutamate neurotransmitter function. Data on glial glutamate transporters in G93A mice are controversial, decrease or invariance having been described in different studies (Canton et al., 1998; Trotti et al., 1999; Sasaki et al., 2001). Recent studies have demonstrated multiple alterations of reactive astrocytes in the spinal cord of ALS patients, such as increased expression of metabotropic glutamate receptors and of neuronal nitric oxide synthase, that may theoretically aggravate glutamate excitotoxicity (Aronica et al., 2001; Catania et al., 2001). Under these circumstances, the fact that glutamine synthetase is not increased in its activity may be favorable by not increasing the amount of glutamine, a quickly convertible precursor of the neurotransmitter pool of glutamate (Fonnum, 1985).

In conclusion, the present report provides new information on disease-related regressive changes of the forebrain cholinergic system in G93A mice, whose possible existence and extent is worth of being investigated in ALS patients too.

Differential neuroprotection by creatine supplementation

The dose of creatine supplementation used for present experiments was the same recently adopted for studies of neuroprotection in rats and mice, including G93A mice (Dedeoglu et al., 2003; Klivenyi et al., 1999; Matthews et al., 1998, 1999). Creatine is transported to body organs, including the brain, where it is assumed by cells through an active membrane transport system (Ipsiroglu et al., 2001). Long-term (4–8 week) oral administration of creatine, resulting in average intake similar to the one produced by the diet used in this study, increased by 15–30% total creatine and phosphocreatine concentration in brain of rats and mice (Ipsiroglu et al., 2001). Rats fed with normal or with creatine-enriched diet did not show any significant difference in daily food intake and body weight increase. Unilateral intrastriatal injection of ibotenic acid in rats resulted in large depletion of markers for cholinergic and GABAergic striatal neurons (Fig. 7), reflecting the widespread excitotoxic death of these neurons, as previously shown (Ciani et al., 2001). Long-term feeding with creatine-enriched diet was remarkably neuroprotective towards the excitotoxic lesion. In the IBO-injected striatum, the marker for cholinergic neurons, ChAT, was decreased by 63% as compared to the contralateral side in normally fed rats, while the decrease was only 32% in rats fed with creatine-enriched diet (Fig. 7A). Similarly, the marker for GABAergic neurons, GAD, was decreased by 68% in the IBO-injected striatum of normally fed rats, while it was significantly less reduced (49%) in rats fed with creatine enriched diet (Fig. 7B). Interestingly, while creatine supplementation did not affect, per se, the striatal ChAT level, the GABAergic marker was significantly increased (+35%) in the saline-injected striatum of rats fed with creatine-enriched diet (Fig. 7B). The levels of enzymatic activities measured in saline injected striata were not different from those present in sham-operated animals assayed in parallel (Figs. 7A, B). In a different model of excitotoxicity, IBO injection in the nucleus basalis magnocellularis (NBM) with degeneration of cholinergic basal forebrain neurons and consequent decrease of cholinergic innervation to the cortex (Casamenti et al., 1998; Contestabile et al., 2004), dietary creatine supplementation was not neuroprotective, as in both groups of rats a similar decrease of ChAT activity in the ipsilateral cortex was recorded (Fig. 8).

In G93A SOD1 mutant mice, the progression of the ALSlike disease can be monitored by the decrease of the cholinergic marker, ChAT, not only in the affected regions of the spinal cord where cholinergic motor neurons degenerate, but also in some forebrain areas receiving cholinergic innervation from basal forebrain cholinergic neurons (Crochemore et al., 2005). Creatine supplementation results in increased survival of G93A mice and in delay of some disease-related symptoms (Klivenyi et al., 1999; Zhang et al., 2003) and it may, therefore, favorably affect the loss of the cholinergic marker. In the present experiment, 2% creatine supplementation starting at the age of 40 days resulted in a small, but significant, increase in the survival of transgenic mice (mean lifespan of normally fed G93A mice: 132.4 ± 2.6 days; mean lifespan of creatine-supplemented G93A mice: 142 ± 3 days; $n = 7$, $P < 0.05$, Student's t test). Some G93A mice were sacrificed at the age of 110 days, together with age-matched wild type littermates, as at this stage of progression the disease is already characterized by significant decrease of ChAT activity in the spinal cord, as well as in some forebrain areas (Crochemore et al., 2005). The levels of ChAT activity in wild type mice were not changed by the different dietary regimen in any of the regions examined and, accordingly, data from these animals were pooled together. In the lumbar spinal cord, ChAT activity was similarly decreased in G93A mice, either fed with normal or creatine-supplemented diet, compared to wild type animals (Fig. 9A). In the olfactory cortex and the hippocampus of the same mice, instead, the decrease in ChAT activity present in G93A mice at 110 days of age was completely counteracted by the long-term dietary supplementation with creatine (Figs. 9B, C). Interestingly, also in mice, creatine supplementation increased GAD activity in the striatum, but not in the hippocampus of both wild type and transgenic mice (Fig. 10).

With the present report, we demonstrate differential response to dietary creatine supplementation in different types of acute excitotoxic insults as well as in an animal model of a human degenerative disease. In our model of excitotoxicity involving the degeneration of intrinsic striatal neurons, the neuroprotective effect of long-term creatine supplementation was similar to the one determined by different methods in similar types on neurodegenerative insults involving the striatum (Malcon et al., 2000; Mattews et al., 1998; Zhu et al., 2004). The experimental approach allowed to evaluate the relative

damage, and the degree of protection granted by creatine, regarding different neuronal population of the striatal complex. The present data suggest that one of the ways through which creatine is beneficial towards excitotoxic lesions, at least regarding the striatum, may be related to increased inhibitory activity at synaptic connections in the neuronal circuits involved in the excitotoxic mechanism. Theoretically, indeed, this may be the consequence of the increased activity of the GABA synthetic enzyme, GAD, in the striatum, which we have observed both in rats and mice. The data on increased GAD activity in the striatum of creatine-supplemented rats may suggest that creatine supplementation has a role in favoring recovery of GABAergic neurons surviving the lesion rather than an actual neuroprotective role. This would not explain, however, the similarity of the results concerning striatal cholinergic neurons, whose neurochemical marker, ChAT, displays the same degree of protection of GAD, without undergoing any diet-related upregulation. Hypotheses regarding the mechanisms of neuroprotection from dietary creatine supplementation have been, so far, mainly focused on enhanced energy stores granted by increased phosphocreatine availability for ATP synthesis, stabilization of creatine kinase, protection of mitochondrial integrity and direct antioxidant activity (Klivenyi et al., 1999; Lawler et al., 2002; Matthews et al., 1998; O' Gorman et al., 1997; Wendt et al., 2002; Wyss et al., 2000; Zhang et al., 2003; Zhu et al., 2004). These hypotheses are in agreement with the well established role of energy deficit/mitochondrial impairment in excitotoxic neurodegeneration (Beal et al., 1993; Bordelon et al., 1997; Greene et al., 1993; Stout et al., 1998; Tsuji et al., 1994) (see however the negative evidence concerning creatine kinase in a knockout mouse model) (Klivenyi et al., 2004), and the mechanisms listed above likely contribute to the neuroprotective effect of creatine. It is, however, difficult to explain, on the sole basis of these hypotheses, why different types of excitotoxic insults are ameliorated or not by creatine administration and why different neuronal populations respond or not to the dietary supplementation. An example of the first case is given by a previous report demonstrating a protective effect of creatine against excitotoxic lesions caused by intrastriatal infusion of NMDA, but not against kainic acid or AMPA, notwithstanding the similarity in the striatal lesion size (Malcon et al., 2000). The present results give an example of the second case, as we demonstrate here that long-term

creatine supplementation protects striatal, but not basal forebrain, neurons against ibotenic acid excitotoxicity. These intriguing results suggest that, in addition to a general role of metabolic type, creatine supplementation may have differential effects related to the nature, the brain topography and the connectivity of different neuron types. In this respect, a possible effect on GABAergic system, which is suggested by our data on GAD activity increase in the striatum of creatine-supplemented rats, may be relevant in order to investigate at the neurochemical level these possible differences. Interestingly, indeed, a similar increase of GAD activity was not detected in the cortex of creatine-supplemented rats (data not shown). A specific effect of long-term creatine supplementation on GAD activity, apparently restricted to the striatum, also emerged from measurement of the enzymatic activity in transgenic and wild type mice. Researches are presently being carried on, to see whether increased potency of GABA synthesis actually results in increased GABA availability at synaptic sites and whether similar alterations can be detected in other brain regions. Long-term creatine administration has been demonstrated to delay disease symptoms and to prolong mean lifespan in the G93A mouse model of ALS (Klivenyi et al., 1999; Zhang et al., 2003). Concerning survival, the results confirm here a small, but significant, beneficial effect of dietary creatine supplementation. A new observation regards a primary neurochemical marker of the disease, i.e. the decrease of ChAT activity related to degeneration of the cholinergic motor neurons in the spinal cord, as well as an accessory neurochemical marker recently discovered by us (Crochemore et al., 2005), i.e. the decrease of ChAT activity in forebrain regions that are targets of the cholinergic neurons of the basal forebrain. As it has recently been demonstrated that both these markers are significantly affected at an intermediate stage of the disease (110 days of age), this stage was used to characterize protective effects of the creatine creatine supplemented diet. By using the neurochemical parameter to evaluate the effect of choline supplementation, no significant alteration in the cholinergic decrement associated with the disease was demonstrated in the lumbar segment of the spinal cord, while the decrease of cholinergic activity found in the two forebrain regions examined was completely reverted by the dietary supplementation. Previous reports have correlated the increased survival granted by creatine, which may vary in its extent likely depending on subtle differences

among different founders of litters from the same strain ((Klivenyi et al., 1999; Zhang et al., 2003) (and present results), with protection and delaying of motor neuron death (Klivenyi et al., 1999). This was, however, demonstrated at 120 days of age in a group of transgenic mice whose normal lifespan reached 143 days instead of the 126 – 132 days documented by others, (Crochemore et al., 2005; Facchinetti et al., 1999; Zhang et al., 2003) (and including present results). It may be that a more rapid progression of the disease renders more difficult to put in evidence stages of partial protection concerning the primary targets of the neurodegenerative process, i.e. the spinal cord motoneurons and their distinctive neurochemical marker. Alternatively, this difference could be explained by the different way adopted to monitor spinal motor neuron death, i.e. direct cell counting vs. quantitative evaluation of the decrease of a specific neurochemical marker, as previous observation suggested that the matching between these two procedures is not absolute (Crochemore et al., 2005). The complete reversion of the cholinergic impairment detected in forebrain areas of G93A mice at 110 days of age (Crochemore et al., 2005), on the other hand, demonstrates that other neurochemical correlates of the disease can be ameliorated by creatine supplementation, in parallel with the prolonged lifespan. Even if present data do not allow us to postulate a causal relationship among the two events, it is of interest the fact that also in G93A mouse model a neurochemical parameter, whose alterations characterize symptomatic stages of the disease (Crochemore et al., 2005), is kept at its normal levels by long-term dietary creatine supplementation.

Taken together, the present data emphasize the usefulness of studying alterations of neurochemical parameters marking specific neuronal populations, in evaluating neuroprotective effects of long-term creatine supplementation in animal models of acute and chronic neurodegenerative diseases.

ODC/polyamine system alterations in ALS-G93A mice

Novel data about ODC/polyamine systemchanges in the spinal cord and brain regions from transgenic SOD1 G93A mice, an animal model for ALS are reported in this work. It clearly appears that there is a remarkable up-regulation of ODC enzymatic activity, only

occurring at a late symptomatic stage of the motor syndrome in the affected regions of the spinal cord and the brainstem. The increase of ODC activity is paralleled by a similar increase in levels of putrescine, the immediate product of the ODC enzymatic reaction. While one could expect to find also increased levels of spermidine and spermine, the higher order direct derivatives of putrescine, the present data demonstrate that this is not the case except for a small increase of spermidine levels in the affected spinal cord tracts, that only reaches statistical significance in the cervical spinal cord. As previously described in rats and humans (Virgili et al., 2001; Ekegren et al., 2004), the spermidine/spermine ratio in mice is regionally very different, being higher in the spinal cord and brain stem and lower in cortex and cerebellum. The fact that these ratios are maintained at both pre-symptomatic and symptomatic stages do not support the occurrence of generalized, pathology-linked, biochemical alterations. Rather, it is confirmed the major susceptibility of the ODC-putrescine step to become upregulated in response to acute and chronic stressful conditions, as it has been reported in various rodent models of neurodegeneration and by our group in aged rats (Paschen, 1992; Virgili et al., 2001; Babu et al., 2003). However, the dysregulation of the ODC/polyamine system reported here is not in agreement with the result of a recent post-mortem study on patients deceased by ALS (Ekegren et al., 2004). These authors, indeed, reported no significant changes in polyamine levels in spinal cord tissue autopsied from ALS patients. These discrepancies between data from the animal model and from human post-mortem analysis are a critical point that deserves to be better investigated. In particular, it should be verified whether the conflicting results are due to different methodological procedures about tissue manipulation and analytical measurements or whether they reflect specific differences between the human disease and the animal model. The fact that only ODC up-regulation and increase of putrescine level at symptomatic stages of the disease progression were found and the fact that these alterations were only restricted to the regions where motor neuron degeneration primarily occurs, can encourage to think that what was observed is a specific correlate of the neurodegenerative process. In this experimental model, the lack of increase in spermidine and spermine as a consequence of putrescine accumulation could reflect a reduced activity of S-adenosylmethionine

decarboxylase (SAMDC), the other important rate limiting enzyme in polyamine biosynthesis. A reduced SAMDC activity, in parallel with increased ODC activity and putrescine levels but in the absence of any change in spermidine and spermine levels, has been found after various conditions of severe neuronal stress (Paschen, 1992). However, these findings cannot be generalized because a marked increase of SAMDC activity has been reported by others in post-mortem studies of aged human brain or in brain tissue from patients affected by Alzheimer's disease (Morrison et al., 1993a,b). As an alternative, or additional explanation for putrescine accumulation in the absence of spermidine and spermine increase, the role of spermidine/spermine-N-acetyltransferase (SSAT) in polyamine homeostasis can be considered. This enzyme, known to be stimulated during aging and by various noxious stimuli (Baudry and Najm, 1994; Ferioli et al., 1996; Rao et al., 2000), is responsible for the acetylation of polyamines. Acetylated polyamines are in turn a substrate for polyamine oxidases (PAO), responsible for polyamine degradation and for the inter-conversion of spermidine and spermine back to putrescine (Thomas and Thomas, 2001). Indeed, recently generated transgenic mice overexpressing SSAT showed a marked accumulation of putrescine in many brain areas (Pietila et al., 1997). In the absence of an apparent increase of the two physiologically more relevant polyamines, spermidine and spermine, it is interesting to address the possible role of putrescine accumulation in neurodegenerative processes, such as those accompanying ALS and other motor disorders. That ODC induction and putrescine levels elevation could be involved in neuron damage seems to be suggested by the close correlation between putrescine production and neuronal injury. Moreover, it has been reported some evidence supporting a putative protective effect of difluoro-methylornithine (DFMO), a specific ODC inhibitor, in various in vivo and in vitro models of neurodegeneration (Trout et al., 1993; Babu et al., 2003). On this basis, the present data add evidence to the tight correlation between ODC/putrescine system induction and neuropathology because a dramatic increase of ODC activity and putrescine level was measured in the spinal cord, the region most affected by motor neuron degeneration, of diseased mice and a lower to not significant effect moving to higher CNS regions, from brain stem to cerebellum and cortex.

A critical question regarding the putative involvement of polyamines in

neurodegeneration is whether changes in their levels, due to change in enzymatic biosynthetic activities or in other processes of their homeostatic regulation, are causative agents of the neurodegenerative process as originally proposed (Paschen, 1992). Recent results from ODC or SSAT transgenic mice, rather support the view that polyamine enhanced catabolism and putrescine accumulation may be neuroprotective responses to injury (Kauppinen and Alhonen, 1995; Kaasinen et al., 2000). The present data cannot clarify this issue but it can be of some interest the fact that alterations in ODC enzymatic activity and in polyamine levels are quite absent in a pre-symptomatic stage (55 days of age) of the G93A mice disease. By contrast, these alterations are highly detectable at an advanced pathological stage (125 days of age), when a large proportion of spinal cord motor neurons have died (Chiu et al., 1995; Morrison B.M. et al., 1998). To further understand the cellular basis of the ODC/polyamine system response, it could be interesting to address in future studies the issue of the involved cell types, as astroglial and microglial activation and proliferation parallel neuronal degeneration during the disease progression. When activated, both these cell populations are thought to be able to exert beneficial or deleterious effects on stressed neurons by releasing a variety of trophic or cytotoxic substances (McGeer and McGeer, 1995; van Rossum and Hanisch, 2004). Astrogliosis and microgliosis are reactive responses, often associated with neuroinflammatory states, observed in acute insults and neurodegenerative diseases (McGeer and McGeer, 1995; Eikelenboom et al., 2002). Under these conditions, it has been reported that ODC can be expressed in brain and retinal astrocytes (Bernstein and Muller, 1999; Biedermann et al., 1998) and increased ODC mRNA has been measured in microglial cells submitted to the activatory action of endotoxins (Soulet and Rivest, 2003). Concerning the G93A mutant ALS mice model, astrogliosis was found to be maximal at early symptomatic stages of the disease progression while it was not further increased at more advanced symptomatic phases (Hall et al., 1998; Crochemore et al., 2005). By contrast, microglial cells have been shown significantly increased also at advanced symptomatic stages of the disease in G93A mice and in ALS patients (Hall et al., 1998; Turner et al., 2004). It seems then reasonable to hypothesize, in view of future studies, a possible role of polyamines in contrasting or sustaining neurodegeneration mediated through a regulation

of glial cell populations.

Proteomics of insoluble protein complexes

As described before, numerous reports have shown that protein aggregation is a hallmark of several neurodegenerative diseases such as Alzheimer's disease (Kosik et al., 1984), Huntington's disease (Di Figlia et al., 1997), Kennedy's disease (Merry et al., 1998) and amyotrophic lateral sclerosis as well (Watanabe et al., 2001). The present project is based on a modified insoluble protein (P3) fraction extraction protocol from Wang et. al. (2002) in order to make it compatible with 2D electrophoresis. Our P3 fractions showed strong SOD1 immunoreactivity, just as Wang's P3 fractions (Fig. 15A-F). We hypothesized that this strong SOD1 immunoreactivity, showing also high molecular weight bands (or spots in the case of 2D electrophoresis) was due to SOD1 mediated protein aggregates. We also hypothesized that by studying this insoluble fraction through 2D gel electrophoresis we would be able to identify which proteins could aggregate with SOD1. We could not exclude however, that this insoluble fraction is also composed of proteins that normally aggregate even without mutant SOD1 being present. Basso et al. (2006) have shown in a Triton insoluble fraction isolated from G93A mutant animal spinal cords, separated by 2D electrophoresis, that part of the SOD1 immunoreactive spots are actually mono and oligo-ubiquitinated SOD1. We aimed at identifying more proteins by comparing the insoluble fractions extracted from G93A mutant animals, wild-type and non-transgenic animals and we hypothesized that the observed differences in G93A animals when compared to the two controls could be causally linked to the presence of mutant SOD1. The present proteomic data show that SOD1 immunoreactive bands with a low-to-high molecular weight range are present in insoluble fractions (P3) from symptomatic animals but not from asymptomatic animals. No high molecular bands could be detected in the spinal cord extract (whole homogenate) either. A possible explanation for the absence of high molecular SOD1 immunoreactive bands in 6 week-old G93A animals could be that SOD1 mediated aggregates have yet not started to form or they are present to a minimal amount that cannot be detected by western blotting ECL whose sensitivity limit is around 10

picograms of protein (ref). 2D gel analysis shows that there are protein level differences in the insoluble protein fraction (P3) between mutant transgenic mice and controls even at an early presymptomatic stage of the disease (6 weeks). Interestingly, more proteins were detected as statistically significant changed at 6 weeks of age that at 11 weeks of age.

Because SOD1 immunoreactivity is much stronger in P3 fractions extracted from symptomatic animals and assuming that SOD1 aggregates sequester or bind tightly other proteins we expected to observe more protein changes in 11-week old animals. An explanation could be that other types of protein aggregates that form in mutant G93A expressing mice are not directly mediated by mutant SOD1 but already form in asymptomatic mice (6 weeks). It could also be possible that after the presymptomatic stage, there is some kind of compensatory effect that changes the proteome composition of the insoluble protein fraction mainly in mice that express mutant hSOD1. A third explanation could be that SOD1 mediated protein aggregates are very resistant even to urea lysis and therefore a small fraction of the sequestered proteins are released during urea solubilization.. In fact, even after urea solubilization, high molecular SOD1 immunoreactive bands are still observed in P3 fractions from symptomatic animals (Fig. 15A-F). Some of these higher molecular bands can be explained as mono and oligoubiquitinated SOD1 (Basso et al. 2006), but the presence of 75kDa and higher molecular bands is more difficult to be explained.

By using non-transgenic and wild-type transgenic animals as controls, we aimed at identifying insoluble fraction components that were present only because of overexpression of mutant hSOD1, ruling out the effects of the sole overexpression of hSOD1.

39 protein spots were identified as statistically significant differences: 29 protein spots at 6 weeks and 10 protein spots at 11 weeks. However only 16 protein spots were sequenced: 11 protein spots at 6 weeks and 5 protein spots at 11 weeks. 23 protein spots could not be sequenced because of limitation of MALDI-ToF's sensitivity.

The unsequenced spots appeared very faint on the analytical gels and could not be sequenced from the preparative gels. Four-hundred micrograms of protein were loaded for every preparative gel but probably for these spots, a greater protein load was needed

or even pooling from several preparative gels could have aided protein spot identification. It is important to remember that from one spinal cord, aproximately fifty micrograms of insoluble protein fraction were isolated, thus for a preparative gel an average of eight animals were needed. This was an important factor, because the number of available animals was limited.

ON changes actually refer to proteins that could only be detected in G93A animals, not meaning that they are not present in the insoluble fractions from the other animals. It is likely that by loading a greater amount of protein, the ON protein spots could have been detected in non-transgenic and wild-type transgenic animals.

The reason a non-linear gradient was used for the first dimension is because proteins separate more evenly, especially within the pH 5-7 range. This is very useful for analysis and sequencing purposes. The price to pay for this enhanced separation is not being able to determine the exact pI of protein spots. pH 3-10 gradients were used to be able to separate as many as possible proteins from the whole insoluble fraction. By using narrower pH ranges (pH 4-7, pH 5-6), specific fractions could be separated much better but all proteins outside these pH ranges would have been lost during isoelectrofocusing (IEF). It is important to notice that by using a phH 3-10 gradient, there can be resolution limitations, as it was the case with the identified protein spot containing both QCX8H2 and HSC70. These two proteins posses extremely similar molecular weight and isolectric points.

VATA1, VATB2, QCX8H2, HSC70, VDAC2, GBB1, TBB4, CRMP2 were the only successfully sequenced proteins belonging to statistically significant changes, besides GFAP. As described before, VATA1 and VATB2 are involved in vesicle trafficking, QCX8H2 in energy metabolism, HSC70 in stress-response and protein folding, GBB1 in signal transduction, TBB4 in cytoskeleton architecture, VDAC2 in ion transport and in apoptosis and CRMP2 in axonal growth. HSC70 has previously been reported to be localized in some protein inclusions in sporadic ALS cases (Watanabe et al., 2001). Also, it is interesting to notice that CRMP2 has been found in neurofibrillary tangles in Alzheimer's disease (Gu et al., 2000)

Therefore the identified proteins belong to very different functional roles. Except for TBB4,

CRMP2 and GFAP, these proteins were found only as downfold changes, that is, they are less present in the insoluble fraction from mutant animals when compared to controls. Whether this reflects technical limitations of the adopted procedure or an actual effect related to the presence of mutant SOD1, cannot be resolved by our present observations.
TBB4 being part of the cytoskeleton is a very abundant protein. GFAP is very abundant especially in advanced disease stages because of astrocyte activation and gliosis. Interestingly GFAP was identified especially in mutant animals as different protein spots with different molecular weight and isolelectric point, suggesting these are actually GFAP fragments. It could be possible that GFAP is therefore cleaved or degraded preferentially in mutant animals already during the early stages of the disease. CRMP2 seems to be ubiquitously expressed but has an important role in axonal outgrowth.

The 23 unsequenced spots could cast more light into the mechanisms of this disease, especially at 6 weeks, when 11 proteins were detected as ON changes but could not be identified. Overall, considering the unsequenced spots, more protein spots differences were found at 6 weeks, a situation quite surprising and suggesting that differential protein expression may result from the presence of the mutated SOD1 gene without being directly related to the disease itself. Overexpression of wild-type hSOD1 could also cause aggregate formation and this is why by using two different controls (non-transgenic and wild-type transgenic animals) we aimed at understanding better the effect of mutant SOD1 overexpression on the insoluble fraction.
The present proteomic data need to be further validated by western blotting and immunohistochemistry. Probing with a specific antibody for 1D and 2D western blottings can further help understand how these protein levels change in insoluble fractions. It can also be interesting to study protein distribution and expression in whole tissue (spinal cord) from animals at asymptomatic, symptomatic and end-stage animals.

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