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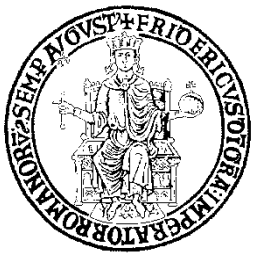

School of Medicine
PhD Program in Neuroscience
XVII cycle

The Cu,Zn-superoxide dismutase (SOD1) exerts neuroprotective effects in Amyotrophic Lateral Sclerosis through the Ca^{2+}/ERK1/2/Akt prosurvival pathway

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Academic Year 2014/2015

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Summary

SUMMARY

Amyotrophic lateral sclerosis (ALS) is a human adult-onset neurodegenerative disease characterized by progressive weakness, muscles atrophy, spasticity and paralysis resulting in respiratory arrest. These symptoms are related to degeneration and loss of upper motor neurons in cerebral cortex and lower motor neurons in brainstem and spinal cord. Most of cases of ALS are sporadic (sALS), while in the 5-10% of cases the disease is familiar (fALS) with autosomic dominant inheritance. In particular, in more than 15% of cases, the fALS is caused by mutations in the gene coding for the Cu,Zn-superoxide dismutase SOD1. Today, more than 125 different mutations in this gene are known, but how SOD1 caused motor neurons degeneration is unclear. Interestingly, most of mutants partially preserve their enzymatic activity, suggesting that SOD1 mutations are associated with a gain of toxic function rather than a loss of function. For instance, G93A mutation, that has been studied very intensely, leaves the enzyme activity intact. At last, while wt SOD1 secretion is compromise in ALS, mutant SOD1 is not secreted and accumulates in motor neurons within the endoplasmic reticulum and Golgi apparatus. Furthermore, after secretion, SOD1, independently from its dismutase activity, induces phospholipase C (PLC)/ protein kinase C (PKC) transductional pathway. Very interestingly, SOD1 is released by microglial cells and protect against 6-hydroxy-dopamine (6OHDA) toxin. Accordingly, wt SOD1 administration in transgenic $SOD1^{G93A}$ mice ameliorates motor symptoms of disease with an unknown mechanism.

Thus, starting from the hypothesis that the neuroprotective effects of SOD1 could be due to its transductional property, we studied the effects of SOD1 and ApoSOD, which lacks dismutase activity, in both NSC-34 motor neurons and primary motor neurons exposed to the cicad neurotoxin L-beta-methylaminoalanine (L-BMAA), a model to reproduce ALS *in vitro*, and in NSC-34 motor neurons exposed to chemical hypoxia, a model of Ca^{2+} and ROS-dependent neurodegeneration. We showed that the exogenous administration of both SOD1 and ApoSOD, but not of human recombinant $SOD1^{G93A}$, prevented cell death induced by the exposure to 300 μM L-BMAA for 48 hrs or to 45 min chemical hypoxia. On the other hand, MnTMPyP pentachloride, a SOD mimetic drug, failed to protect neurons, thus suggesting that SOD1 exerts neuroprotection independently from its most common enzymatic function. Interestingly both the pharmacological inhibition and the knocking down of

MEK by PD98059 and siMEK, respectively, counteracted SOD1-induced neuroprotection. Analogously, the well known inhibitor of PI3'K LY294002 and the dominant negative form of Akt prevented SOD1-induced neuroprotective effect on neuronal death induced by L-BMAA and chemical hypoxia. Accordingly, wt SOD1 and ApoSOD elicited a phosphorylation of ERK1/2 and Akt in motor neurons through an early increase of intracellular Ca^{2+} concentration, thus suggesting that a Ca^{2}+-dependent activation of ERK1/2 and Akt is mainly involved in SOD1-induced neuroprotective effects.

Finally, SOD1 prevents ER stress induced by both L-BMAA and chemical hypoxia, as observed by a reduction of GRP78, CHOP, caspase-12 and caspase-3 levels in motor neurons pre-incubated with SOD1 and then exposed to toxic *stimuli*.

Collectively, our data suggest that SOD1 is able to activate Ca^{2+}/ERK1/2/Akt pro-survival pathway in ALS thus exerting a neuroprotective effects independently from its enzymatic function.

Introduction

I.A. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease or Charcot's disease, belongs to the motor neuron disorders, that comprise a spectrum of syndromes with large variety in both clinical characteristics and disease course, such as progressive muscular atrophy, primary lateral sclerosis, spinal muscular atrophies, and frontotemporal dementia (**1**) (**Fig. 1**).

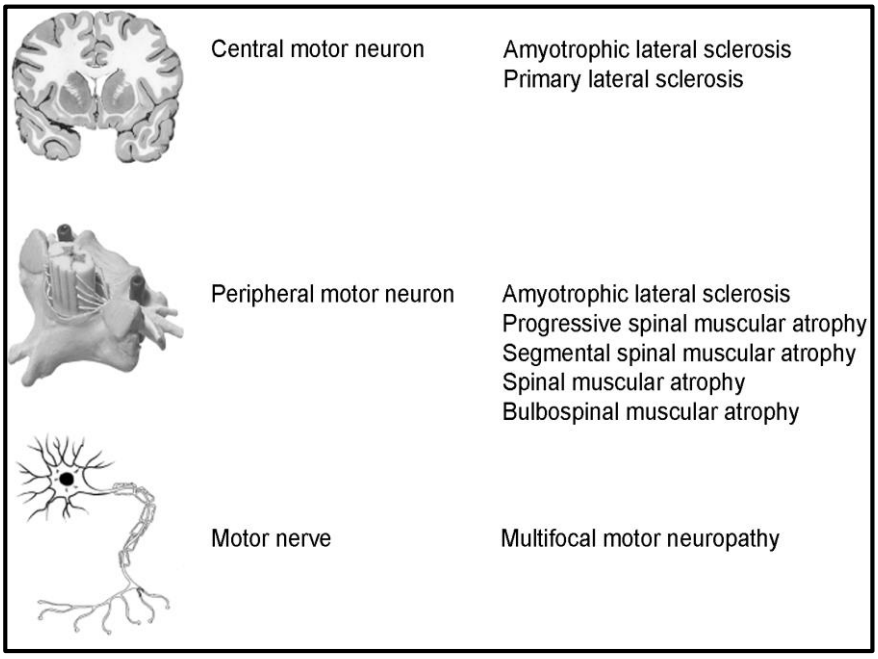

Figure 1. The spectrum of motor neuron disorders

The name comes from the Greek, and was used first in 1874 by the French neurologist J.M. Charcot to describe a fatal neurological syndrome with progressive muscle weakness, spasticity, and fasciculation. This name well describes the disease: indeed, "amyotrophic" means muscle wasting due to loss of trophic signals and "lateral sclerosis" describes the hardening of the outer part of the spinal cord as it is turned into connective tissue, where the corticospinal axons, connecting upper and lower motor centers, have degenerated. Charcot also pointed out the prognosis and that there was, and still is, no therapy for the disease. Today, about 150 years after the first clinical characterization of ALS, the mechanism(s) that lead(s) to motor neuron death are yet unknown.

ALS is a human adult-onset neurodegenerative disease characterized by progressive weakness, muscles atrophy, spasticity and paralysis resulting in respiratory failure, the primary cause of death in patients, or other pulmonary complications, such as pneumonia (**2**). These symptoms are related to degeneration and loss of upper motor neurons in cerebral cortex and lower motor neurons in brainstem and spinal cord (**3-5**). These neurons die in a progressive manner without plateaus and the disease, often, starts in one limb, and then spreads to the other limbs and the rest of the body.

Motor neurons arise from the motor cortex and extend from the brain stem throughout the spinal cord, forming the pathway to control voluntary movement. Upper motor neurons carry impulses to lower motor neurons and those innervate muscles. Motor neuron axons are amongst the most unusual cells in the body because of their very large size and their role as the critical link between the motor areas of the brain and the muscles. The loss of these motor neurons causes the muscles under their control to weaken and waste away, leading to paralysis (**Fig. 2**).

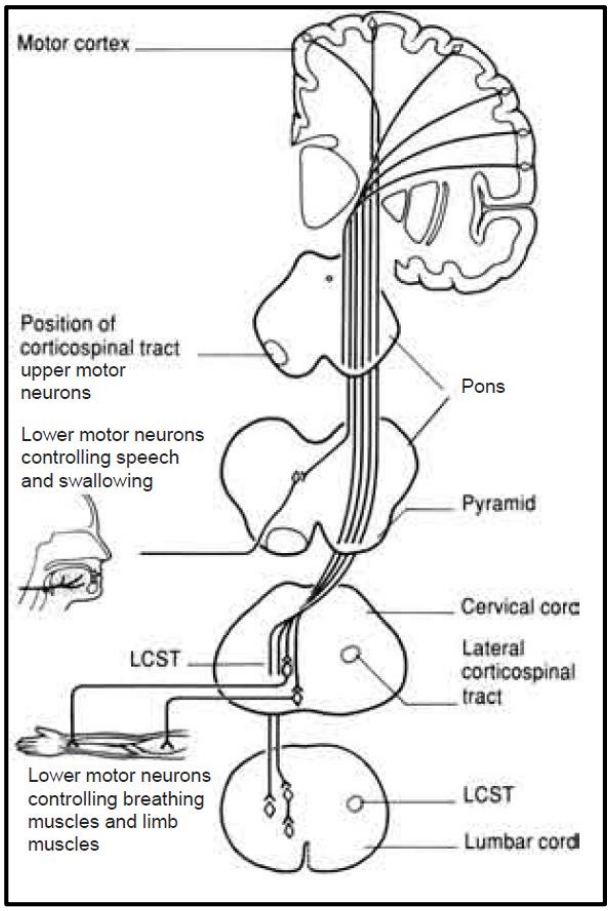

Figure 2. The human motor neuron system

I.A.1. Epidemiology

Amyotrophic lateral sclerosis is found all over the world and in all ethnic groups. The incidence appears to be homogeneous in most studied population; in particular, ALS affects about one-to-two individuals per 100,000 per year (**6**-**8**), with males being affected somewhat more frequently than females (**9**, **10**). However, in recent studies, this difference has been lower and a trend showing the same incidence in men and women is now apparent (**11**). Alterations in smoking habits and occupation and better clinical evaluation of women may explain this phenomenon (**12**).

In the 90-95% of cases, ALS affects patients who have no known relative with the disease, and it is defined sporadic ALS (sALS); instead, in about 5–10% of cases, ALS patients have a first- or second-degree relative with ALS, and the disease is then classified as familial ALS (fALS) (**13**, **14**). In the familial cases, the male-to-female ratio is 1:1 (**15**). Close relatives to ALS patients have an up to 10-fold elevated risk of developing the disease (**16**).

The average age of onset for sALS is reported to be 55–65 years of age (**17**, **18**), but it varies considerably; the age of onset for fALS, instead, is ten years earlier (**15**). Obviously, juvenile forms exist, with onset before the age of 25 (**19**), often with a family history (**20**, **21**). Only 5% of ALS patients are younger than 30 years old, and there are cases in which the disease did not develop until the patient was over 80 years of age (**18**). The incidence of ALS increases with age up to about the age of 75, and then starts to drop (**22**). The mean survival after onset is reported to be about 3 years (**23**, **24**), but with large variability; for examples, recent epidemiological studies indicate that 10% of patients survived for more than 10 years (**25**).

Moreover, a high incidence of an ALS-like syndrome has been reported on the island of Guam in the Western Pacific (**26**). This form of ALS is associated with the exposure to a neurotoxin, the L-β-methylamino-L-alanine (L-BMAA) (**Fig. 3**); indeed, many animal species (including mice, rats, and monkeys) fed high levels of L-BMAA develop motor symptoms similar to ALS (**27**).

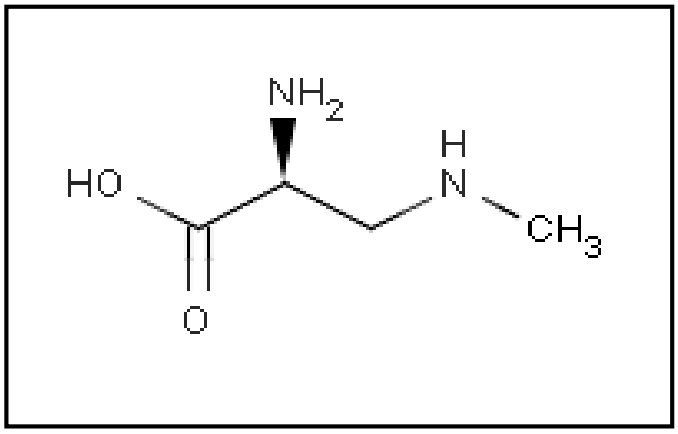

Figure 3. L-BMAA structure

L-β-methylamino-L-alanine is a non proteic aminoacid produced by *cyanobacteria*, a blue-green *algae* common to lakes, oceans, and soils around the world (**28**, **29**), and found in the seeds of the fresh false sago palm, *Cycad circinalis*. The people of this region process cycad seeds into flour, but the L-BMAA concentration in the flour is low and should not be sufficient to cause symptoms (**30**). However, a theory of bioaccumulation through consumption of flying foxes (bats) is thought to result in sufficient concentrations to give toxicity (**31**).

L-BMAA was detected in 29/30 samples of blue-green *algae* tested from a variety of water sources in Scotland, Northern Ireland, the Netherlands, Israel, India, Australia, the United States, and elsewhere (**32**). It was also detected in samples from the Baltic Sea and oceanic blooms, suggesting that significant quantities may be released into the world's oceans (**33**). However, the concentrations found were very low and no BMAA has been found *post mortem* in affected brain areas of ALS patients (**34**, **35**). Additionally, it has been noted that fish and animals may be exposed to L-BMAA from consumption of cyanobacterial blooms in sources of drinking water (**28**).

The environmental risk factors of neurodegeneration in ALS were largely unknown until recently. Indeed, the endemic occurrence of ALS, such as in the high-risk areas in the Pacific, could not be fully explained by genetic factors (**36**-**38**). Cycad nuts, mineral content of the drinking water and the soil content became suspected as long-term acting environmental neurotoxic risks. Attention was especially focused on the fruits of the local growing cycad palms (**39**, **40**), best of all because geographical incidence rates for ALS are strongly correlated with concentrations of cycasin, found in traditional food prepared from the toxic seed of the cycad plants (**41**, **42**). The

pathogenic effect of cycasin seems to be due to a decrease of neuronal DNA-repair, persistent up-regulation of *tau* mRNA expression, and enhancement of excitatory neurotoxicity (**43**).

Other putative environmental risk factors for ALS include exposure to radiation, electrical shocks, welding or soldering materials, paint- or petroleum industry and the dairy industry. Also the chemical agents of warfare, such as sarin and cyclosarin, which have effects on the nervous system, seem to be risk factors associated with ALS. Indeed, veterans of the Gulf War (1991–1993) have presented a higher incidence of ALS at younger ages (**44**, **45**) and service in general in the US Armed Forces was later associated with an increased risk of ALS (**46**). However, a large follow-up study was not able to replicate these findings and, instead, an increased risk of brain cancer was found in Gulf War veterans (**47**).

Moreover, a great study about the causes of death in Italian football player, performed in order to verify the use of illegal substances in Italian football, was conducted (**48**). This study showed a significantly higher incidence of ALS in the players: 18 developed ALS and the expected number of cases was 0.7 according to Italian registers (**49**). The same elevated risk was not found neither for professional cyclists and basketball players (**50**) nor for amateur football players or people performing fighting sports (**51**). As possible causes of the increased risk, history of multiple micro-traumas to the brain and spinal cord (**52**), strenuous physical activity, use of performance-enhancing drugs (**53**), and exposure to toxins from five pesticides used on the fields (**49**) have been put forward.

Exposure to pesticides as also been linked to ALS in agricultural workers (**54**). In addition, exposure to heavy metals and occupation have been proposed to increase the risk of ALS (**55-58**), but, when pooled, these associations are not very strong (**59-61**). Dietary habits do not seem to influence the risk of ALS (**62**). Furthermore, reports of head trauma preceding ALS (**63**, **64**) and high levels of physical activity (**65**, **66**) have been inconsistent. The only widely accepted risk factor for developing ALS is smoking. Although inconsistently reported (**67**, **68**), meta-analyses of pooled data indicate a modest increment of risk for present and past smokers (**69-71**).

However, none of the exogenous risk factors for ALS has been reported consistently. Although malignancies were reported in 10% of patients with motor neuron disease in a population study (**72**), this association remains unexplained.

Long-latency viral infections are another potential etiology of ALS (**73**, **74**). Enterovirus nucleic acids have been detected by reverse transcriptase-PCR (polymerase chain reaction) in 15/17 spinal cords of sporadic ALS patients in a French study, but not in more than 20 spinal cords examined in other studies (**75-79**).

In conclusion, exogenous risk factors are inconsistently reported in ALS. This might indicate that these risk factors do not cause the pathology. Alternatively, it might reflect a complex interaction between several environmental factors and specific genetic susceptibilities. Authors of several epidemiological studies have tried to find exogenous risk factors to explain sALS, but the results have been inconclusive.

I.A.2. Clinical signs and symptoms

Amyotrophic lateral sclerosis is a disease of the motor system. In classical ALS, both lower motor neurons in the spinal cord and brainstem, and upper motor neurons in the precentral gyrus should be affected. The neurons die in a progressive manner without plateau and the main feature of the disease is the rapidly progressive muscle weakness without sensory signs leading to limb weakness.

ALS usually begins in the fifth and sixth decades of life, starts in one limb and, then spread to the other limbs and the rest of the body. The skeletal muscles suffer when they are left without neuronal input.

At onset, fatigue, cramps, muscle weakness and wasting of one or more limbs or fasciculation of the tongue (bulbar onset) occur (**80**, **81**). Bulbar involvement causes difficulties in swallowing and respiration, and is a frequent cause of death in later stages of disease.

In a typical patient, the muscles that control eye movement, bowel and bladder function are spared. Usually, symptoms related to the loss of lower motor neurons appear first and then patients show symptoms related to the loss of upper motor neurons. Loss of lower motor neurons results in muscle weakness, painful cramps and muscle fasciculations followed by muscle atrophy and loss of tendon reflexes. Features that arise from dysfunction of the upper motor neurons are spasticity, and brisk tendon reflexes, like the extensor plantar responses, the Babinski sign. When loss of both upper and lower motor neurons occurs, spasticity or weakness may predominate before

the amyotrophy develops. Moreover, pathologically increased reflexes are more common than loss of reflexes (**82**).

ALS inevitably leads to death, usually because of respiratory failure or other pulmonary complications, such as pneuomonia (**83**).

Traditionally, ALS is considered a pure motor neuron disease, but recent studies show also evidence of degeneration of different parts of the central nervous system (CNS) at *post mortem* examination. Multiple systems show widespread degeneration after long treatment in a respirator (**84**-**87**), but patients without respirator care can also be affected in non motor neuron areas (**88**, **89**), thus suggesting the idea that ALS is a multi-system disease, at least in the end stage.

The majority of ALS patients have cognitive and/or behavioral impairment and many patients fulfill the criteria for frontotemporal dementia (FTD) (**90**-**92**). This also suggests that neurons different form motor neurons can be affected (**93**, **94**). An association between ALS and FTD has been suspected for a long time; indeed, there are subsets of patients suffering from FTD or ALS or even coincident ALS-FTD in the same person (FLTD). Several studies identified a locus on chromosome 9 that is linked to both diseases, but no mutations have yet been found.

Collectively, these studies suggest that ALS does not affect only motor neurons, but different part of the CNS, especially the frontotemporal lobe.

I.A.3. Pathogenesis and pathological features

I.A.3.1 Pathogenesis

The cause of motor neuron degeneration in ALS is unknown. Sporadic ALS is considered a complex multi-factorial disease, caused by the interaction of largely unknown genetic and environmental factors (**95**, **96**).

The discovery that mutations in several genes cause ALS allowed different theories and hypothesis about ALS pathogenesis: for example, excitotoxicity and oxidative stress have been investigated as pathogenetic mechanisms after the finding that SOD1 mutations cause familial ALS. In particular, exitotoxicity, caused by overstimulation of glutamate receptors, is involved in several neuronal conditions, such as stroke, epilepsy, and neurodegenerative disease. Upon stimulation, glutamate

activates AMPA (amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and NMDA (N-methyl-D-aspartate) receptors, resulting in an increase of intracellular Ca^{2+} concentration. Motor neurons show high levels of glutamate and AMPA receptors, thus they are more sensitive to exitotoxicity (**97**). The consequent increase of intracellular Ca^{2+} concentration, finally, cause the activation of damaging enzymes and mitochondrial damage, resulting in an increase of ROS production. Another example of excitotoxicity involvement in ALS is represented by L-BMAA, a neurotoxin that cause ALS/Parkinsonism in Guam: the exposure to this neurotoxin, indeed, is associated with high levels of glutamate.

The involvement of SOD1 in fALS also suggests a role for oxidative stress, a particular condition characterized by high levels of toxic oxygen-derived species, such as reactive oxygen species (ROS). Indeed, it was hypothesized that the loss of SOD1 activity cause an increase of oxidative stress in motor neurons, but it was clarified that SOD1 does not loss its function in ALS. Actually, it is yet unclear whether oxidative stress is a pathogenetic mechanism or a consequence of the degenerative disease. However, it is considered a risk factor for ALS, because high levels of oxidative stress markers are found in ALS patients.

In the pathogenesis of ALS, also mitochondrial involvement was suggested, because mitochondria are the most important organelle for energy production; they show high levels of ROS, and are involved in apoptosis. Autopsy examinations of muscle, liver, lymphocytes and part of the CNS, in addition, reveal structural alterations in mitochondria. These alternations affect calcium uptake and alteration of protein expression (**98**). However, mitochondrial affections could determine ALS in different ways: increased production of ROS, reduced oxidative phosphorylation, increased sensitivity to exitotoxicity, release of pro-apoptotic proteins, and interference with axonal transport (**99**).

Other pathogenetic hypotheses include protein aggregation: indeed, a particular feature of ALS is represented by different inclusions of misfolded protein that accumulates in the cytosol causing motor neurons degeneration. For this cause, it is suggested also the involvement of endoplasmic reticulum (ER) stress in the pathogenesis of ALS. ER stress include all the conditions in which the accumulation of misfolded proteins in the endoplasmic lumen exceed the capability for folding and degradation.

Furthermore, the hypothesis that viral infections or ensuing inflammatory reactions underlie ALS pathogenesis is supported by the analogy of poliovirus and poliomyelitis, and the reported presence of enterovirus nucleid acids and reverse transcriptase activity in cerebrospinal fluid of ALS patients.

Finally, genome-wide association studies have identified mutations in several candidate genes as risk factors for sporadic ALS and may direct future studies to dissect pathways in ALS pathogenesis.

I.A.3.2 Macroscopic pathological signs

ALS patients generally show atrophy and wasting of muscles, both limb muscles and internal muscles, such as the diaphragm. The spinal cord is macroscopically atrophic, especially the cervical and lumbar enlargements. The ventral motor roots are atrophic and discoloured compared to the dorsal sensory roots, which are normal. In cases of very long duration, atrophy can be seen in the precentral gyrus, but otherwise the brain is often normal (**100**). ALS and FTD are being recognized more and more as overlapping syndromes (**101**). Patients with concomitant ALS and FTD may also show wasting of the frontal parts of the brain (**102**).

I.A.3.3 Histopathology

The pathological hallmark of ALS is degeneration of the motor system with massive loss of ventral horn cells along the whole spinal cord. Motor neurons are also lost in the brainstem and in the motor cortex and both white and gray matter are affected. This is accompanied by astrogliosis in large areas of the brain and spinal cord, including the ventral horn (**103**). These reactive astrocytes are in close contact with motor neurons but are also found at longer distance from the degenerating cells (**104**, **105**).

The same type of reactive astrogliosis is seen in animal models carrying mutant SOD1 or TDP-43 (Transactive response DNA-binding Protein 43 kDa). In particular, animal models with either high or low SOD1 expression, even before the onset of symptoms, and animal models with expression of mutant and wild type TDP-43 develop astogliosis (**106**-**109**).

SOD1-positive aggregates are also found in glial cells in ALS patients with SOD1 mutations (**110**), but, recently, SOD1-containing aggregates were found in glial cells in all types of ALS patients, and in both sporadic and familial cases, indicating that there is a general SOD1-dependent glial component in ALS (**111**). Whether these astrocytic changes are a secondary reaction to degenerate neurons, or are the primary toxic insult or a combination of the two is not known and is currently the subject of intense research in the field.

The residual motor neurons show shrinkage, and many are filled with lipofuscin. The degeneration is mainly restricted to the motor neuron system. The hallmarks of ALS, such as atrophy of the ventral horn cells and ventral roots and myelin pallor in the anterolateral columns, are also seen in myelin preparations of spinal cord segments from ALS patients. Striated muscles show denervation atrophy. Oculomotor nuclei, abducens nuclei, and neurons in control of the anal and urethral sphincters are traditionally reported to be spared, but there have been reports of degeneration of these neurons too. Functional defects of the urinary tract are frequently reported for patients carrying the $SOD1^{D90A}$ mutation (**112**).

As in other neurodegenerative disorders, several types of inclusion pathology can be seen in ALS patients. These types of structures with misfolded and aggregated proteins strongly suggest that protein stability, misfolding, and aggregation is central to the disease process. SOD1 is found in some of these structures linking SOD1 misfolding to ALS. Anyway, none of the different inclusions found in ALS patients is pathognomonic for ALS.

The main inclusions and aggregates associated with ALS are listed below:

1. **Bunina bodies**.

These small, granular, eosinophilic inclusions can be found in clusters or singly in the cytoplasm of the ventral horn cells, but are not detected in axons (**113**). Bunina bodies stain positively for cystatin C (**114**) and transferrin (**115**). They appear as electron-dense granular material containing vesicular structures and filaments by electron microscopy (**116**). It is known that 65–95% of ALS cases show Bunina bodies at autopsy and, being extremely rare in other neurological conditions, they are considered nearly pathognomonic of ALS. Bunina bodies can be found in sALS and non-SOD1 fALS (**117**), but have not been regularly detected in SOD1-reletated ALS.

2. Ubiquitinated inclusions.

Present in almost all ALS patients but rarely in other conditions, ubiquitinated inclusions in motor neurons in the spinal cord and in the brainstem are important for *post mortem* diagnosis of ALS. These inclusions are refractory to standard stains, like hematoxylin and eosin staining (H&E), but immunohistochemical staining of ubiquitin shows fibrillar inclusions in motor neuron cell bodies or dendrites. Based on their appearance, they are divided into more loosely formed skein-like inclusions (SLIs) or compact Lewy body-like hyaline inclusions (LBHIs) (**107**). The ubiquitinated inclusions in patients with SOD1 mutations stain strongly for SOD1 (**118**, **119**) and aggregated SOD1 isolated from transgenic mice expressing mutant SOD1 is also partially ubiquitinated (**120**). Recently, a major constituent of the ubiquitinated inclusions in sALS, in some forms of fALS, and in FTD was identified as TDP-43 (**121**), a DNA/RNA binding protein of largely unknown function. The molecular forms of TDP-43 found in the inclusions include hyperphosphorylated C-terminally truncated variants. Following the discovery of TDP-43-containing inclusions in ALS patients, RNA biology has gained much focus to understand how these aggregates are linked to possible disease mechanisms. In addition, a protein with similar DNA/RNA binding properties as TDP-43, fused in sarcoma/translated in liposarcoma (FUS/TLS), is mutated in a few SOD1- and TDP-43-negative fALS families. Similar ubiquitinated inclusions containing the FUS/TLS protein are found in these patients, further linking ALS to RNA metabolism (**122**).

3. Hyaline conglomerate inclusions.

These are large inclusions (10–15 μm) with a glassy appearance by H&E. They stain for phosphorylated and non-phosphorylated neurofilaments and for other cytoplasmic proteins (**123**). They have also been detected in other neurological patients and in controls (**124**).

4. Basophilic inclusions.

These large basophilic inclusions are specific for juvenile forms of ALS. They are not limited to the motor system and are found in other brain regions too. A recent study showed that these inclusions stain positively for FUS/TLS and that patients carry FUS/TLS mutations (**125**). The inclusions are TDP-43 negative.

5. Spheroids.

It is known that neurofilament biology is involved in ALS. Mice overexpressing different human neurofilament genes develop progressive motor

neuron degeneration resembling ALS (**126**, **127**). Phosphorylated neurofilaments accumulate into large inclusions in axons of the ventral horn cells in ALS patients (**128**). These inclusions are called spheroids and are not specific to ALS but are probably more frequent in ALS patients than in controls.

6. Brannstrom bodies.

Inclusions in ALS patients without SOD1 mutations have shown immunoreactivity to SOD1 in some studies (**129**, **130**), and this is a rarely reported phenomenon (**131**, **132**). Due to antibodies without the capability to distinguish between the majority of native SOD1 and the minute amounts of misfolded SOD1 intracellularly, small aggregated structures may be difficult to identify. By the use of antipeptide antibodies that only detect misfolded SOD1, small punctuate aggregates of SOD1 were discovered in neurons and glial cells not only in mutant SOD1-mediated ALS, but also in fALS and sALS patients not carrying SOD1 mutations (**133**), indicating that SOD1 has a role in ALS in general. Similar findings were presented shortly afterwards in another material where oxidative modifications of wild-type SOD1 in sALS patients caused the protein to misfold and expose elements encoded by exon 4 that are normally hidden in the interior, as demonstrated by binding of a conformation-specific antibody (**134**). However, these misfolded SOD1 species were most likely soluble.

I.A.4. Diagnosis and treatment

Actually, there is no single test or biomarker assay to set the diagnosis of ALS; thus, the clinical signs and symptoms due to the motor neuron death and the progressive nature of the disease are used for diagnosis. The criteria were defined by the World Federation of Neurology (El Escorial criteria) (**135**-**137**) and are based on clinical, neurophysiological, and neuropathological examinations. Anyway, there is a long list of tests that should be performed to exclude all possible ALS-mimicking disease (**138**).

Therefore, diagnosis of ALS is difficult and often takes more than one year, with the median time to diagnosis reported to be 14 months (**139**). This is a serious problem, since therapeutic intervention early in the disease process is often more efficient and early diagnosis makes enrollment in clinical trials possible. Many

conditions show clinical manifestations that are similar to those of ALS; thus, misdiagnosis has been reported to be around 10% (**140**, **141**), with inappropriate or delayed therapy as a result.

Several attempts have been made to identify a biomarker for ALS by different methods, such as metabolomic profiling, proteomic profiling, and different enzyme-linked immunosorbent assays (ELISA) in different biological samples, such as blood plasma and cerebrospinal fluid (CSF), and different imaging techniques (**142**). Examples of proposed biomarkers are cystatin C (**143**) and neurofilament light chain (NF-L) in CSF (**144**); the latter has come to be used clinically to some extent.

Another problem concerning ALS is the pharmacological therapy. Indeed, today, only one drug, riluzole, is available for treatment of ALS. Molecularly, riluzole has anti-glutamate properties, but its precise mechanism is still unclear. However, it reduces the synaptic glutamate levels by inhibiting gluatamate release, and it also blocks ionotropic glutamate NMDA (N-methyl-D-aspartate) receptors. In laboratory tests, riluzole shows anti-convulsant properties (**145**) and thus it was approved for ALS therapy in the mid-1990s (**146**). Riluzole can prolong the life of most ALS patients by at least 2–3 months (**147**), but there are indications that its effect, when administered from an early point in the disease process, may be better (**138**). Subsequent to initiation of clinical trials, riluzole proved effective in animal models of ALS (**148-150**), but the effect could not be replicated in a well-controlled study with large cohorts of mice (**151**).

This, and the fact that many studies with positive effects in ALS animal models were not replicated in clinical trials, have forced those in the field to conduct better-planned and better-controlled preclinical trials (**152**).

Since no curative treatment is yet available, much effort has been put into symptomatic relief for the patients. Examples are listed below:

- botulinum toxin injections and radiotherapy seem to reduce hypersalivation (**153-155**);
- external coughing machines are useful for assistance in clearing of the upper airways from tenacious secretions (**156**);
- treatment of malnutrition (**157**) with a percutaneous endoscopic gastrostomy (PEG) tube is used when the patient can no longer eat by himself/herself (**158**);

- and psychopharmaceuticals are used for treatment of sudden and involuntary mood swings.

Finally, stem cell technology is often mentioned as a promising treatment strategy for ALS and other incurable (neurological) diseases. Several stem cell transplantation techniques are being tested in laboratory models of ALS, but are far from being ready for clinical trials (**159**-**161**). Despite the state of art, cell transplantation therapies are offered at high fees. A clinic in Beijing, China, offers a transplantation procedure of fetal olfactory ensheathing cells that, according to their advertisement, "can prevent or reverse the deterioration of ALS".

Anyway, the keystone of ALS treatment remains symptomatic (**162**).

All ALS patients eventually develop respiratory insufficiency: nocturnal hypoventilation is usually the first sign of respiratory dysfunction and symptoms may negatively influence quality of life long before respiratory ensues (**163**). Non-invasive home mechanical ventilation is a treatment option for (nocturnal) hypoventilation. The primary objective of non-invasive ventilation is symptomatic treatment of (nocturnal) hypoventilation and thereby improving quality of life and prolonging survival (**164**).

Today, many hospitals have special ALS care teams with, for example, physicians, nurses, dentists, physical therapists, speech therapists, and dieticians, all specialized in the care of ALS patients. These kinds of teams have been shown to improve survival in clinical trials (**165**).

I.A.5. Genetics of Amyotrophic Lateral Sclerosis

The genetics of ALS is complicated and genetic risk factor has been studied extensively. In the ALS Online Genetic Database (**166**), there are currently 71 reported ALS-associated loci (http://alsod.iop.kcl.ac.uk/).

In 90-95% of cases, the disease is defined sporadic (sALS), while in about 10% of cases the disease is familial (fALS) and patient has at least one or two first or second-degree relatives with the disease (**167**). A recent meta-analysis of 33 studies reporting fALS rates in different populations showed a low rate of only 5% (**168**). This percentage is probably too low due to inadequate family history, incomplete penetrance, misdiagnosis, and false paternity. Moreover, a recent twin study indicated

a genetic component also for sALS (**169**) and genome-wide association studies (GWAS) conducted on sALS patients have supported this idea (**170-171**).

Familial ALS is clinically and genetically heterogeneous with multiple autosomal dominant and recessive forms. Dominant fALS is characterized by large intra- and interfamilial variability of age of onset and progression. Incomplete penetrance has been observed in pedigrees (**172**) and confirmed by mutation analysis (**173**). Before linkage analysis and molecular diagnosis of ALS through molecular analysis became available, autosomal recessive fALS was considered very rare; however, actually, at least three forms of recessive fALS are recognized. Altogether, the now established forms of fALS account for probably 20-30% of fALS.

Molecular genetic analysis of fALS has led to the discovery of several genes involved in ALS, classified as "major genes" and "susceptibility genes" (**Table 1**).

Classification	Gene	Localization	Inheritance	Ref.
Major genes				
ALS1	*SOD1*	21q22	AD/AR	73,75
ALS2	*alsin*	2q33-34	AR	126,127
ALS3		unkown	AD	
ALS4		9q34	AD	128
ALS5		15q12-21	AR	129
ALS6		18q21	AD	131
FTDP	*tau*	17q21.2	AD	132, 138
FTD		9q21-22	AD	155
Susceptibility genes				
Neurofilament heavy chain	*NF-H*	22q12.2		187
Neurofilament light chain	*NF-L*	8p21		191,193
Peripherin	*PRPH*	12q12-13		182
Glutamate transporter	*EAAT2*	11p13-12		215
Glutamate receptor	*AMPA*	5p33		207
Apolipoprotein E	*ApoE*	19q13.2		219
Ciliairy neurotrophic factor	*CNTF*	11q12.2		230
Debrisoquine hydroxylase	*CYP2D*	22qq13.1		233
Apurinic apyrimidinic endonuclease	*APEX*	14q11-12		236
Mitochondrial DNA	*COX*			246
Manganese superoxide dismutase	*SOD2*	6q25		248
P2 Blood Group	*P2*	22q11		262

Table 1. Genetics of ALS

In particular, the "major genes" cause ALS with a clearly monogenic inheritance pattern. They may either predominantly lead to ALS (ALS1-ALS6) or cause multisystem neurodegeneration (tauopathies and ALS with dementia and parkinsonism) with ALS as an occasional symptom. The "susceptibility genes", instead, may trigger the cascade of neurodegeneration or may act as susceptibility factors for neurodegeneration, interacting with environmental or other genetic risk factors.

The "major genes" include:

- **ALS1/SOD1.** In 1991, the first ALS gene (ALS1) for an autosomal dominant form of fALS was mapped to chromosome 21q (**174**), and, two years later, the cytosolic copper-zinc superoxide dismutase (SOD1) gene was shown to be the ALS1 gene (**175**). Actually, more than 110 different mutations in all five exons of *SOD1* gene have been identified (**176-177**). All these SOD1 mutations are associated with autosomal dominant fALS, except two, D90A and D96N that can cause both dominant and recessive ALS (**178-180**).
- **ALS2 or Alsin.** The gene for ALS2 was identified in Tunesian and Kuwaiti families and named alsin or ALS2 (**181-183**). Alternative splicing of this gene produces a short and a long transcripts, and deletions could affect both transcripts resulting in the ALS2 phenotype. Symptoms occur in the first or second decade of life, and include progressive spasticity of the limbs and the facial and pharyngeal muscles. Survival is relatively long (range from 15 to 20 years) (**184**).
- **ALS3.** One locus on chromosome 18q21 was found to be linked to ALS in a GWAS involving a large European family (**185**), but the gene is still unknown. Affected individuals suffer from adult-onset classical ALS, inherited in an autosomal dominant manner.
- **ALS4.** A second form of autosomal dominant fALS, named ALS4, has been localized to a locus on chromosome 9q34 in an extended family with juvenile onset (mean age at onset 17 years) and slow progression without bulbar involvement. Later, the gene was identified as senataxin (**186**), and cause an autosomal dominant form of juvenile ALS.

- **ALS5.** The ALS5 gene was mapped to chromosome 15q and twelve sequence variants were detected in the spatacsin gene in 10 unrelated families (**187**). This form of autosomal recessive fALS is clinically similar to ALS2 (**188**).
- **ALS6.** The same region on chromosome 16p12-16q21 was identified by three independent groups as being linked to autosomal dominant, classical adult-onset ALS (**189**, **190**). Later, the mutated gene was identified as fused in sarcoma (FUS), also known as translocated in liposarcoma (TLS) (**191**). About 5% of fALS cases have been reported to carry FUS/TLS mutations and there is an overlap with FTD. FUS/TLS is a functional homolog of TDP-43; both function as DNA/RNA binding proteins. In disease, the proteins accumulate abnormally in the cytoplasm (**192**). This has led to theories about toxicity in ALS and FTD.
- **ALS8.** A region on chromosome 20q13 was linked to fALS in a large Brazilian family of European descent (**193**). Further investigation led to the discovery of a point mutation in the gene encoding vesicle-associated protein B (VAPB) (**194**). Mice expressing mutant VAPB develop normally and do not develop motor phenotypes, but TDP-43-positive inclusions were seen at 18 months (**195**).
- **ALS10.** The clinical and pathological overlap of ALS and FTD has been known for a long time, and families exist with persons diagnosed with ALS, FTD, or both in the same patient (**196**). Several loci have been linked to ALS-FTD. When the intracytoplasmic inclusions in both ALS and FTD were shown to contain TDP-43, an intensive hunt for mutations in the gene was started. Soon, a number of mutations were found in families with ALS, FTD, and both. About 4% of fALS cases and < 1% of sALS cases carry TDP-43 mutations mostly without cognitive dysfunction (**197**).

Considering that mutations in ALS genes occur in familial as well as sporadic ALS patients, probably more than 10% of ALS can be explained by the multiple autosomal dominant and recessive forms. Since there is still no evidence for any specific environmental cause for sporadic or familial ALS, most remaining cases of the disease are thought to result from interaction of several genes and environmental factors. In this broader complex genetic context, these genes are referred to as

'susceptibility genes' as mutations in these genes may lead only to ALS in the presence of other genetic or environmental risk factors.

The "susceptibility genes" include:

- **Neurofilaments**. Abnormal accumulation of intermediate filaments (IF) in the perikarya and proximal axons of motor neurons is a common pathological hallmark of ALS, suggesting that abnormalities in neurofilament organization may be involved in the pathogenesis of the disease (**198**). Neurofilaments are the principal intermediate filament type expressed by motor neurons. They are formed by the co-assembly of three subunits: NF-L (light subunit), NF-M (medium subunit), and NF-H (heavy subunit). NF-L is necessary for filament assembly, whereas NF-M and NF-H form links with other neurofilaments in axons (**199**). It is unclear whether the IF accumulations in ALS occur as a secondary feature of ALS associated neurodegeneration or represent the primary cause of disease. Several lines of evidence suggest that neurofilament genes play a causal role, and it seems that IF variants are likely modifying risk factors in sporadic ALS and probably modulate disease expression in SOD1-related ALS (**200**).
- **Excitotoxicity genes**. The term excitotoxicity refers to a phenomenon in which the excessive or prolonged activation of excitatory amino acid receptors results in damage and, eventually, death of the involved neurons. The activation of these receptors leads to depolarization and neuronal excitation and is normally transitory. Excitatory damage of neuronal cells appears to be mediated by sustained elevations of intracellular calcium levels and these excitotoxic mechanisms are implicated in the pathogenesis of ALS. Moreover, in the cerebrospinal fluid (CSF) of ALS patients, the levels of glutamate, the major excitatory transmitter in the motor neuron system, are increased (**201**), whereas glutamate transport is reduced in the brain and spinal cord of ALS patients (**202**). Motor neurons are vulnerable to excitotoxicity mediated by the glutamate AMPA receptors (**203**, **204**) and ingestion of excitotoxins may contribute to the pathogenesis of the ALS-parkinson-dementia complex of Guam (**205**, **206**).
- **Apolipoprotein E.** The frequencies of apolipoprotein E (*ApoE*) ε2, ε3 and ε4 genotypes in familial and sporadic ALS are not different from those in the general population (**207**), thereby excluding *ApoE* mutations as a primary

cause of ALS. However, the *ApoE* genotype was reported in some studies to have an effect on the age of onset and clinical presentation of sporadic ALS. The ε3/ε3 genotype may prolong survival in ALS patients, whereas the ε4 allele may predict an earlier age at onset (**208**). However, a significantly higher proportion of ALS patients with the ε3/ε4 genotype had bulbar symptoms, while the ε2/ε3 genotype seemed to predispose to limb onset of the disease. Therefore, as in Alzheimer's disease, a protective role in ALS of ε2 and ε3 alleles, and a deleterious role of the ε4 allele have been suggested (**209**-**211**). The exact mechanism of the deleterious effect of the ε4 allele is unknown, but it may simply result from the absence of the protective ε2 and ε3 alleles.

- **Ciliary neurotrophic factor (CNTF).** Reduced levels of ciliary neurotrophic factor (CNTF), a survival factor in spinal motor neurons, might contribute to the development of ALS. Indeed, it is demonstrated a decrease in CNTF levels in the corticospinal tract neurons of ALS patients (**212**) and a progressive motor neuron degeneration in knockout mice for *CNTF* (**213**). Finally, the frequency of the homozygous state for a mutation in the *CNTF* gene leading to absent CNTF is slightly increased in sporadic ALS patients (**214**).
- **Cytochrome P450 debrisoquine hydroxylase (CYP2D6).** Protein polymorphisms in the cytochrome P450 debrisoquine hydroxylase gene (*CYP2D6*) are associated with Parkinson's disease (**215**) and three different studies suggested the role of the CYP2D6 (b) allele as a risk factor for ALS (**216**, **217**). In the Chamorro population, the frequency of this mutant allele was higher than in Caucasians, but no differences were found between healthy Chamorro people and patients with Guamanian ALS- dementia-parkinsonism complex.
- **Apurinic apyrimidimic endonuclease (APEX).** The DNA repair enzyme apurinic/apyrimidinic endonuclease (APEX nuclease) might be a risk factor for ALS as reduced levels of APEX nuclease were found in the frontal cortex of ALS patients (**218**). In two different studies, it is demonstrated that APEX gene mutations result in a decreased level and activity of the enzyme in ALS (**219**-**221**). APEX nuclease may also play a role in the Guamanian ALS-dementia-parkisnonism complex, since a metabolite of cycasin, methylazoxymethanol (MAM), a potential environmental genotoxin in Guamanian ALS, was found to cause significantly reduced neuronal APEX levels and activity. Although

APEX mutations do not seem to account for a large proportion of ALS, the role of DNA damage in the etiology of ALS may be important.

- **Additional candidate genes.** Various additional "candidate genes" for ALS as a monogenic entity or a multifactorial disease have been proposed. These candidate genes include genes involved in free radical removal, despite SOD1 is involved in ALS through mechanisms probably not related to antioxidant activity. For example, the copper chaperone for superoxide dismutase (CCS), a cytosolic protein that mediates the delivery of copper to SOD1, seems to have a role in ALS (**222**); indeed, experiments with CCS knockout mice show a marked reduction in SOD1 activity (**223**). Another example is the monoamine oxidase (MAO), a generator of free radicals. Indeed, it is demonstrated the association with MAO-B and a later age-at-onset of ALS, suggesting a role for oxidative damage in ALS (**224**).

  A role in ALS has been proposed also for different genes. For examples, Bcl-2 gene, encoding a protein that inhibits apoptosis, seems to attenuate neurodegeneration in SOD1-mediated ALS models (**225**); moreover, the levels of c-jun, a transcription factor, are increased in the spinal cords of patients with sporadic ALS (**226**, **227**).

  Finally, an important role is played by the blood group phenotype P2. It is over-represented in ALS patients (43% of U.S. veterans with ALS, more than twice as frequent as expected) (**228**), and appears extremely frequent (66%) in the indigenous Chamorros of the islands Guam and Saipan (**229**). Close structural similarity of P2 to GM1 ganglioside may explain the characteristic immunologic features of ALS patients, such as the increase in IgM antibodies against GM1 ganglioside by cross-reactivity against the P-system blood group antigens (**230**).

  Other genes that might involve predisposition to ALS are the interleukin-2 receptor beta chain gene, localized to the chromosome 22q11.2-q12, which is a locus for many neodysplasias of the lymphoid system, and the leukemia inhibitory factor gene (LIF) on chromosome 22q12.1-q12.2 (**231**). Recently, knockout mice for the hypoxia-response element (HRE) of the vascular endothelial growth factor (VEGF) gene were shown to develop an ALS-like condition, but VEGF mutations have not yet been identified in human ALS (**232**).

I.A.6. Pre-clinical models used to study Amyotrophic Lateral Sclerosis

The exact mechanisms by which all the above described gene products are involved in ALS pathogenesis are the subject of many ongoing researches. Different studies have identified some involved cellular pathways, including protein misfolding, RNA processing, oxidative stress, excitotoxicity, axonal transport, mitochondrial dysfunction and abnormal secretion of proteins.

In the attempt to clarify the molecular mechanisms that lead to ALS, many different *in vitro* and *in vivo* models are developed to study the disease.

In vitro models are extremely helpful to study human diseases, because they allow to analyze different cell types independently from each other and to perform dynamic studies on isolated cells. Moreover, diseased cells can be combined with healthy ones to better understand which cell type is the most critical in the different stages of the disease.

In vivo models, similarly, are extremely useful because animal models replicate human disease. Nevertheless, how accurately animal models replicate all ALS human clinical symptoms remains an unanswered and troublesome question.

I.A.6.1 *In vitro* models

Some *in vitro* models developing to study ALS deriving from animal models. Indeed, neural cells are impossible to obtain from patients and their extraction from *post-mortem* tissues is limited due to the difficulty to isolate living cells from adult brain or spinal cord, especially motor neurons. However, such *post-mortem* tissue biopsies are mostly used to perform histological and immunohistochemical, genetic and proteomic studies.

The most used *in vitro* models useful to study ALS include: organotypic cultures, spinal cord cell cultures, NSC-34 motor neurons, *Xenopus* oocytes, and neuronal precursor cells. More recently, three-dimensional *in vitro* models are developed to study the disease.

Organotypic slices could be obtained from the spinal cord of embryos and post-natal animals. (**233**, **234**). These cultures could be used for more than 2 months (**235**) to reproduce the *in vivo* situation perform various analysis. Indeed, organotypic slices

are useful for dynamic studies with different drugs, for immunohistochemical staining and electrophysiological recordings (**236**, **237**).

A valid alternative is represented by spinal cord cell cultures, isolated from E12-14 mice embryonic developmental stage. At this stage, the spinal cord of mice embryos is easy to remove and motor neurons, astrocytes and microglia can be extracted and maintain in culture (**238**, **239**). Motor neurons will be easy to identify because of their large cell body (>20μm in diameter) and dendritic trees, and can be stained for expression of typical markers, such as choline acetyltransferase (**240**, **241**). The isolation of individual cells is extremely useful to study various intracellular mechanisms from proliferation to mRNA expression, mitochondrial function, protein aggregation, intermediate filament assembly or axonal transport.

However, neurons cannot proliferate, and, thus, it is necessary to perform cell extraction from embryos for each new experiments. Moreover, primary neuron cultures are difficult to maintain in culture for more time and cells cultured on plastic dishes cannot recapitulate the *in vivo* environment. Furthermore, another disadvantage to use primary cells from embryos is that some features of the adult phenotype may not be expressed at this early stage (**242**).

To greatly facilitate *in vitro* studies, motor neuron-enriched embryonic mouse spinal cord cells were fused with mouse neuroblastoma to generate the hybrid cell line NCS-34 (**243**). This cell line contains small proliferative and undifferentiated cells and larger multinucleate cells. These cells express properties of motor neurons, such as choline acetyltransferase, acethylcholine synthesis and neurofilament expression. They respond to agents affecting voltage-gated ion channels, cytoskeletal organization and axonal transport similarly with primary motor neurons, but failed to reproduce synaptic connections (**244**).

To reproduce an ALS phenotype, primary and secondary motor neurons can be induced to express multiple copies of the gene of interest or can be exposed to L-BMAA to reproduce the Guam form of ALS. Moreover, NSC-34 motor neurons are useful to study cellular morphometry (axon length), gene expression using RT-PCR, apoptosis, signaling pathways, calcium imaging, mitochondrial membrane potential, glutamate uptake, excitotoxicity, and to screen antioxidant molecules capable of rescuing them from expression of mutated SOD1 (**245**).

For electrophysiological recording, the oocyte of *Xenopus Laevi*, an African frog, are useful because of its large size (about 1 mm in diameter) which facilitates its

handling. The oocytes, usually, are used to study various ion channels, transporters and receptors. In studies related to ALS, for examples, oocytes are used to analyze ion channels or transportes, such as the human glutamate transporter GLT1 (EAAT2), under controlled voltage clamp in presence or not of mutant SOD1 (**256**).

The described *in vitro* model are particularly useful to study familial ALS, because, it is possible induce in each model the expression of a gene with a mutation that reproduces ALS. To study sporadic ALS, in which the mutation is unknown, instead, mouse embryonic stem cells (ESCs) or neuronal precursor cells (NPCs), which can be extracted from adult tissues, are more useful. Indeed, the potential of mouse ESCs to differentiate into motor neurons has been well established (**257**). Thus, it is possible to obtain ESCs from easily accessible tissues, such as skin (**258**), and to induce their differentiation into motor neurons. Moreover, neurons, astrocytes and oligodendrocytes could be differentiated from NPCs purified from familial and sporadic ALS patient *post-mortem* spinal cord samples. These cultures are useful, for example, to study the toxic properties of patients-derived astrocytes on motor neurons (**259**).

A good alternative to ESCs and NPCs is represented by the induced pluripotent stem cells (iPS cells), obtaining from somatic cells. To obtain iPS cells, a small biopsy of a few millimeters in diameter collected from the patient's skin is required. From this biopsy, dermal fibroblasts will be extracted and expanded to generate iPS cells. Other methods to reprogram cells are now being intensively tested, such as the use of secreted recombinant reprogramming factors present in the culture media. The iPS cells are morphologically and phenotypically similar to ESCs, and, thus, it is possible to obtain motor neurons starting to the IPs cells (**260**, **261**). Actually, it is not clear whether motor neurons and glial cells derived from ALS patients-iPS cells will efficiently recapitulate the disease *in vitro*, because the technology is still recent.

Finally, more recently, motor neurons derived from iPS cells or NPCs ALS patients are used to develop three-dimensional *in vitro* models of ALS. For examples, these cells have been cultured on methylcellulose scaffold enriched with laminin and they showed reduction of apoptosis, enhance of survival, differentiation in neuronal and glial cells and neurite extension (**262**, **263**). Thus, the combination of ALS patient NPC-derived neural cells in tissue-engineered reconstructed spinal cord models is a promising strategy to develop the next generation of *in vitro* models of ALS.

I.A.6.2 *In vivo* models

Actually, different invertebrate and vertebrate animals are used to obtain *in vivo* models able to reproduce ALS and the human features of the disease. Each model organism, of course, has its own advantages and disadvantages and the choice of an appropriate model depends on the investigated question. For this cause, often, it is useful to perform experiments in two or more model systems to better characterize different aspects of the disease. Moreover, in this way, it is possible to reproduce human disease.

Invertebrate model organisms include the simple yeast (*fungi*), fly, and nematode. These model organisms have highly manipulable genomes allowing for rapid generation of transgenic lines to provide insight on gene functions and protein network interactions. The obvious disadvantage of using invertebrate model organisms is that they are evolutionarily far from mammals and that many physiological functions are not conserved. Furthermore, their organs are extremely undeveloped and simple compared to other animals. The limited cellular diversity also represents a major disadvantage.

Actually, different ALS models are obtained in *C. elegans*. The first ALS model was obtained by introducing human wild type and various fALS SOD1-linked mutations (A4V, G37R and G93A). Moreover, in order to study TDP-43 function and neurotoxicity, transgenic *C. elegans* model was generated allowing pan-neuronal expression of the wild-type TDP-43 human protein (**264**). Obviously, the nematode is a too simpler organism unable to reproduce a human disease; however, several investigations using these models may reveal insights into SOD1 and TDP-43 functions, potentially reveal neurotoxic mechanisms relevant to ALS and other neurodegenerative diseases, and ultimately lead to the development of novel therapeutic targets.

The fruit fly *Drosophila melanogaster* is a powerful genetic tool to study neurodegenerative diseases, because *Drosophila* is a complex organism, with a functioning brain and nervous system, capable of many behaviors, like learning, motility, and visual acuity. In the context of ALS, *D. melanogaster* is used to obtain SOD1, TDP-43 and FUS transgenic models. These models mimic human disease: for example, SOD1-mutated flies show progressive motor dysfunction, electrophysiological defects and abnormal accumulation of SOD1 and stress respond

in surrounding glial cells (**265**). Studies in TDP-43-mutated *D. melanogaster*, instead, support the notion that loss of normal TDP-43 function may contribute to the pathogenesis of ALS and FTLD. Moreover, ubiquitous or tissue-specific overexpression of human TDP-43 also recapitulated key hallmark features of ALS pathology, including premature lethality, neuronal loss, neuromuscular junctions architecture defects and locomotor deficits. (**266**, **267**). Finally, FUS/TLS transgenic flies show locomotor dysfunction and premature lethality; the overexpression of mutant FUS/TLS, instead, caused an accumulation of ubiquitinated proteins, a pathological hallmark of ALS.

Invertebrate model organisms, however, are too simple to reproduce a human disease. The use of vertebrate models, instead, offer the best opportunity to define landmarks of disease progression and to understand the functional consequences of gene mutations. The great advantage of using vertebrates to model human diseases is clearly the possibility they offer for evaluation of new treatments. Indeed, testing new drugs on mice or other vertebrate models is often mandatory and asked by the Food and Drug Administration, for safety reasons, prior to approve new drug treatments. This step is also fundamental for paving the way towards human clinical trials, with both larger and smaller vertebrate model organisms. Other advantages of using vertebrate models include:

- the ability to make efficient targeted gene knockouts by homologous recombination;
- they are evolutionary closer to human;
- the developmental overview is similar for all mammals;
- the availability of material at all stages of development;
- their brains are more similar to human;
- they respond to injury and can be conditioned, great advantages to study learning, neuronal connectivity and plasticity;
- and they provide a valuable source of primary cells for culture.

On the contrary, the high maintenance cost, a relatively slow life cycle development, lower number of progeny, genetically identical offspring more difficult to obtain and the difficulty to manipulate embryos (intrauterine development) represent the major disadvantages of using vertebrate as disease model organisms.

The main vertebrate models used to study ALS are mice, rats, zebrafish, dogs and pigs. Obviously, in the laboratories, mice and rats represent the most used animal models.

Over the past century, the mouse *Mus musculus* has become the premier mammalian model organism for experimental studies and genetic research, because of its close genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed, ease of handling and relatively high reproductive rate. There are many mouse models commercially available for genetic research, including thousands of unique inbred strains and genetically engineered mutants.

The use of mouse models has been of particular importance in studying the pathogenesis of ALS. The initial description of SOD1 gene mutations in familial ALS patients, in 1993, first led to the hypothesis that the disease resulted from compromised enzymatic activity due to the loss of the enzyme function (**268**). However, this loss of function hypothesis was rapidly refuted, because SOD1 knockout (KO) mice do not develop disease (**269**). In contrast, transgenic mice ubiquitously overexpressing various SOD1 gene mutations with different biochemical properties, even in the presence of endogenous mouse SOD1 gene, develop a neurodegenerative disease that is quite similar to the human illness. Of particular interest, transgenic mice overexpressing wild type human SOD1 or specifically expressing mutant SOD1 only in neurons or only in glial cells do not develop disease (**270**, **271**).

Although more than 150 mutations in SOD1 are known and many transgenic mice are developed, the $SOD1^{G93A}$ mouse is, currently, the most widely used experimental model in ALS research and drug testing. Unlucky, several pharmacological approaches tested so far have produced only modest beneficial effects. Riluzole, a glutamate antagonist and the only drug useful for ALS treatment, extended the life span of $SOD1^{G93A}$ mice by 10 to 15 days without affecting disease onset (**272**). This mouse model, thus, is useful to test new drugs potentially useful for ALS treatment. For example, it has been shown that treatment with the endoplasmic reticulum (ER) stress-protective agent salubrinal attenuated disease manifestations and delayed progression in a $SOD1^{G93A}$ mouse model (**273**), suggesting a role of ER stress in ALS.

Other mouse models useful to study ALS are mice TDP-43 models, mice intermediate filament models, and mice ALS2 models.

Embryonic lethality is observed in homozygous mouse knockouts for TDP-43 (**274**, **275**). The TDP-43 deficient embryos die at embryonic day 7.5, thereby demonstrating the essential function of TDP-43 protein in development. Mice heterozygous for TDP-43 disruption only exhibit subtle muscle weakness with no evidence of motor neuron pathology. However, many of the transgenic mouse lines overexpressing wild type or mutant TDP-43 reported to date have showed some ALS features, including early paralysis leading to premature death (**276**, **277**).

Several transgenic mouse lines and knockout mice implicating different neurofilament subunits have been extensively studied over the past years (**278**). Even though genetic mutations in intermediate filament (IF) genes are not major causes of ALS, it is of potential relevance to ALS that transgenic mice with altered stoichiometry of neuronal intermediate filament develop pathological features of the disease (**279**, **280**). Of particular interest was the finding that overexpression of wild type peripherin, a type III intermediate filament, in NEFL knockout mice caused age-dependent selective motor neurons degeneration (**281**). This mouse model is also characterized by the formation of perikaryal and axonal intermediate filament inclusions resembling spheroids in motor neurons of human ALS. The precise mechanism by which accumulation of intermediate neurofilament leads to neurodegenerative disorders is not fully understood. Neurofilament and peripherin proteins are two types of intermediate filaments detected in the majority of axonal inclusion bodies, called spheroids, in motor neurons of ALS patients (**282**). Multiple factors can potentially cause the accumulation of intermediate filament proteins, including de-regulation of intermediate filament protein synthesis, proteolysis, defective axonal transport, abnormal phosphorylation, and other protein modifications.

Finally, six different groups have reported the generation of an alsin knockout mouse (**283**, **284**). Despite an age dependent loss of motor coordination revealed by rotarod and grip strength performances of the ALS2 KO mice, no major motor deficits consistent with ALS or other motor neuron diseases were present in these models. All ALS2-deficient mice appear to be grossly normal, viable and fertile with lifespan expectancy similar to wild type littermates. However, some differences are noteworthy and may explain the heterogeneity of the phenotype. The diversity of the apparent phenotypes among different ALS2 KO mouse models may be due in part by different gene targeting strategies used to generate each mouse models, ES cell lines used leading to differences in the genetic background, housing conditions or approaches

taken to evaluate the mice. Interestingly, thorough molecular analysis of one of these *ALS* KO mouse model revealed the presence of a number of novel ALS2 isoforms expressed in the central nervous system of these animals (**285**). These results suggest that other alternatively spliced ALS2 isoforms may exist and that some of these novel ALS2 mRNA species still can be transcribed in ALS2 knockout animals and may compensate for the loss of the full-length protein.

The rat *Rattus norvegicus* offers some advantages over the mouse and the other organisms. First, rats are physiologically more similar to humans compare to mice. The size of the animal also confers a valuable advantage and enhances its use as a disease model. For example, the size of the rat brain offers unique possibilities for the application of microsurgical techniques, intrathecal administration of drugs, stem cell transplantation, serial sampling of the cerebrospinal fluid (CSF), *in vivo* nerve recordings, and neuroimaging procedures. However, even though its size is considered as an advantage over mice, the higher cost of maintenance (bigger cages, food, less animals can be housed per cages) and limited housing capacity in animal facilities directly related to its size also confers the principal limitation of using rats as a model.

Transgenic rat models of ALS have also been generated, including SOD1 transgenic rats and TDP-43 transgenic rats.

In rats, overexpression of G93A or H46R mutant SOD1 led to an ALS-like phenotype (**286**). These transgenic rat models reproduce the major phenotypic features of human ALS, such as selective motor neuron loss, ubiquitination, hyaline inclusions, vacuolation, and neuroinflammation. However, several differences between the rat and mouse ALS models can be denoted, including a more rapid progression of disease and the transient appearance of vacuoles in the transgenic SOD1 rats.

Moreover, in rats, the overexpression of mutant TDP-43 only, but not the wild type protein, caused widespread neurodegeneration, in contrast to what observed in mice. The transgenic mutant TDP-43 rat model exhibited progressive degeneration of motor neurons, neurodegeneration was not only restricted to motor neurons. However, TDP-43 mutation affected motor neurons earlier and more severely than other neurons in the CNS at end stage of the disease. However, this rat model mimic TDP-43 pathological features both seen in the different mouse models and in ALS patients, indicating that this model could be used in future pharmalogical studies in order to identify novel therapeutic avenues to treat TDP-43 related disorders.

I.B. Superoxide dismutase

The superoxide dismutase (SOD) belongs to a metal-protein family that catalyzes the dismutation of superoxide anion (O^{2-}) in molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (**fig. 4**). Thus, SOD plays a key role in the antioxidant defense of organisms against toxicity induced by reactive oxygen species (ROS).

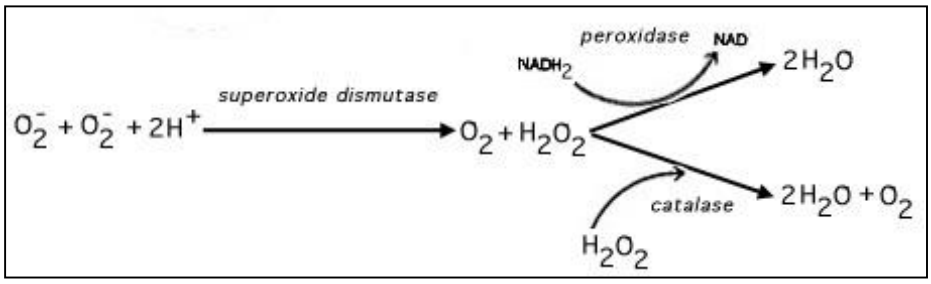

Figure 4. Dismutation of superoxide anion

The name of the protein derives from its discovery; indeed, about 50 years ago, copper was associated with hematic and hepatic proteins, named erythrocuprein and hepatocuprein (**287**). Then, in 1969, Fridovich and McCord discovered the activity of SOD by studying the reduction of cytochrome c (**288**).

Actually, different isoforms of SOD are known: they are proteins cofactored with copper and zinc, manganese, iron or nickel (**289**, **290**). Thus, there are three major families of superoxide dismutase, depending on the metal cofactor: Cu,Zn, which binds both copper and zinc, Fe and Mn isoenzymes, which bind either iron or manganese; finally, another family of SOD uses Ni as cofactor. These families of enzymes are different for localization, distribution and specie-specificity (**291**).

The discovery of SOD and its function has important physiological implication; indeed, superoxide anion is produced by the metabolism of molecular oxygen and its half-life is very brief, because superoxide anion reacts with different organic molecules and causes their oxidation. Thus, SOD represents the first defense mechanism against ROS in all aerobic organisms. In this context, in 1979, Mc Cord suggested an enzyme-based theory of obligate anaerobiosis: the aerobic existence of an organism depend on its capability to produce SOD; thus, an organism lacking SOD could survive only in anaerobic environment (**292**). However, other evidence suggests

a tolerance range, defined as maximum amount of atmospheric oxygen that allows the survival of anaerobic organisms lacking SOD (**293**).

I.B.1. Isoforms of superoxide dismutase

The superoxide dismutates are metal-enzyme containing a metal-ion (copper, iron or manganese). The genes that code for the three major families of SOD derive by two not correlated ancestral genes: from one gene derives the families of Fe-SOD and Mn-SOD, diffused in aerobic organisms, from bacteria to plants and humans. From the other gene derives the family of Cu,Zn-SOD, diffused only in eukaryotes. In **table 2**, the main characteristics of the different isoforms of SOD are represented.

Today, it is known the cellular distribution of the different isoforms of SOD: Cu,Zn-SOD (SOD1) is the eukaryotic cytosolic isoenzyme; Mn-SOD (SOD2) is located in the mitochondrial matrix of some eukaryotes and mammals; Fe-SOD is located in the cytosol of prokaryotes and some eukaryotes. Finally, the EC-SOD (extracellular SOD or SOD3) is located at extracellular level (**fig. 5**). In humans, there are three isoforms of SOD: SOD1, SOD2 and SOD3.

However, exceptions to this general rule exist; for example, some prokaryotes express the Cu,Zn-SOD (**294-296**); moreover, the Mn-SOD is the mitochondrial isoform in mammals, but in humans it is the main isoform located in the cytosol of hepatic cells.

Name	Molecular weight	Structure	Localization	Distribution
Cu,Zn-SOD	32,000	Dimeric	Cytosol	Eukaryotes/ Prokaryotes
Mn-SOD	40,000 - 80,000	Dimeric – tetrameric	Cytosol, mitochondria	Mammalians
Fe-SOD	40,000 - 80,000	Dimeric - tetrameric	Cytosol, chloroplast, mitochondria	Prokaryotes

Table 2. Molecular weight, structure, localization and distribution of the three isoforms of SOD.

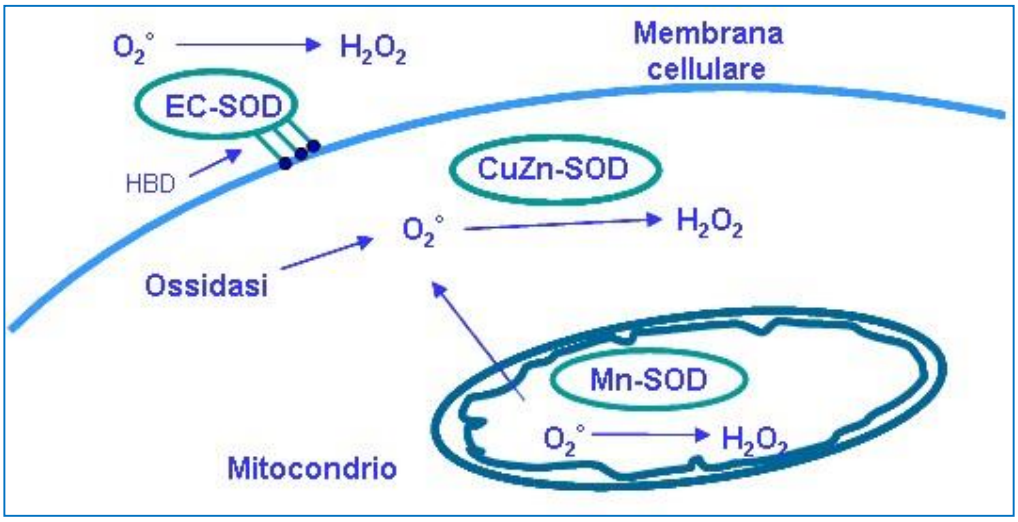

Figure 5. Isoforms of SOD and their localization

I.B.1.1. Mn-SOD and Fe-SOD

The molecular structure of Mn-SOD (**fig. 6**) and Fe-SOD (**fig. 7**) are not well-defined as the molecular structure of Cu,Zn-SOD; indeed, it is known only the primary structure of four Mn-SOD (**297**-**299**), while it is known eleven primary structures of Cu,Zn-SOD (**300**-**302**). Moreover, the complete aminoacid sequence of Mn-SOD is yet unknown.

The isoenzymes containing iron and manganese show molecular subunits of about 23.000 Dalton: the Fe-SOD exists only as dimer, while the Mn-SOD could exist as dimeric or tetrameric enzyme (**303**). These isoforms could contain one or two atoms for dimer and this difference, maybe, is caused by the loss of metal during purification processes. The crystallography analysis about Fe-SOD suggests two binding sites for metal (**304**). In addition, the ligands of manganese are localized in the same position of Fe-SOD (**305**), and many evidence indicate that Mn-SOD and Fe-SOD are structurally homologous (**306**).

Since the little knowledge about these two enzymes, the reaction mechanism is similar to the reaction catalyzed by Cu,Zn-SOD. However, the exact catalytic activity is not completely known, because Mn-SOD and Fe-SOD are not structurally correlated with Cu,Zn-SOD.

The catalytic activity follows the same general scheme of Cu,Zn-SOD, because it involves the couples Fe^{3+}/Fe^{2+} and Mn^{3+}/Mn^{2+}. Moreover, for the Mn-SOD, it is

hypothesized that catalytic activity is involved in the production of final species (**307**, **308**) and the catalytic efficiency is lower rather the catalytic efficiency of the other SOD.

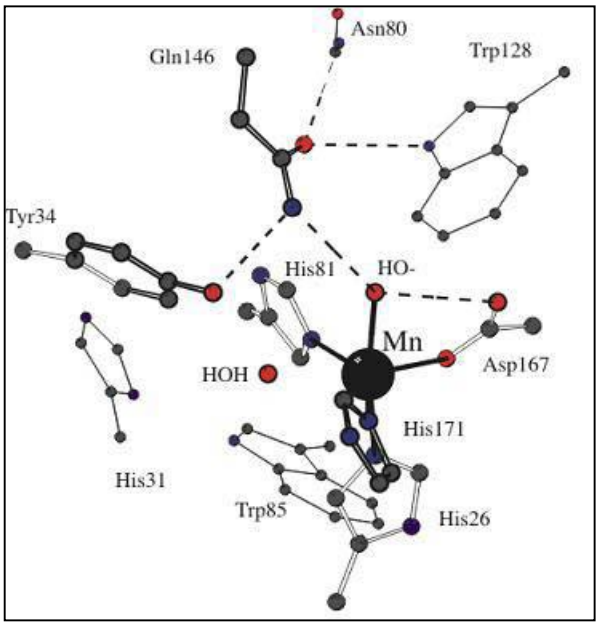

Figure 6. Binding site for metal of Mn-SOD

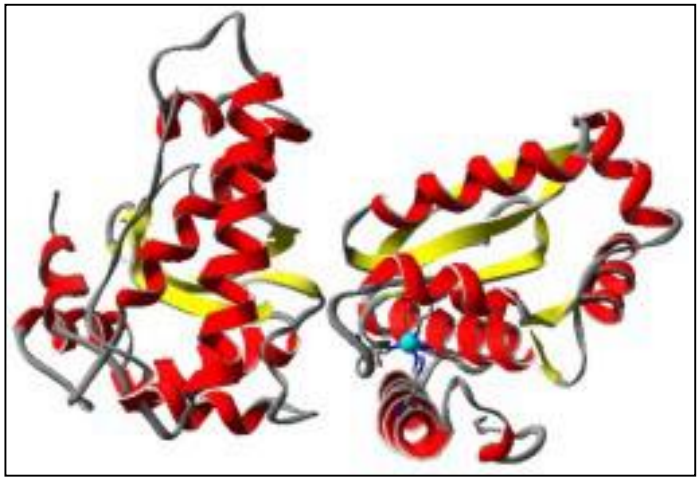

Figure 7. Three-dimensional structure and binding site for metal of Fe-SOD.

I.B.1.2. Cu,Zn-SOD

Cu,Zn-SOD (SOD1) (**fig. 8**) is a dimer of about 32.000 Dalton, located mainly in the cytosol of eukaryotes. The two identic subunits are composed of 153 aminoacids with a theoretical molecular weight of about 16 kDa, and are associated with not-covalent interaction and each one contains 1 g for atom of copper and zinc. The two ions are binding by an imidazole ring of a histidine of the protein.

The gene coding for SOD1 in humans is located on the long arm of chromosome 21 at 21q22.11, and, today, it is well-characterized (**309**). The gene is 11 kb long and has five exons and four introns (**310**). The main transcript can be both 0.7 kb and 0.9 kb, due to different polyadenylation at the 3' end. Additional splice forms have be found (**311**): these alternative splice forms are unstable and may be immediately degraded.

The three-dimensional structure of bovine SOD1 from erythrocytes was solved in 1982 (**310**), and many different structures of SOD1 variants have been solved both by nuclear magnetic resonance (NMR) and by X-ray crystallography. The first human SOD1 structure was solved ten years later (**312**).

Immunocytochemistry analysis and cellular fractionation confirm the cytosolic localization of SOD1 in same different cell lines. Moreover, SOD1 is located also in different subcellular compartments, such as nucleus, lysosomes, mitochondrial intermembrane space, endoplasmic reticulum and Golgi apparatus (**313**).

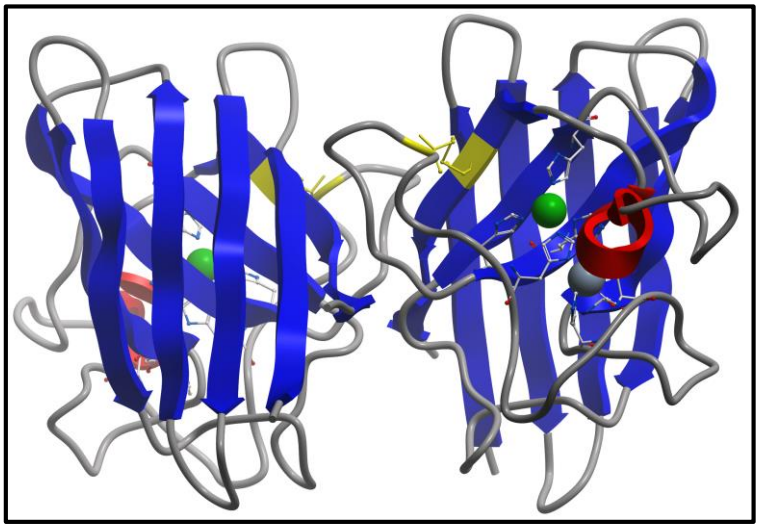

Figure 8. Three-dimensional structure of SOD1.

The dismutation reaction follows two step at the same speed: in the first step, the superoxide anion is reduced in molecular oxygen with the reduction of Cu^{2+} to Cu^{+}; in the second step, hydrogen peroxide is reduced by another superoxide anion and, in this way, the catalytic site is regenerated (**fig. 9**).

The enzymatic function was assigned to copper bound to the enzyme, since all activity was lost upon removal of metal. Instead, the zinc ion is needed for structural stability of the protein.

The exact catalytic mechanism was determined by pulsate radiolysis analysis and, then, it is confirmed for Mn-SOD and Fe-SOD (**314**).

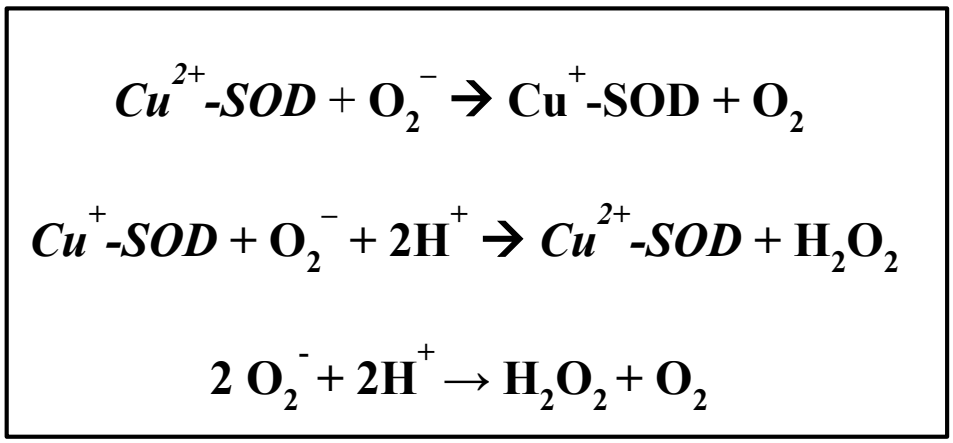

Figure 9. Catalytic reaction of SOD1

SOD1 is expressed in almost all eukaryotic cells at high levels and it is also found in some bacteria (**294**-**296**). In humans, SOD1 is mainly located in the liver and in the kidneys; in the central nervous system, that is affected in ALS, SOD1 expression, instead, is lower (**315**-**316**). In particular, immunohistochemistry analysis reveal the expression of SOD1 in spinal cord: the most intense staining was found in the ventral horn.

SOD1 is mainly a cytosolic enzyme (**317**), but some SOD1 could be found in other cellular compartments: for example, it is found in the mitochondrial intermembrane space (**318**), where the high production of superoxide during oxidative phosphorylation requires SOD1 and SOD2 (that is the main mitochondrial isoform).

In addition, SOD1 has also been found in the nucleus of different cell types and in body fluids, such as serum and cerebral spinal fluid (CSF) (**319**, **320**).

I.B.2. Secretion of Cu,Zn-superoxide dismutase

Since SOD1 is considered a constitutive enzyme located in the cytosol, recent evidence indicate that SOD1 could be secreted in different cell lines (**321**) through microvesicles. In particular, the secretion mechanism is an ATP-dependent process; indeed, it is inhibited by brefeldin-A, 2-deoxyglucose and sodium azide, drugs that reduce intracellular pool of ATP (**322**).

Thus, SOD1 could have a protective role against oxidative stress, and this effect could be important in the nervous system, where a high oxidative metabolism generates a great amount of ROS. In addition, it is demonstrated that neuronal cells secrets SOD1 in absence of oxidative stress. Thus, this secretion could have a paracrine modulatory action on neurons.

SOD1 export in neuronal cells involves vesicles located in cytosol and dendrites and needs on SNARE (*solubile NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptor*) complex activation. Indeed, it is demonstrated that the pre-incubation of rat adenohypophysis GH3 cells with the botulin toxin (BoNT/A), that cleaves SNAP-25 (**323**), inhibited SOD1 release.

Finally, SOD1 release was observed in basal condition and after depolarization mediated by high potassium concentration (**324**), and cytometry analysis reveals that SOD1 could bind cellular membrane of neuronal cells (**325**).

I.B.3. Transductional effects of Cu,Zn- superoxide dismutase

Western blotting analysis and cytometric experiments indicate that SOD1 is able to induce the activation of PLC (phospholipase C)/ PKC (protein kinase C) pathway in human neuroblastoma SK-N-BE cells through an increase of intracellular Ca^{2+} concentration (**325**). The increase of intracellular Ca^{2+} concentration is partially reduced by free Ca^{2+} in the culture medium. Thus, it is hypothesized that this increase involves intracellular Ca^{2+} stores.

In addition, ω-conotoxin and L-nimodipine, that are respectively an N-type and L-type voltage-dependent calcium channels inhibitors, partially inhibits Ca^{2+} increase, suggesting the involvement of voltage-dependent calcium channels (VGCCs) in intracellular Ca^{2+} concentration increase induced by SOD1 (**326**).

The activation of PLC/PKC pathway is independent on catalytic activity of SOD1, because this pathway is activated also by ApoSOD, the enzyme lacking catalytic activity, while mimetic drugs of SOD1, such as MnTMPyP, fail to have the same effect. Moreover, the pre-incubation of neuronal cell with U73122, a PLC inhibitor, completely inhibits PLC activation by SOD1 (**325**).

Finally, in the last few years, it is reported that SOD1 is able to induce the phosphorylation of kinases downstream PLC/PKC pathway, such as p-ERK1/2 and p-Akt (**327**).

Thus, SOD1 is not only a scavenger of ROS, but it could be involved in other biological functions related to intracellular Ca^{2+} concentration dependent on ERK1/2 and Akt activation.

I.B.3.1. MAP kinases activation

MAP kinases (MAPK, mitogenic-activated protein kinases) belong to a family of ubiquitous proline-directed, protein-serine/threonine kinases, which are involved in signal transduction pathways that control intracellular events, including major developmental changes in organisms, and apoptosis (**328**). The serine-threonin kinase ERK1/2 (extracellular signal regulated kinase) belongs to this family (**fig. 10**).

ERK1 and ERK2, in particular, are proteins of 43 and 41 kDa that are nearly 85% identical overall, with much greater identity in the core regions involved in binding substrates (**329**-**331**). The two phosphoacceptor sites, tyrosine and threonine, which are phosphorylated to activate the kinases, are separated by a glutamate residue in both ERK1 and ERK2 to give the motif TEY in the activation loop (**332**). Both the kinases are ubiquitously expressed and requires, for their activation, three upstream families of kinases, named Ras, Raf, and MEK (MAP kinase extracellular signal regulated kinase).

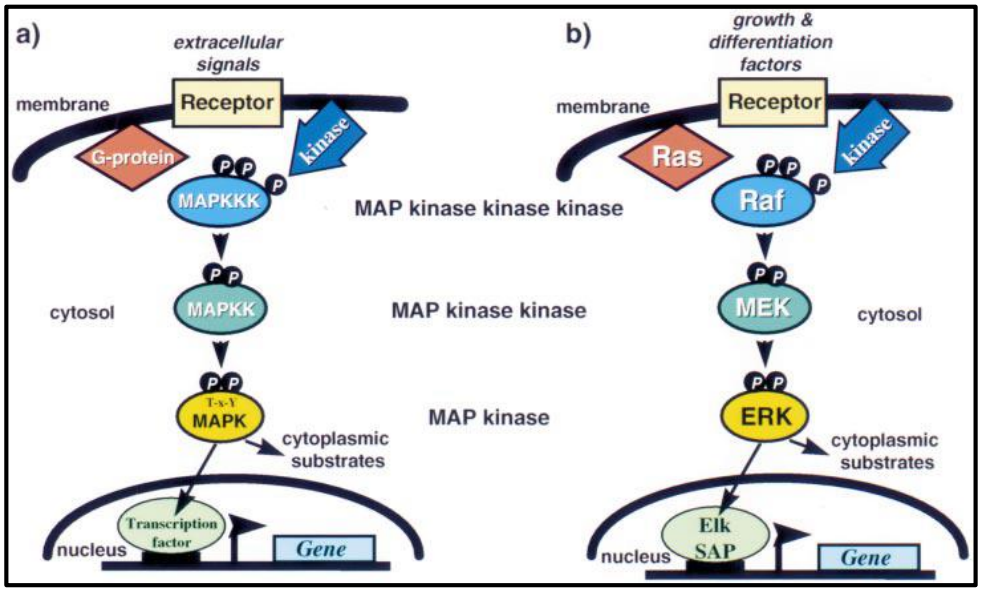

Figure 10. Schematic representation of the structure of MAPK pathways: (a) General MAPK pathways; (b) the ERK pathway in particular (Kolch W., 2000).

All the developmental signals able to induce MAPK activation are transmitted by specific receptors, such as tyrosine-kinase receptors (RTKs), cytokines receptors, and G-protein coupled receptors. These receptors, in particular, induces the activation of Ras, a small GTPase that acts as molecular switch in the transductional signal cascade of MAPK. After activation, indeed, Ras switches from its inactivated state bound to GDP (guanosine diphoshapte) to its activated state bound GTP (guanosine triphosphate). The first effectors of Ras is a protein-kinase belong to Raf family, named Raf-1. This protein is able to bind Ras in a GTPasic dependent manner: activate Ras binds Raf-1 and causes its translocation in membrane, where Raf-1 is activated (**333**). Then, Raf induces MEK phosphorylation through MEK kinase (MEKK) (**334**, **335**). Finally, MEK induces ERK1/2 phosphorylation on the acceptor sites, represented by threonine and tyrosine residues (**335**). After phosphorylation, ERK1/2 translocates in the nucleus and induces the activation of several transcription factors.

I.B.3.2 PI3K/Akt activation

The serine/threonine kinase Akt, also named protein kinase B or PKB, belongs to the cAMP-dependent protein kinase A/protein kinase G/ protein kinase C (AGC)

super family of protein kinases that show structural homology within catalytic domain and have similar activation mechanism (**336**, **337**) (**fig. 11**).

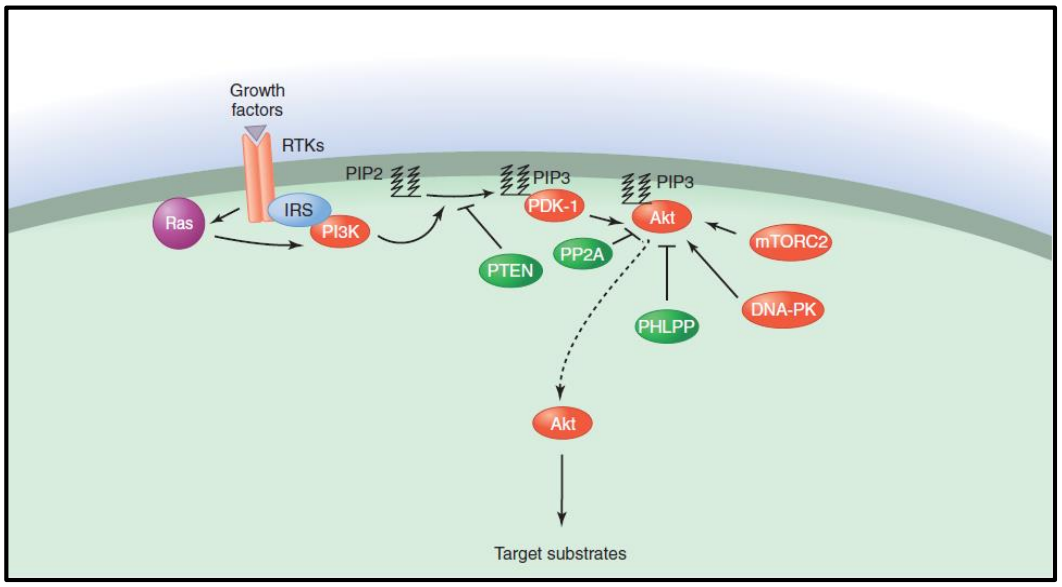

Figure 11. PKB/Akt activation downstream of RTKs via the P13K pathway (Hemmings B.A., 2012).

There are three isoforms of Akt, named Akt1, Akt2, and Akt3, with a conserved domain structure, represented by an amino terminal pleckstrin homology (PH) domain, a central kinase domain, and a carboxyl terminal regulatory domain with hydrophobic motif. The PH domain interacts with membrane lipid products, such as phosphatidylinositol (3,4,5) triphosphate (PIP3) produced by phosphatidylinositol-3-kinase (PI3K). Thus, Akt is the downstream kinase of PI3K, activated by tyrosine kinases receptors (RTK) or G-protein coupled receptors (**338**). In particular, PIP3 does not activate Akt directly, but induces Akt traslocation to the plasma membrane causing the alteration of its conformation and following the phosphorylation by phosphoinositide-dependen kinase-1 (PDK1).

The activation of Akt depends on site-specific phosphorylation: the main sites of phosphorylation are threonine 308, activated by PDK1 (**339**), and serine 473, activated by a mechanism yet unknown. It seems that serine 473 phosphorylation site could be autophosphorilated (**340**) or phosphorylated by other kinases, such as integrin-linked kinase (ILK) (**341**).

The activation of Akt is also regulated by a phosphatases, such as PTEN (phosphatase and tensin homolog), which removes phosphate from the 3-OH position (**342**), causing the inactivation of Akt.

Akt plays a key role in the signal transductional pathways, because it is involved in metabolism, cell growth, trasncritional regulation and cell survival (**343**).

I.B.4. Cu,Zn-superoxide dismutase and Amyotrophic Lateral Sclerosis

In 1993, a link between SOD1 and fALS was found for the first time. Today, more than 150 mutations in the gene coding for SOD1 associated to ALS are described and reported in the ALS Online Genetic Database (ALSOD). Obviously, not all described mutations in SOD1 are necessary pathogenetic (**344**), but these studies reveal anyway that SOD1 is extremely sensitive to mutation.

The ALS-linked mutations are located all over the aminoacid sequence of SOD1, and they are found in the five exons of the gene. Most of these mutations are single aminoacid exchanges, but insertions and deletions are also described.

The discovery of a link between SOD1 and ALS first suggest that oxidative stress could have a key role in the pathogenesis of the disease: indeed, it was hypothesized a loss of function of SOD1, one of the most important enzymes involved in the defense against ROS.

Actually, it is known that mutations in SOD1 are associated with a gain of toxic function rather than a loss of function. Indeed, most of the mutant SOD1, such as $SOD1^{D90A}$, $SOD1^{G93A}$ and $SOD1^{G41D}$ (**345**, **346**), preserve catalytic activity, and SOD1 mutations usually are inherited in a dominant way (**347**, **348**), preserving at last 50% of enzymatic activity. In addition, transgenic overexpression of SOD1 in mice generates an ALS phenotype (**349**, **350**), while SOD1 knock-out mice do not develop the disease (**351**). Finally, mutated SOD1 aggregates in the cytosol, at level of endoplasmic reticulum and Golgi apparatus (**352**), causing mitochondrial dysfunction, ER stress and motor neuronal death.

Since mutations in SOD1 are associated with about the 20% of cases of fALS, recent evidence suggests an important role for SOD1 both in fALS and in sALS. Indeed, inclusions containing misfolding SOD1 are found in the CNS of both sALS

and fALS patients without SOD1 mutations (**353**), and there are evidence for soluble misfolded SOD1 in the spinal cord of sALS patients. Interestingly, it has been recently reported that wild type (wt) SOD1 can acquire properties of ALS-linked mutant SOD1 species, indicating a shared pathophysiological pathway between sALS and fALS (**354**). Other studies showed that wt SOD1 may acquire toxic properties upon oxidative damage and that wt SOD1 expression dramatically exacerbated disease in transgenic mice expressing mutant SOD1 forms, such as $SOD1^{A4V}$, $SOD1^{G85R}$, $SOD1^{L126Z}$, and $SOD1^{G93A}$ (**355**).

SOD1 is one of the most stable proteins known; thus, several researches has been tried to explain the involvement of misfolded SOD1 in neurodegenerarion. The aggregates found in ALS patients with SOD1 mutations are immunoreactive for SOD1 and are a hallmark of the disease. Moreover, in control patients a SOD1 strains is observed diffusely in the cytoplasm, indicating that SOD1 is involved in the pathogenesis of ALS and, maybe, the enzyme loses its structure. In addition, nine mutations cause premature stop codons generating a pathogenic truncated SOD1, as observed in transgenic mice with truncated variants that develop ALS (**356**). Moreover, when SOD1 loses its post-traslational modification, the enzyme lost its stability, becoming one of the most unstable proteins (**357**). Mutations could affect SOD1 stability and folding in different ways. For example, the location of the mutated residue and how different chains side are between the normal aminoacid and the aminoacid introduced by mutation affect the stability; moreover, most mutation could destabilize the Apo state (**358**, **359**). A common way to form aggregates of SOD1 is represented by disulfide crosslinking. SOD1 has four cysteinse (**360**, **361**) and all these aminoacids have been found to be mutated in ALS patients (**362**, **363**). Anyway, SOD1 is not sensitive to aggregation compared to other proteins involved in neurodegeneration, but particular conditions, such as low pH and reducing condition (**364**, **365**) could induce SOD1 aggregations.

Thus, how SOD1 misfolding and aggregation is toxic to motor neurons is yet unknown. It is possible that the expression of mutant SOD1 in motor neurons will be not sufficient to cause degeneration, suggesting the expression of mutated SOD1 in other neuronal cells to develop the disease. This hypothesis, however, does not explain the involvement of misfolding and aggregates. The misfolded SOD1 could interact with other important proteins or could impair SOD1 recruitment to outer membrane of mitochondria, or could affect the ubiquitin proteaseom pathway (**352**, **366**). Indeed,

recent evidence indicate that SOD1 could be traslocated trough ER-Golgi network and chromogranins, a protein expressed in neurons, interneurons and astrocytes, could act as chaperone-like protein to promote misfolded SOD1 secretion and it has been demonstrated that extracellular mutant SOD1 can induce motor neuron degeneration.

Finally, it has been proposed that endoplasmic reticulum (ER) stress response might exert a critical role in the disease pathogenesis. The ER is the site of synthesis and folding of secretory and membrane bound proteins. The capacity of the ER to process proteins is limited and the accumulation of misfolded proteins may activate different ER stress pathways. In particular, several mutated SOD1 cause alteration in calcium homeostasis, redox stress and energy deprivation, all conditions that result in ER stress (**367**).

I.C. Intracellular organelles in neuronal function: the endoplasmic reticulum and its functions

The endoplasmic reticulum (ER) is an important cellular organelle, located in the cytoplasm of all eukaryotic cells. It is involved in several cellular functions, such as regulation of calcium homeostasis, secretion of different proteins, and biosynthesis of lipids. The ER is organized into a netlike labyrinth of branching tubles and flattened sacs extending throughout the cytosol. The tubules and sacs are all thought to interconnect, so that the ER membrane forms a continuous sheet enclosing a single internal space, named ER lumen. The ER membrane separates the ER lumen from the cytosol, and it mediates the selective transfer of molecules between these two compartments (**368**).

The ER plays a key role during protein folding: indeed, the ER Ca^{2+}-dependent molecular chaperons act to allow the correct folding of new proteins and so only the correct folded protein can be translocated from ER to other cellular compartments. The folding of proteins involves also PDI (protein disulphide isomerase), an enzyme that catalyzes the formation of disulphide bound among polypeptidic chains (**369**). In this context, another important role of the ER is represented by post-translational modifications, such as glycosylation and lipidations, which are fundamental for the correct folding of the proteins. All the misfolded or unfolded proteins are exported in the cytosol and degraded trough the ubiquitin-proteasome system. The ER is involved also in lipids biosynthesis, and controls cholesterol and membrane lipids production. Finally, the ER regulates the signal transduction through the releases of Ca^{2+}.

Ca^{2+} is a universal second messenger controlling a wide variety of cellular reactions and adaptive responses (**370**). Ca^{2+} is the most versatile intracellular messenger discovered so far, since it is involved in the regulation of almost all known cellular functions and reactions. Ca^{2+} concentration differs in the different cellular compartments: for example, in the extracellular fluid Ca^{2+} concentration is about 1 mM and represents about the 50% of total calcium; instead, in the cytosol, Ca^{2+} concentration is about 100 nM. This marked difference is due to the binding of Ca^{2+} to different cysolic molecules or to its presence in organelles. Indeed, Ca^{2+} ions are presented in two different forms: cytosolic free Ca^{2+} and Ca^{2+} bound to proteins. In order to cross membranes and reach the cytosol, calcium ions require being free.

The intracellular Ca^{2+} concentration is finely regulated by different mechanisms that control the passage of the ion trough plasma membranes and trough membranes of organelles, which act as intracellular store of Ca^{2+}. The ER represents the main store of intracellular calcium. Indeed, in the ER lumen the highest intracellular free Ca^{2+} is found (about 500 µM) (**371**). Other organelles that act as calcium store are mitochondria, Golgi apparatus, secretory granules and lysosomes. Except ER and mitochondria, however, the rule of the other intracellular calcium stores is not yet known. Anyway, the homeostasis of intracellular calcium concentration involves several ionic pumps, named Ca^{2+}-ATPases, which coupled ATP (adenosine triphosphate) hydrolysis to Ca^{2+} ions transport against concentration gradient.

In particular, the ER has different ionic channels and Ca^{2+}-ATPases in membrane that controls Ca^{2+} influx and efflux through the lumen. The ionic channels are channel-receptors that connect the lumen of the organelle to the cytosol. When Ca^{2+} interacts with specific receptors, the ionic channels are opened and allowed the release of Ca^{2+} from the stores. The ionic channels are divided in two classes: the IP_3-dependent (inositol-1,4,5-triphosphate) channels and the Ry-dependent (ryanodine, an alkaloid used to experimental aims) channels (**372**).

The IP_3 receptors (IP_3R) are activated by IP_3 production, resulting by phospholipase C (PLC) activity. PLC, indeed, catalyzes the hydrolysis of PIP_2 (phosphatidyl-inositol-4,5-bisphosphate) in IP_3 and DAG (diacylglycerol). When released by the membrane, IP_3 spreads in the cytosol and binds a specific receptor, permitting the release of Ca^{2+} from its intracellular stores. In this context, Ca^{2+} regulates channel activations: indeed, it is a positive regulator in presence of low Ca^{2+} concentration, and a negative regulator in presence of high Ca^{2+} concentration. This mechanism first potentiates the activation of channel-receptor and Ca^{2+} efflux; then, when cytosolic Ca^{2+} concentration is too high, the receptor is inactivated to allow the restore of intracellular Ca^{2+} concentration (**fig. 12**).

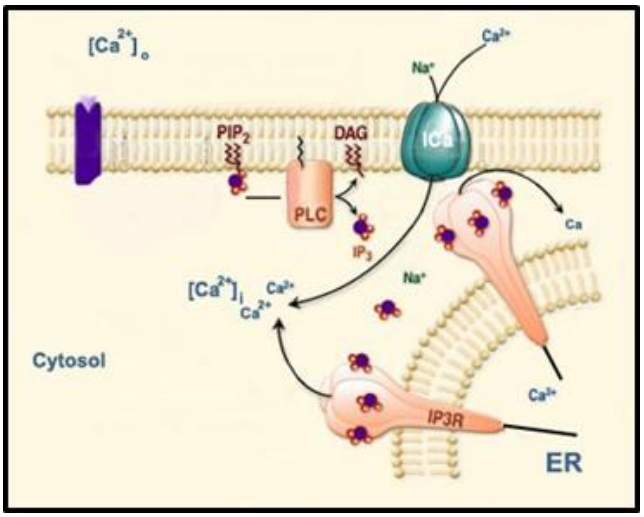

Figure 12. IP_3 binds its receptor and induces Ca^{2+} release from ER. Ca^{2+} translocates into the cytosol also through the activation of voltage-dependent calcium channels located on the plasma membrane.

The ryanodin receptors (RyR) exist in three isoforms, named RyRI, RyRII, and RyRIII, codified by different genes. The three isoforms differ for localization: indeed, RyRI is the isoform of muscles; RyRII is expressed in cardiac tissue and in CNS; finally, RyRIII is expressed ubiquitously. This channel is a cationic channel poorly selective, because it allows the passage of several divalent cations. The activation of RyR is concomitant to voltage-dependent calcium channels activation. These channels act as "voltage-sensors", because, after depolarization, they undergo conformational change that is transmitted to RyRI. In this way, RyRI is activated and allowed Ca^{2+} flux (**372**). The other isoforms, RyRII and RyRIII, are activated by cytosolic Ca^{2+}, through a particular mechanism named "calcium-induced calcium release" (CICR) (**fig. 13**). The endogenous regulator of RyR seems to be cADP-ribose (cyclic adenosine diphosphate-ribose).

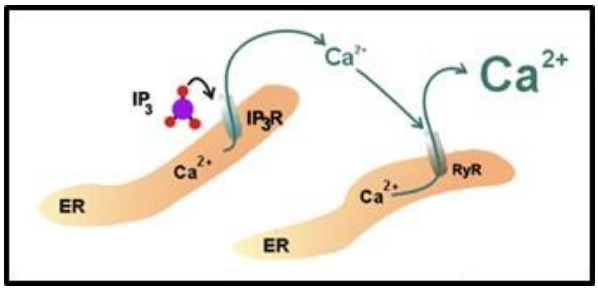

Figure 13. Molecular mechanism of CICR: Ca^{2+} released into the cytosol activates RyR, potentiating Ca^{2+} release.

When both the channels are opened, the Ca^{2+} in the ER diffuses into the cytosol, causing an increase of intracellular calcium concentration. In the ER, Ca^{2+} is bound to different proteins of the ER lumen, which have low affinity for the ion. A family of ATPases, named SERCA (sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPases), finally, restores the ER calcium concentration, permitting the cross of two Ca^{2+} ions for each hydrolyzed ATP molecule. SERCA are different from other Ca^{2+}-ATPases for their sensibility to tapsigargin (Tg), which is a specific SERCA inhibitor (**372**) (**fig. 14**).

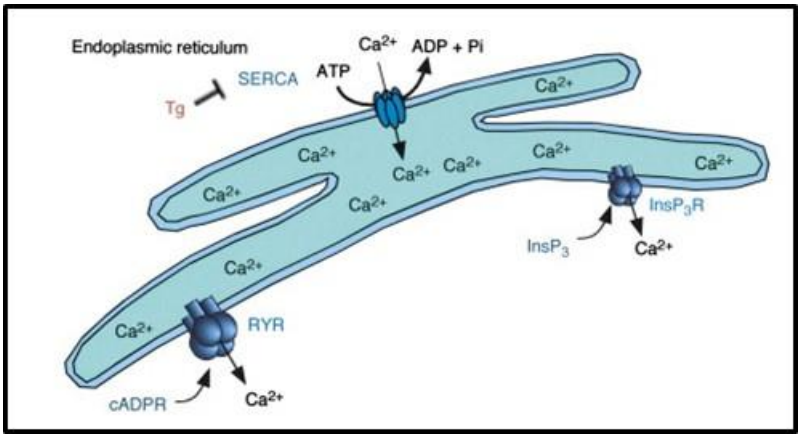

Figure 14. IP_3R, RyR and SERCA control of calcium concentration in the ER. (Churchill, *et al.*, 2002).

I.C.1. Endoplamic reticulum stress

ER stress refers to a state in which the accumulation of misfolded proteins in the ER lumen exceeds the capacity for folding and degradation. Several stimuli and pathological events could determine ER stress. The main events responsible of ER stress include:

- glucose deprivation that determine a failure in post-transductional modifications;

- alteration in cellular redox regulation, causing hypoxia and increase of oxidant molecules and misfolded proteins;

- alteration in calcium homeostasis in the ER; indeed, in absence of Ca^{2+}, molecular chaperons are not activated, causing an increase of misfolded proteins;

- high fat diet;

- viral infections that cause an increase of viral proteins in the ER, conducing to activation of apoptosis and other cell death mechanisms;

- disease, especially neurodegenerative disease, characterized by nuclear inclusions that cause accumulation of unfolded proteins in the ER for reduction of proteasome activity.

The main cellular defense to ER stress is represented by the unfolded protein response (UPR). UPR could have two consequences: adaptation to new conditions or apoptosis. The adaptive response is associated with the activation of several transcriptional pathways, resulting in the expression of genes able to increase ER ability to promote protein folding or able to induce the ER-assisted degradation (ERAD) (**369**). These mechanisms contribute to eliminate unfolded proteins in the ER. Thus, all correctly folded proteins are traslocated to the secretory pathway, while the unfolded proteins are retrotranlocated to the ubiquitin-proteasome system, activated by ERAD (**373**). Concomitantly, in the nucleus, in order to support the translation of mRNA coding for proteins involved in UPR, the translation of other mRNA are inhibited. In this way, the entry of new proteins in the ER is reduced.

During UPR, several pathways associated with cellular stress are activated. These pathways involve MAPK, JNK (Jun N-terminal kinase), p38MAPK, and, sometimes, NF-κB (nuclear factor-κB) mediated signaling.

When UPR fails, cells induce their death trough caspase-dependent and independent mechanisms. The caspase-dependent cell death is defined apoptosis; the caspase-independent cell death, instead, includes necrosis and autophagy, a particular catabolic mechanism associated with lysosomal degradation of macromolecules, organelles and other damaged cellular compartments. Usually, autophagy is a prosurvival mechanism, especially in nutrients deprivation and hypoxia. It could be activated also by ER stress or in all situations that induce non apoptotic cell death (**369**).

Alterations of calcium homeostasis in the ER could induce UPR and modify cellular events, resulting in cell survival or cell death. For example, a great increase of Ca^{2+} concentration in the ER could induce apoptosis through the activation of BCL-2 (B-cell lymphoma 2). BCL-2 belongs to a family of protein located in the membrane of ER that includes pro- and anti-apoptotic members. These proteins act at mitochondrial level, and most of them are involved during the ER stress, because are

able to maintain calcium homeostasis and control cell death induced by different death-inducing substances, such as tunicamycin, brefeldin-A, tapsigargin, and other oxidative molecules (**369**).

The anti-apoptotic proteins, such as BCL-2 and BCL-XL, cause a reduction of basal calcium levels in the ER; the pro-apoptotic proteins BAX (BCL-2 associated X protein) and BAK (BCL-2 antagonist/killer), instead, mediate the opposite effect, causing an increase of Ca^{2+} concentration in the ER. Many evidence, indeed, demonstrated that $BAX^{-/-}$ cells and $BAK^{-/-}$ cells are characterized by a low Ca^{2+} concentration. How the proteins of BCL-2 family act in regulating calcium concentration is unclear. It is hypothesized an involvement of IP_3R that are modulated by BCL-2 and BCL-X_L, independently on IP_3 concentration: these two proteins permit the efflux of calcium to the ER. In particular, BCL-2 is an anti-apoptotic protein that inhibit only apoptosis induced by ER stress; indeed, it is unable to prevent apoptosis induced by all the other mitochondria-dependent mechanisms (**369**).

The ER calcium concentration is regulated also by BI1 (BAX inhibitor 1), a transmembrane protein located in the ER that interacts with the pro-apoptotic members of BCL-2 family. Precisely, BI1 interacts with BAX and so blocks cell death induced by oxidative stress and by the overexpression of BAX. The protective action against ER stress mediated by BI1 are demonstrated in transgenic knockout $BI1^{-/-}$ mice, which have an increased sensitivity to kidney damage induced by tunicamycin and to neuronal cell death. Moreover, the proteins of BCL-2 family require BI1 in order to regulate basal calcium concentration in the ER; it is showed, indeed, that, in $BI1^{-/-}$ cells, BCL-X_L alone is unable to reduce Ca^{2+} concentration in the ER. Actually, it is yet unclear whether BI1 require IP_3R for its function. Finally, in the ER, the pro-apoptotic protein BAP31 (B-cell receptor-associated protein 31), by binding BCL-2 and BCL-X_L, activates apoptosis and induces an increase of ER calcium concentration (**369**).

The alteration in the ER calcium concentration are important in regulating cell death: indeed, whether ER recognizes a low amount of free Ca^{2+}, in stress conditions, the ER cannot induce Ca^{2+} release into the cytosol and, consequently, pro-apoptotic effectors are not activated.

In the molecular mechanisms of cell death, ionic channels seem to be involved, as shown by mutations in the genes coding for ER Ca^{2+} channels. For example, in *C.*

elegans, it was demonstrated that mutations in IP_3R or in RyR prevent neuronal cell death.

Finally, calcium is involved also in ER stress-induced autophagy. In ER stress conditions, indeed, pro-autophagic proteins are activated; these proteins include PKCθ (protein kinase Cθ), and CaMKKβ (calmodulin-dependent kinase kinase-β) (**369**). Furthermore, high Ca^{2+} levels induce the activation of some Ca^{2+}-dependent proteases, such as calpain, expressed in almost all tissues concomitant to their inhibitor calpastatin. In physiological condition, calpain mediates different cellular processes, such as turnover and removal of membrane receptors, enzyme activation, and mitosis (**372**). Marked levels of calcium induce the dissociation of calpain-calpastatin complex, resulting in irreversible activation of calpain. Instead, in ER stress condition, calpain induces apoptosis through the cleavage of BAX and BID, which are activated, BCL-2 and BCL-X_L, which are inactivated, and several caspases, included caspase-12.

Finally, it is possible that alteration in calcium concentration could affect not only calcium release from ER, but also the transductional events that mediate UPR and that are important in the regulation of cell death (**369**).

I.C.2. The unfolded protein response

The unfolded protein response (UPR) is the first defense against misfolded proteins accumulated in the ER. The activation of UPR involves several proteins that induce several transductional mechanisms: these proteins could induce an increase of ER chaperones, could block mRNA translation in order to prevent the entry of new proteins in the ER, and could accelerate retrograde export of proteins from the ER to the cytosol in order to induce ubiquitination and proteasome-dependent degradation.

In the UPR, GRP78 (glucose-regulated protein 78 kDa), also named BiP (binding immunoglobulin protein), plays a key role. GRP78, in particular, is a molecular chaperone resident in the ER. In physiological conditions, GRP78 is associated with three transmembrane proteins of the ER, named IRE1, PERK, and ATF6. These three proteins are considered as "stress sensor" of the organelle, because their bound with GRP78 inhibit their activation. In ER stress conditions, misfolded proteins accumulated in the ER lumen, bind GRP78, resulting in the dissociation of

GRP78 bound to the three stress sensors. In this way, the three proteins are activated and UPR can start in order to attenuate ER stress (**374**).

Precisely, the serine/threonine kinase PERK (PRKR-like ER kinase) is activated by oligomerization and autophosphorylation. When activated, PERK phosphorylates the initiation factor of the translation eIF2α (eukariotic initiation factor-2α), causing its inactivation. The inactivation of eIF2α determines the global repression of protein synthesis and so reduces the cargo of new proteins in the ER. Oligomerization and autophosphorylation induce also the activation of IRE1 (inositol-requiring kinase 1); IRE1, once activated, induce alternative splicing of the pre-mRNA coding for XBP1 (X-box-binding protein 1), resulting in the production of XBP1-s (spliced X-box-binding protein 1), a very stable transcriptional factor. XBP1-s migrates into the nucleus and activates the transcription of several genes involved in UPR and ERAD. Moreover, IRE1 and XBP1 promote cleavage and degradation of several mRNA coding for secretory proteins, causing a further reduction of proteins in the ER. Finally, ATF6 (activating transcription factor 6), after its dissociation to GRP78, translocates into the Golgi apparatus. Here, proteases cleave ATF6 on a juxtamembrane site, causing its release into the cytosol. Once in the cytosol, ATF6 can translocate into the nucleus, where activates the expression of some target genes, included GRP78, PDI, and EDM1 (ER degradation-enhancing α-mannosidase-like protein 1). Collectively, the activation of these genes causes an increased activity of molecular chaperons in the ER and allows misfolded proteins degradation (**369**) (**fig. 15**).

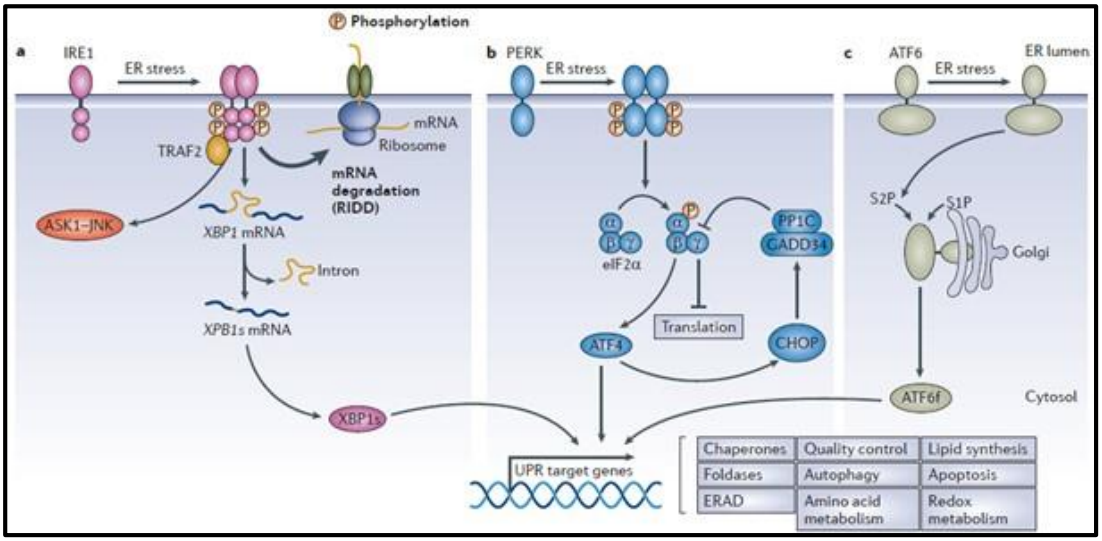

Figure 15. During UPR, IRE1 (a), PERK (b), and ATF6 (c) activate different pathways in order to restore the ability of ER to promote the correct folding of the proteins (Hetz, *et al.*, 2013).

Thus, UPR, in the first phase of ER stress, tries to restore the normal functions of the ER and shows a protective rule. However, whether the stress conditions are not solved and the ER function is serious compromised, apoptosis is activated in order to eliminate damaged cells.

Actually, it is known that almost three different molecular pathways are involved in the apoptotic process: the first pathway requires the activation of the gene coding for CHOP; the second one require caspase-12 activation; finally, the third one induces the expression of JNK kinase (**375**). While the activation of CHOP and caspase-12 are considered specific ER stress events, JNK pathway is activated in several type of cellular stress (**376**) (**fig. 16**).

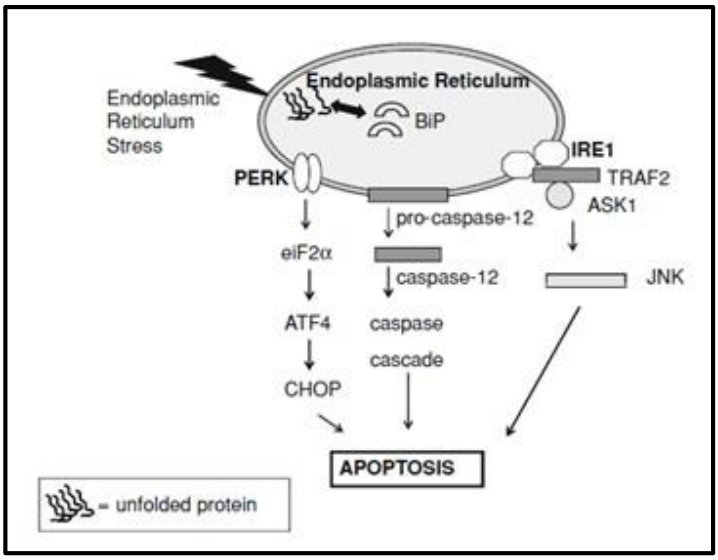

Figure 16. Pro-apoptotic transductional pathways activated by prolonged ER stress conditions (Van der Kallen C.J.H., et al., 2009).

CHOP (C/EBP homologous protein), named also GADD153 (growth arrest and DNA damage inducible protein 153), is a transcription factor of 29 kDa expressed ubiquitously. In physiological condition, CHOP are expressed in the cytosol at very low levels; in ER stress condition, instead, the transcription of the gene coding for CHOP increases and the protein accumulates into the nucleus. It has been demonstrated that PERK/eIF2α pathway plays a key role for the activation of CHOP in ER stress conditions. Indeed, the phosphorylated eIF2α up-regulates the transcription factor ATF4 (activating transcription factor 4), which induces an increase

of CHOP (**374**). Moreover, it has been demonstrated that also ATF6 induces also CHOP transcription; finally, IRE1/ASK1/p38MAPK can increase CHOP activity at post-transcriptional level, after the phosphorylation mediated by p38MAPK. Another CHOP activator is ATF2, a transcription factor induced by hypoxia and required for CHOP expression in absence of aminoacids. The over-expression of CHOP causes cell cycle arrest and induces apoptosis activation; several studies, indeed, demonstrate that CHOP can activate apoptosis by increasing BIM expression and by inhibiting BCL-2. In addition, CHOP promotes the expression of several genes coding for ER proteins, increasing the amount of proteins in the organelle and exacerbating oxidative stress (**368**). In ER stress conditions, the overexpression of GRP78 determines a reduction of CHOP that affects negatively apoptosis. Moreover, in transgenic $CHOP^{-/-}$ mice, ER stress-induced apoptosis was reduced. These results confirm that CHOP plays a key role in the activation of apoptosis during ER stress (**375**).

In mice, the second pathway involved in apoptosis activation requires caspase-12 activation. The caspase-12 is a cysteine-pretease belong to the caspases family that acts as pro-apoptotic proteins. In particular, capsapse-12 is activated in ER stress condition after IREI1 activation (**369**), and, usually, it is not involved in cell death induced by ER-independent stimuli (**377**). In physiological condition, caspase-12 is bound to ER membrane as inactive enzyme associated with GRP78. In ER stress condition, instead, the Ca^{2+} mobility induces calpain activaton, determining cleavage of capsase-12 and its activation. Caspase-7 can activate pro-caspase-12 too, by traslocating from cytosol to ER (**378**). Once activated, caspase-12 induces the activation of downstream caspase-9 and caspase-3, DNA fragmentation, and promotes apoptosis (**374**, **376**). In humans, polymorphism analysis reveal that caspase-12 is not fundamental for apoptosis activation; indeed, in most of men, the gene of caspase-12 is inactivated by nonsense mutations (**369**). However, these men can activate apoptosis, suggesting that caspase-12 is substituted by different caspases activated by ER stress. Moreover, several studies demonstrated that rat caspase-12 has a high sequence homology (about 48% of identity) with human caspase-4, which is involved in ER stress-induced apoptosis (**377**), suggesting that in humans caspase-4 can substitute caspase-12.

Finally, JNK pathway is involved in cell death mechanisms, but this pathway is not specific for ER stress. Anyway, in ER stress condition, JNK, activated by the bound between IRE1 and adapter protein TRAF2 (TNF receptor-associated factor 2),

phosphorylates the pro-apoptotic protein BIM and inhibits the anti-apoptotic protein BCL-2, inducing apoptosis.

I.C.3. ER stress and neurodegenerative diseases

The ER is the first compartment of the secretory pathway associated with protein synthesis, post-translational folding, and modification. Thus, all situation that determine accumulation of misfolded protein in the ER lumen cause ER stress.

ER stress is a pariticular condition that could be involved in the pathogenesis of several neurodegenative and non-neurodegenerative disease or could be conseguence of pathological processes.

Thus, ER stress plays a key role in neurodegenerative disease characterized by misfolded protein accumulation or protein inclusions, such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease (AD), juvenile Parkinson's disease (PD), Huntington's disease (HD), and cerebral ischemia.

In addition, many evidence indicates that ER stress plays a key role also in several non-neurodegenerative diseases, such as myocardial ischemia, cardiac hypertrophy, cardiac failure, arteriosclerosis, diabetes, some cancers and autoimmune disorders (**369**). All these diseases is caused by specific alterations, but, in each one, UPR activation and apoptosis are observed (**table 3**).

Disease	Neurodegeneration	ER stress induced mechanisms	Pharmacological targets
Alzheimer's Disease (AD)	Yes	· The exact mechanisms of ER stress are yet unknown; anyway, in the nervous system, the expressions of proteins involved in the UPR are increased. · Mutated presenilin-1 induces ER stress and affects UPR, reducing IRE1, PERK, and ATF6 activity	Presenilin, PERK-eIF2α
Parkinson's Disease (PD)	Yes	· In physiological conditions, parkin prevents cell death induced by ER stress. · ER stress induce an increase of parkin. · Mutations in parkin are associated with PD.	Parkin, α-sinuclein, and other proteins
Amyotrophic lateral sclerosis (ALS)	Yes	· Mutated SOD1 affect ERAD and activates ASK1.	SOD, ASK1
Huntington's Disease (HD)	Yes	· Polyglutamine expansions induce UPR and inhibit proteasome activity.	Huntingtin
Cerebral ischemia	Yes	· Ischemia induces, in neurons, ER stress, UPR, and apoptosis through CHOP and ASK1 activation	PERK- eIF2α, ASK1
Cardiac diseases	No	· ER stress induced cardiomyocytes degeneration. · Myocardial infarction (heart attack) induces an increase of UPR markers.	ASK1
Atherosclerosis	No	· Oxidated lipids and homocystein induce ER stress in vascular cells	IRE1 pathway
Type 1 diabetes	No	· Alteration in PERK pathway causes type 1 diabetes (Wolcott-Rallison syndrome).	PERK-eIF2α
Type 2 diabetes	No	· Obesity, often associated with type 2 diabetes, induces ER stress dell'ER and promote insulin-resistance.	XBP1, JNK
Cancer	No	· The increase of proteins involved in UPR in cancer cells has a protective effect.	GRP78, XBP1, PERK
Autoimmune disorders	No	· The great amoun of protein in the ER could promote the production of autoantigens. · GRP78 seems to be an autoantigen.	GRP78, HLA-B27, and other proteins

Table 3. Neurodegenerative and non-neurodegenerative diseases associated with ER stress.

I.C.3.1 Amyotrophic Lateral Sclerosis

ER stress seems to be involved in ALS, a disease characterize by several protein inclusions. It is hypothesized, indeed, that protein aggregates could exhaust proteasome activity, resulting in a secondary accumulation of misfolded proteins in the ER. In particular, Turner and Atkin, in 2006, proposed an integration of ER stress, UPR and Golgi apparatus (GA) dysfunctions in mutant SOD1-induced neurotoxicity in ALS.

In this model, secretory pathway is maintained in presence of wt SOD1, which is exported to act in the extracellular space. In ALS, mutated SOD1 interacts with the GA, causing its fragmentation by an unclear mechanism. GA fragmentation, then, results in increased retrograde flow of Golgi-associated membranes and proteins, including mutated SOD1, into the ER. In this way, misfolded SOD1 and impaired retrotranslocation due to proteasomal inhibition may induce aggregation and binding to ER-resident chaperones, such as GRP78. The activation of GRP78, finally, results in UPR and apoptosis. Thus, these pathways may contribute to motor neuron degeneration and experimental manipulation of UPR and ERAD components could help to better understand ER stress role in ALS (**374**) (**fig. 17**).

On the other hand, different evidence indicated that mutated SOD1 is unable to block proteasome activity, but is able to bind derlin-1, which is a component of ERAD. This interaction induces ER stress, because it interferes with ERAD and contributes to motor neurons death (**379**).

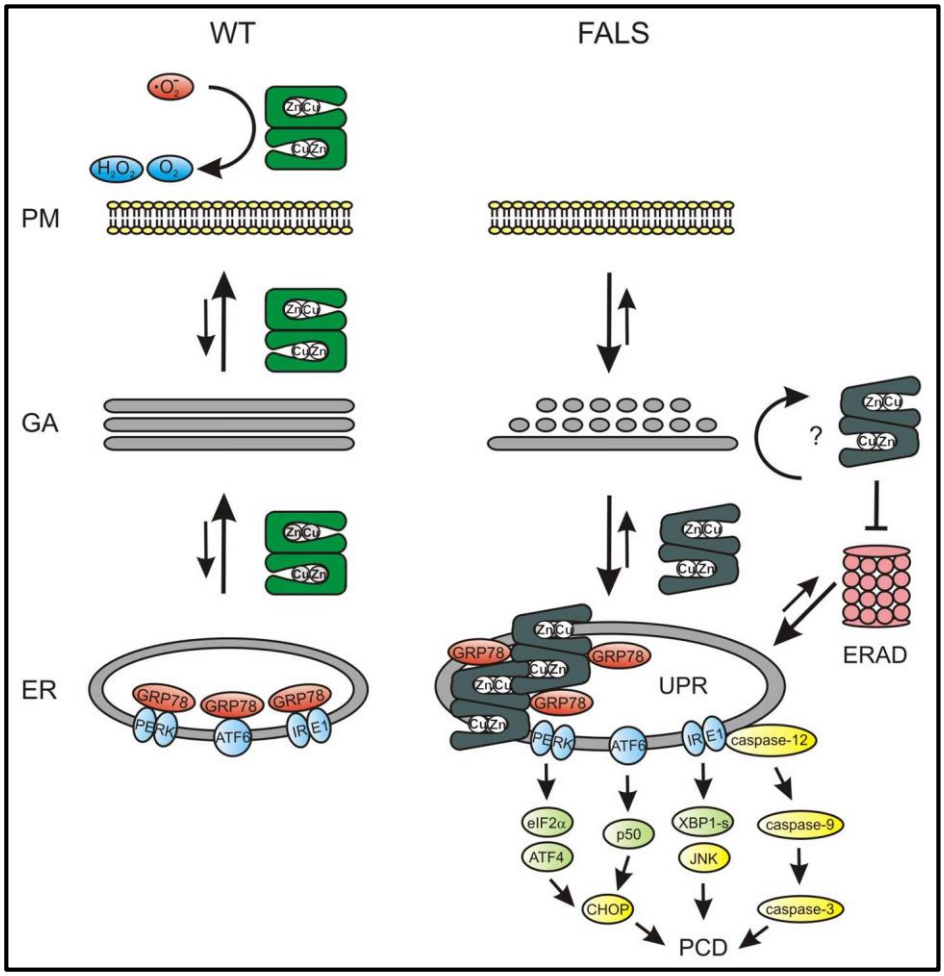

Figure 17. A proposed model for mutant SOD1-mediated disruption of the ER-Golgi system in Fals (Turner B.J., Atkin J.D., 2006)

I.C.3.2. Cerebral ischemia

Ischemic stroke is the third cause of death and a major cause of long-lasting disability in industrially developed countries, only surpassed by heart disease and cancer. It is a pathological condition resulting from occlusion or hemorrhage of blood vessels supplying oxygen and essential nutrients to the brain.

In all cases, stroke ultimately induces death and/or dysfunction of brain cells, as well as neurological impairments that reflect the location and size of the ischemic brain area. Even though a large number of compounds have been proven to reduce ischemic injury in experimental animal models, clinical trials have reported disappointing results because of toxic side effects. Actually, the only FDA (US Food and Drug Administration) approved treatment is to provide tissue plasminogen activator (tPA) to re-open occluded blood vessels. However, due to a narrow time-

window of 4,5 hours after the stroke onset (**380**), this treatment is only appropriate for every small number of patients. Thus, research on the discovery of novel mechanisms and the development of new drugs for treating cerebral ischemia are imperative.

Cerebral ischemic event triggers a set of complex pathological mechanisms, eventually leading to death and/or dysfunction of brain cells, including excitotoxicity, oxidative and nitrosative stress, inflammation, and apoptosis. Each of these mechanisms have a different timing and cause injury to neurons, glia and endothelial cells (**fig. 18**).

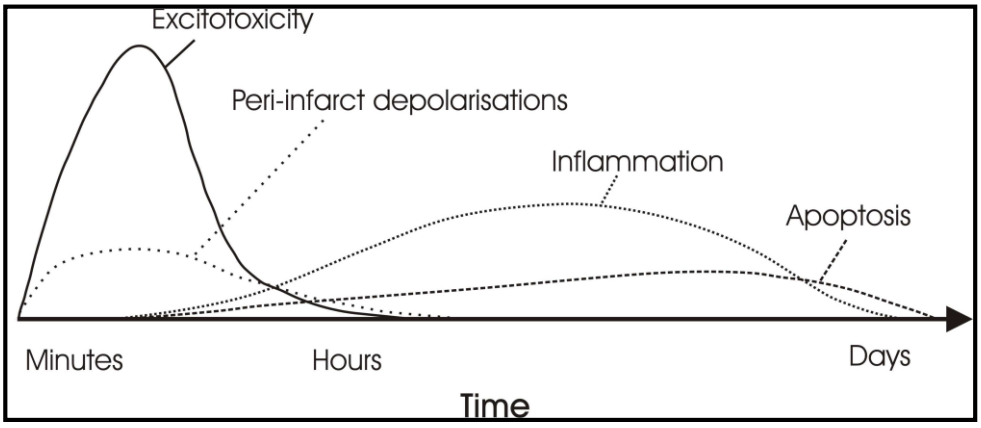

Figure 18. Damaging cascades of events after cerebral ischemia (Dirnagl, *et al*., 1999).

Within the core of the ischemic area, where blood flow is most severely restricted, excitotoxic and necrotic cell death occurs within minutes. Instead, in the periphery of the ischemic area, where collateral blood flow can buffer the full effects of the stroke, the degree of ischemia and the timing of reperfusion determine the outcome for individual cells. In this ischemic penumbra cell death occurs less rapidly via mechanisms such as apoptosis and inflammation (**381**, **382**).

Ischemia determines energy depletion. Thus, after ischemic damage, important ion pumps found on the plasma membrane of neurons, such as Na^{+}/K^{+}-ATPase and CA^{2+}-ATPase, cannot longer function. Thus, in affected neurons, depolarization, release of potassium into the extracellular space and entry of sodium into the cells occur (**383**). Consequently, calcium dependent proteases, lipases and DNases are activated, eventually leading to catabolism and death of many cells in the ischemic

core. In addition, membrane depolarization results in neurotransmitter release, most prominently the release of the excitatory neurotransmitter glutamate, which plays a central role in the pathology of cerebral ischemia (**384**). Increase in synaptic glutamate concentration overactivates its receptors, N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate (mGlu) receptors, which in return cause further membrane depolarization and greater calcium influx, exacerbating excitotoxicity (**385- 387**).

Furthermore, unlike other organs, brain is too vulnerable to reactive oxygen species (ROS), because neurons have low levels of endogenous antioxidants (**388**). Thus, in presence of high levels of intracellular calcium, sodium and ADP, mitochondria produce deleterious high levels of ROS, and consequently, oxygen radicals cause the destruction of cellular macromolecules and participate in signaling mechanisms that result in apoptotic cell death (**389**). Moreover, there is an increased production of superoxide, NO and peroxynitrate, following reperfusion. These free radicals, finally, determine a second oxidative and nitrosative stress, increasing the risk of brain hemorrhage and edema.

Ischemic injury preferentially induces cell death trough an apoptotic-like mechanism rather than necrosis. Because the ischemic penumbra sustains milder injury and preserves ATP, apoptosis predominates in this region (**382**). In ischemia-induced neurons cell death, apoptosis could be activated by the intrinsic or the extrinsic pathways. Indeed, oxygen free radicals, death receptor ligation, DNA damage and protease activation induce cytochrome c release from the outer mitochondrial membrane, an event which is promoted or prevented by Bcl-2 family of proteins, and initiates the intrinsic apoptotic cascade. Instead, extrinsic apoptosis pathway could be activated by inflammatory signals, such as TNF family of ligands (**390**). Eventually, downstream effector caspases are activated targeting the substrates that dismantle the cell by cleaving homeostatic, cytoskeletal, repair, metabolic and cell signaling proteins (**391**).

Finally, inflammation contributes to cerebral ischemic injury. Effects of individual components of the inflammatory cascade, however, can be beneficial depending on the stage of tissue injury, the magnitude of the response and whether the inflammatory components activate neuroprotective pathways (**392-394**). After stroke, immune cells can gain access to brain parenchymal tissue. Infiltration of bone marrow-derived cells into the ischemic brain persists for weeks following stroke, and while the

initial infiltration leads to worsening of tissue damage and exacerbation of neurological deficits, subsequent aspects of the infiltration such as the phagocytosis of debris and the release of cytokines that promote glial scar formation could be crucial for effective wound healing. On the other hand, numerous cytokines and chemokines are produced by activated endothelial cells, microglia, neurons, platelets, leukocytes, and fibroblasts and contribute to ischemic brain injury (**395**, **396**). In particular, IL-1, TNF-α and toll-like receptors (TLRs) are important inflammatory factors with detrimental effects for stroke outcome (**396**-**309**). In contrast, TGF-β was reported to play a neuroprotective role in the pathogenesis of stroke (**400**).

In ischemic neurons, the depletion of calcium ions from the endoplasmic reticulum (ER) has been suggested as an initial signal for ER dysfunction. Many studies indicate that a strong release of calcium ions from ER, due to ion channels or Ca^{2+} transport systems, or through the release of Ca^{2+} ions from intracellular stores, is associated with damage to cells, including damage to neurons after ischemia.

In particular, stimuli such as ischemia, hypoxia, hypertension and hypoglycemia, might trigger the accumulation of unfolded protein in the ER lumen, leading to UPR, which involves expansion of ER membranes, accelerated degradation of unfolded proteins, increased translation of folding chaperones, and inhibition of other protein synthesis (**401**, **402**). Then, reperfusion of tissues determines oxidative stress associated with NO and ROS production, causing alteration of redox reactions, and misfolding of proteins, both situations that exacerbate ER stress (**fig. 19**).

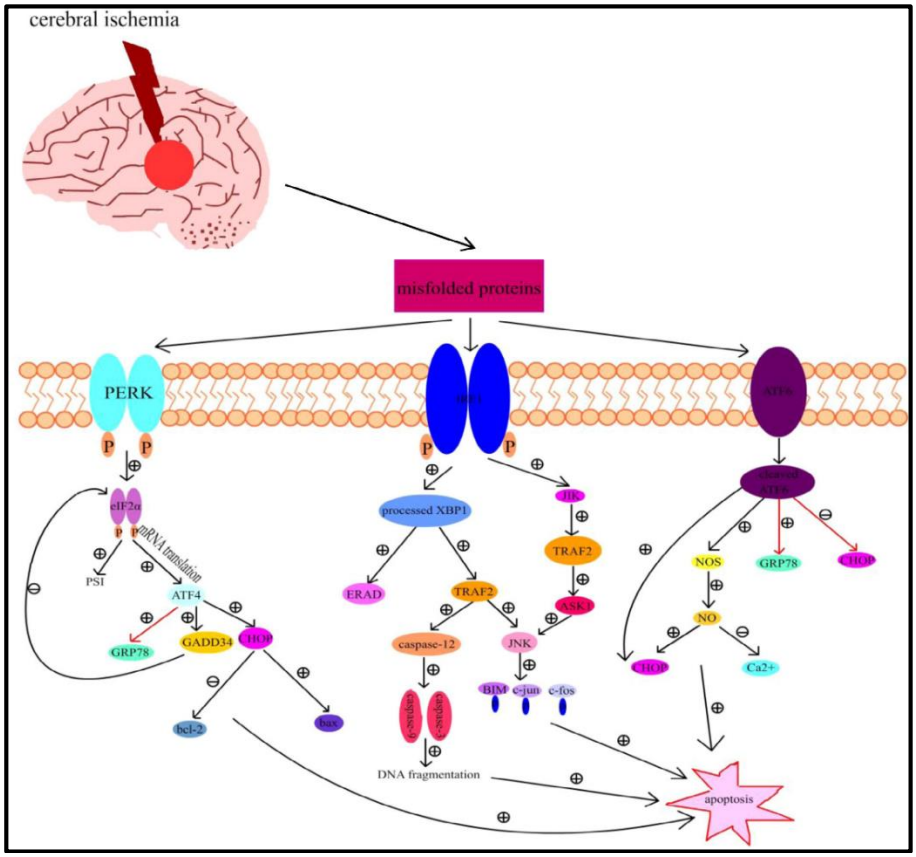

Figure 19. Cerebral ischemia exacerbates ER stress, determining UPR activation and cell death, usually apoptosis.

In mice, for example, ischemia and reperfusion damage activate PERK-eIF2α pathway, resulting in CHOP activation. However, in transgenic CHOP$^{-/-}$ mice, the loss of tissues induced by ischemia are less evident, suggesting that CHOP has a casual role in neuronal cell death induced by ischemic-mediated ER stress. In addition, NO, responsible to tissue damage after ischemia, promotes CHOP expression in primary cultures of neurons and knockout mice for iNOS (inducible nitric oxide synthase, also named NOS2), show a minor activation of CHOP and are less sensitive to cerebral ischemia.

However, ER-stress induced apoptosis plays an important role in the pathophysiology of cerebral ischemia and it is possible that the timing of events for ER stress signaling regulation is important for the balance of life and death, such that ER stress is initially protective, aiming to restore ER homeostasis, whereas prolonged periods of ER stress can be deleterious and damaging. Nevertheless, modulation of ER stress exerts a remarkable protective effect on the ischemic brain and thus may be used as a therapeutic target for ischemic stroke (**376**).

Aim of the study

Aim of the study

Many data demonstrate that SOD1 is secreted in many cellular lines, as human neuroblastoma SK-N-BE cells (**403**) and rat adenohypophysis GH3 cells (**326**), in basal condition and after depolarization mediated by high K^+ concentration.

Moreover, many evidence also shows that SOD1 secretion is compromise in ALS; indeed, mutant SOD1 is not secreted, but accumulates in motor neurons (**366**), best of all in the endoplasmatic reticulum and in Golgi apparatus (**352**). SOD1 aggregates cause mitochondrial dysfunction, ER stress and motor neuron degeneration. Thus, it seems that mutation in SOD1 are associated with a gain of toxic function rather than a loss of function.

In addition, SOD1 administration in $SOD1^{G93A}$ transgenic mice could modify motor symptom of disease (**366**), and, at last, recent evidence showed that SOD1 is released by microglial cells and protect against the toxicity mediated by 6-hydroxy-dopamine (6OHDA) (**404**). However, the molecular mechanism of this neuroprotection is yet unknown.

Furthermore, SOD1 could activate the transductional pathway phospholipase C (PLC)/ protein kinase C (PKC), resulting in an increase of intracellular Ca^{2+} concentration. This activation is independent on dismutase activity, because also ApoSOD, the enzyme without catalytic activity, could activate PLC/PKC pathway, while mimetic drugs, as MnTMPyP, have not the same effect (**325**). Moreover, SOD1 could activate also downstream kinases of PLC/PKC pathway, as p-ERK1/2 and p-Akt (**327**), and drugs, as the insulin-like growth factor 1 (IGF-1), partially prevent motor neuron loss in $SOD1^{G93A}$ transgenic mice through ERK1/2 activation (**405**).

Finally, it is known that endoplasmic reticulum stress plays a key role in neurodegenerative disorders, such as Alzheimer's disease or Parkinson's disease. In the last years, it has been demonstrated that ER stress and the unfolded protein response (UPR) are involved in the pathogenesis of mutant SOD1 linked-ALS (**374**).

Thus, the main aim of this study was to analyze the eventual neuroprotective effects exerted by SOD1 and the molecular mechanism underlies this neuroprotection.

Because SOD1 is released in many cellular lines, firstly SOD1 release in motor neurons has been studied, together with its ability to induce the activation of p-ERK1/2 and p-Akt prosurvival pathways.

Then, the eventual neuroprotective effects exerted by SOD1 in two different neurotoxic models have been studied. In particular, motor neurons will exposed to L-BMAA, a neurotoxin associated with ALS/PDC, in order to obtain an *in vitro* model mimicking ALS. Moreover, motor neurons will also exposed to chemical hypoxia, in order to reproduce *in vitro* a Ca^{2+}- and ROS-dependent model of neurodegeneration.

Because SOD1 is an antioxidant enzyme able to induce ERK1/2 and Akt phosphorylation, in the second part of our study, the ability of SOD1 to exert neuroprotective effects independently from its catalytic activity will be tested together with its transdctional actions. To this aim, the effects exerted by SOD1 will compared to the effects exerted by two different drugs: ApoSOD, the enzyme lacking catalytic activity, and MnTMPyP pentachloride, a SOD mimetic drug.

In addition, because more than 150 mutations in the gene coding for SOD1 associated with ALS are described, the effects exerted by SOD1 will be compared to those exerted by two different recombinant proteins: the recombinant wild type SOD1, and the recombinant $SOD1^{G93A}$. Indeed, the second recombinant protein shows one of the best-characterized mutations associated with ALS.

Finally, in order to better characterize the neuroprotection mediated by SOD1, the ability of SOD1 to prevent ER stress in motor neurons exposed to L-BMAA or to chemical hypoxia will be tested .

Material and Methods

III.1. Reagents

Media and sera for cell culture were purchased from Invitrogen (Milan, Italy); antibiotics for cell culture were from Sigma-Aldrich (St. Louis, MO, USA).

Rabbit polyclonal antibody against SOD1, mouse monoclonal antibody against p-ERK1/2, rabbit polyclonal antibody against ERK1/2, and mouse monoclonal antibody against neuronal nitric oxide synthase (n-NOS) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal p-Akt antibody, mouse monoclonal caspase-3 antibody, mouse monoclonal caspase-12 antibody, mouse monoclonal CHOP antibody, and rabbit polyclonal GRP78 antibody were from Cell Signaling Technology Inc. (Danvers, MA, USA). Mouse monoclonal SMI-32 antibody was from Covance-Signet (CA, USA). Mouse monoclonal antibody against the axonal growth-associated protein-43 (GAP-43), and mouse monoclonal β-tubulin antibody was from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting, ECL reagents, and chemicals were from GE Healthcare (Milan, Italy).

The inactive mutant HA-Aktk179M (Akt D-) plasmids was donated by P. Formisano (University of Naples "Federico II", Naples, Italy), as described by Eves et al., 1998 and Trencia et al., 2003. siRNA-MEK1 and siRNA-CONTROL were purchased from Dharmacon RNA Technologies (Thema Ricerca, Italy).

SOD1, retinoic acid, L-BMAA, oligomycin, 2-deoxy-glucose, EGTA (ethylene glycol tetraacetic acid), ω-conotoxin, L-nimodipin, H_2O_2, LY294002, PD98059, fluorescein, propidium iodure, and all other reagents were from Sigma (Milan, Italy). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was purchased from Calbiochem (San Diego, CA, USA). MnTMPyP pentachloride was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(21-amino-51-methylphenoxy)-ethane-*N*,*N*,*N*1,*N*1-tetra-acetic acid penta-acetoxymethyl ester (fura-2AM) was from Molecular Probes (Invitrogen Italia, Milan, Italy).

III.2. Cell culture

Mouse motor neuronal NSC-34 cells are a hybrid mouse-mouse NSC cell line (neuroblastoma and spinal cord) that resembles motor neurons; indeed, these cells display a multipolar neuron-like phenotype, express choline acetyltransferase and neurofilament triplet proteins, and generate action potentials (**243**). Actually, these cells are considered the best stable motor neuronal cell line model system available.

NSC-34 cells were grown in monolayer in Dulbecco's Modified Eagles Medium (DMEM) containing 4.5 g/L glucose, supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. The cells were kept in a 5% CO_2 and 95% air atmosphere at 37° C.

Before each experiment, NSC-34 cells were differentiated in 10 µM retinoic acid for 48 h.

NSC-34 cells were stably transfected with pTet-ON plasmid, coding for the reverse tetracycline-controlled transactivator, to obtain the line designed NSC-34-ON7, which displays a very low level of basal expression and high inducibility. NSC-34-ON7 was used to construct inducible cell lines expressing the cDNAs encoding human wild type (wt) SOD1 or the human $SOD1^{G93A}$ inserted in the pTRE2 plasmids (**406**). These cells were grown in DMEM supplemented with 10% tetracycline-free FBS, 2 mM L-glutamine, 100 µg/mL hygromicin B and 100 µg/mL geneticin in an atmosphere of 5% CO2 and 95 % air atmosphere at 37° C.

Before each experiment, the induction of human wt SOD1 or $SOD1^{G93A}$ expression was obtained by adding 1 µg⁄mL doxycycline to the culture medium for 48 h. Moreover, NSC-34 $SOD1^{G93A}$ motor neurons were, at the same time, differentiated in 10 µM retinoic acid for 48 h.

III.3. Motor neuron-enriched cultures

Motor neuron-enriched cultures were prepared from spinal cord of 12-14-days-old C57BL/6 wild type mouse embryos. Briefly, mice were anesthetized and then sacrificed by cervical dislocation euthanasia, and spinal cords were isolated from each embryo.

Dissection and dissociation were performed in $MgSO_4$ phosphate-buffered saline (PBS) containing penicillin and streptomycin. Tissues were dissociated by trituration and centrifuged for 2 min at 1000 rpm. Then, tissues were incubated with trypsin for 20 min in a 37° C water bath, agitating. After that, tissues were treated with $MgSO_4$-PBS containing penicillin and streptomycin, DNAse and trypsin. After a centrifugation for 2 min at 1000 rpm, tissues were incubated again with DNAse and trypsin in $MgSO_4$-PBS containing penicillin and streptomycin and then cells were separated by density gradient. Finally, cells were centrifuged for 10 min at 1000 rpm and plated at 8 x 10^4 in glass coverslip pre-coated with poly-D-lysine (20 µg/mL) in Neuron Basal Medium (Gibco, Life Technologies), supplemented with B27 and 2 mM L-glutamine.

Motor neurons were cultured at 37° C in a humidified 5% CO_2 atmosphere and used after 12 days of cultures.

All the experiments on primary motor neurons were performed according to the procedures described in experimental protocols approved by the Ethical Committee of the 'Federico II' University of Naples.

III.4. Immunocytochemistry and confocal image

Mouse monoclonal SMI-32 antibody (Covance-Signet, CA, USA) was used for immunocytochemical experiments, useful to characterize differentiated NSC-34 motor neurons and primary motor neurons.

NSC-34 cells were differentiated in 10 µM retinoic acid for 48 h; primary motor neurons, instead, were cultured on glass coverslips for 12 days. Then, the cells were rinsed twice in cold 0.01 M saline phosphate buffer at pH 7.4 (PBS) and fixed in 4% (w/v) paraformaldehyde (Sigma, Milan, Italy) for 20 min at room temperature (RT). Following three washes in PBS, cells were blocked with 3% (w/v) bovine serum albumin (BSA) and 0.05% Tryton-X (Biorad, Milan, Italy) for 1 h at room temperature (RT). The coverslip were then incubated with the primary antibody anti-SMI-32 (1:1000 diluition) at 4°C overnight. Following three washes in PBS, the coverslips were incubated in the dark with the secondary antibody, Alexa-fluor 488 anti-mouse IgG (Molecular Probes, Eugene, OR; diluition 1:200) for 1 h at RT. After the final wash, the coverslips and the cells were mounted with Vectashield (Vector Labs,

Burlingame, CA) and analyzed with a Nikon Eclipse 400 upright microscope (Nikon Instruments, Florence, Italy), equipped with a CCD digital camera (Coolsnap-Pro, Media Cybernetics, Silver Springs, MD, USA) and Image Pro-Plus software (Media Cybernetics, Silver Springs, MD, USA).

III.5. $[Ca^{2+}]_i$ measurements

[Ca2+]i was measured by single cell computer-assisted video-imaging (**407**) Briefly, NSC-34 motor neurons grown on glass coverslips were loaded with 6 µM Fura-2 acetoxymethyl ester (Fura-2/AM) for 1 h at RT in normal Krebs solution containing the following (in mM): 5.5 KCl, 160 NaCl, 1.2 MgCl2, 1.5 CaCl2, 10 glucose, and 10 Hepes–NaOH, pH 7.4. At the end of the Fura-2/AM loading period, the coverslips were placed into a perfusion chamber (Medical System, Co. Greenvale, NY, USA) mounted onto a Zeiss Axiovert 200 microscope (Carl Zeiss, Germany) equipped with a FLUAR 40X oil objective lens. The experiments were carried out with a digital imaging system composed of MicroMax 512BFT cooled CCD camera (Princeton Instruments, Trenton, NJ, USA), LAMBDA 10-2 filter wheeler (Sutter Instruments, Novato, CA, USA), and Meta-Morph/MetaFluor Imaging System software (Universal Imaging,West Chester, PA, USA). After loading, cells were alternatively illuminated at wavelengths of 340 and 380 nm by a Xenon lamp. The emitted light was passed through a 512 nm barrier filter. Fura-2 fluorescence intensitywas measured every 3''. All the results are presented as cytosolic Ca^{2+} concentration. Assuming that the KD for FURA-2 was 224 nM, the equation of Grynkiewicz *et al*. (1985) (**408**), whose parameters were determined for individual cells as described by Urbanczyk *et al*. (2006) (**409**) was used for calibration.

III.6. NSC-34 motor neurons depolarization

After differentiation in 10 µM retinoic acid for 48 h, NSC-34 motor neurons were exposed to a Normal Krebs solution, containing, in mM, 5.5 KCl, 100 NaCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose and 10 Hepes-KOH pH 7.4. or to a Krebs solution at different K^+ concentration (9 or 55 mM) for 5, 10 e 30 min.

The depolarization mediated by 55 mM K^+ was also evaluated in presence of 50 nM ω-conotoxin (inhibitor of neuronal (N) calcium channels), 1 µM L-nimodipine (inhibitor of long-lasting (L) calcium channels) or 1 mM EGTA (extracellular calcium chelator) for 10 min.

At the end, supernatants and cells were collected separately and analyzed by Western blotting.

III.7. Cell transfection and RNA interference

Transfection of NSC-34 motor neurons was carried out using HyPerFect Transfection Reagent. Small interfering RNA (siRNA) or plasmids were added to HyPerFect Transfection Reagent for 15 min at room temperature, and then the mixture was added to cells plated on 60-mm-diameter plastic dish or on multiple well cluster plate (6 wells) in Optimem medium. After 5 h of incubation, the medium was replaced with fresh medium.

For the silencing, 10 µM siRNA against MEK1 (mitogen-activated protein kinase kinase 1) was used (siMEK1); for the transfection with the dominant negative form of Akt (Akt D-), 2 µg/µL plasmid was used.

III.8. L-BMAA treatment

NSC-34 motor neurons were plated in multiple well cluster plate (6 wells; density: $5x10^5$ cells) and differentiated, as previous described. At the end of differentiation, the culture medium was replaced with DMEM supplemented with 0.2 % FBS, 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin, and the cells were treated with 300 µM *L*-β-methylaminoalanine (L-BMAA) for 48 h in absence or in presence of 400 ng/mL SOD1 (pre-incubated for 10 min).

NSC-34 motor neurons were also pre-incubated with 400 ng/mL ApoSOD or with 400 ng/mL MnTMPyP pentachloride for 10 min and the exposed to 300 µM L-BMAA for 48 h.

At the end of all pharmacological (50 µM PD98059 for 10 min and 10 µM LY294002 for 30 min) and molecular (10 µM siMEK1 and 2 µg/µL Akt D-) treatments, cell viability was measured by MTT assay.

III.9. Chemical Hypoxia

Chemical hypoxia was reproduced by adding to the cells, for 45 to 180 min, 5 µg/ml oligomycin (a oxidative phosphorylation inhibitor) plus 2 mM 2-deoxyglucose (a glycolis inhibitor) in glucose-free medium composed of 145 mM NaCl, 5.5 mM KCl, 1.2 mM MgCl2, 1.5 mM CaCl2, and 10 HEPES, pH 7.4, as described previously (**410**). Control cells were exposed for the same amount of time to Normal Krebs solution composed of 145 mM NaCl, 5.5 mM KCl, 1.2 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, and 10 mM HEPES, pH 7.4.

NSC-34 motor neurons were pre-incubated with 400 ng/mL SOD1, 400 ng/mL ApoSOD or with 400 ng/mL MnTMPyP pentachloride for 10 min and then exposed to chemical hypoxia for 45 min.

At the end of all pharmacological (50 µM PD98059 for 10 min and 10 µM LY294002 for 30 min) and molecular (10 µM siMEK1 and 2 µg/µL Akt D-) treatments, cell viability was measured by MTT assay.

III.10. Western blotting analysis

After treatments, cells and supernatants were washed in phosphate-buffered saline (PBS) and lysed by gentle scraping in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% NONIDET P-40, 1 Mm Na_3VO_4, 0.1% aprotinin, 0.7 mg/mL pepstatin and 1 µg/mL leupeptin. Protein concentration was determined by Bradford method (**411**).

For SOD1 expression, proteins (50 µg) or supernatants (60 µL) were first separated on 12% SDS-polyacrylamide gels and transferred onto Hybond ECL nitrocellulose membranes (Amersham). Membranes were blocked with 5 % nonfat dry milk in 0.1 % Tween 20 (Sigma Aldrich) (2 mM Tris-HCl and 50 mM NaCl, pH 7.5) for 2 h at room temperature, and then they were incubated overnight at 4° C in the

blocking buffer with the 1:1000 polyclonal antibody against SOD1 (Santa Cruz Biotecnology, Inc.).

For GAP-43, nNOS, p-ERK1/2, p-Akt, GRP78, CHOP, caspase-3 and caspase-12 expression, protein (50 µg) were separated on 10% SDS-polyacrylamide gels and transferred onto Hybond ECL nitrocellulose membranes (Amersham). Membranes were blocked with 5 % nonfat dry milk in 0.1 % Tween 20 (Sigma Aldrich) (2 mM Tris-HCl and 50 mM NaCl, pH 7.5) for 2 h at room temperature, and then they were incubated overnight at 4° C in the blocking buffer containing either:

- monoclonal antibody (1:1000) against GAP-43 (Sigma-Aldrich (St. Louis, MO, USA);
- monoclonal antibody (1:1000) against nNOS (Santa Cruz Biotecnology, Inc.);
- monoclonal antibody (1:1000) against p-ERK1/2 (Santa Cruz Biotecnology, Inc.);
- monoclonal antibody (1:1000) against p-Akt (Cell Signaling Technology Inc., Danvers, MA);
- rabbit polyclonal (1:1000) against GRP78 (Cell Signaling Technology Inc., Danvers, MA);
- monoclonal antibody (1:1000) against CHOP (Cell Signaling Technology Inc., Danvers, MA);

All membranes were also incubated in the blocking buffer with the 1:1000 monoclonal antibody against β-tubulin (Sigma-Aldrich).

Finally, after the incubation with primary antibodies, membranes were washed with 0.1% Tween 20, followed by incubation with secondary antibodies for 1 h at room temperature. Immunoreactive bands were detected with the ECL reagent (Amersham). The optical density of the bands (normalized to β-tubulin) was determined by Chemi-Doc Imaging System (Bio-Rad, Hercules, CA).

III.11. Determination of cell viability

III.11.1. MTT assay

Cell viability was evaluated by the MTT assay (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), which measures mitochondrial activity. In this

test, the dye MTT is metabolized by viable mitochondria to a colored product than can be detected using a spectrophotometer at a wavelength of 540 nm.

After toxic treatment, 1 mL MTT were added to each well for 1 h at 37° C; then, 1 mL of dymethil sulfoxide (DMSO) was added for complete solubilisation of the purple formazan crystal and the absorbance was measured at 540 nm. Data obtained were expressed as a percentage of cell viability of control cultures.

III.11.2. Cell death

Cell death was evaluated by measuring the ratio between dead and living cells. To quantify cell death after the experimental procedures, primary motor neurons were washed with normal Krebs and double stained with 36 µM fluorescein diacetate (FDA) and 7 µM propidium iodide (PI) for 20 min at 37 °C in a phosphate buffer solution. Stained cells were examined immediately with a standard inverse fluorescence microscope at 480 and 546 nm (**410**). PI- and FDA-positive cells were counted in three representative high power fields of independent cultures and cell death was determined by the ratio of the number of PI positive cells/PI + FDA-stained positive cells

III.12. ROS production determination

DCFH-DA (2',7'-dichlorfluorescein-diacetate), a cell membrane-permeable fluorescein analogue, was used to detect ROS production (**412**) in differentiated NSC-34 motor neurons. The cells were exposed to 300 µM L-BMAA for 48 h or to chemical hypoxia for 45 min and then were loaded with 10 µM DCFH-DA for 30 min at 37° C in PBS. At the end, the cells were washed with PBS, and the reaction was stopped by addition of 2,6-di-tert-butyl-4-methylphenol (0.2% in ethanol) and 2 mM EDTA. Finally, cells were viewed with a Zeiss fluorescence microscope (Axioscope 2FS plus; Zeiss, Goettingen, Germany) using excitation and emission wavelengths of 488 and 525 nm respectively. Digital images were taken with a CoolSnap camera and analysed with Image-Pro Plus 4.5 software. Image acquisition and processing was performed equally for all experimental conditions; for quantification of results, background fluorescence was subtracted from the data.

III.13. SOD1 inactivation and measurement of SOD activity

SOD1 was incubated with H_2O_2 (200 mM) in 25 mM sodium bicarbonate buffer (pH 7.5) for 5, 30, 60, and 120 min at room temperature. The reaction was then stopped by adding catalase (1000 U/mL) for 30 min at 37° C.

Finally, SOD1 activity was measured by SOD assay kit, purchased from Sigma. This kit uses Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD; therefore, the IC50 (50 % inhibition activity of SOD) can be determined by a colorimetric method: the absorbance at 440 nm is proportional to the amount of superoxide anion and the SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 440 nm.

III.14. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical comparisons between controls and treated experimental groups were performed using the one-way ANOVA, followed by Newman Keul's test. $P < 0.05$ was considered statistically significant.

Results

IV.1. Characterization of NSC-34 differentiation

Motor neuronal NSC-34 cells are a hybrid mouse-mouse cell line obtained from spinal cord and neuroblastoma cells that resembles motor neurons; indeed, these cells display a multipolar neuron-like phenotype, express choline acetyltransferase and neurofilament triplet proteins, and generate action potentials (**243**).

Because only differentiated cells show neuritic processes, and axons, NSC-34 cells were differentiated by the addition of 10 μM retinoic acid for 48 h and used in all experimental paradigms described above.

Fig. 20A depicts NSC-34 cells before (wt NSC-34) and after differentiation (differentiated NSC-34) with 10 μM retinoic acid. In particular, undifferentiated cells showed a rounded morphology, while, after differentiation, showed long neuritic processes with a small rounded cell body. Moreover, in **fig. 20B**, immunocytochemical experiments showed a high expression of non-phosphorylated neurofilament protein SMI-32, a well know marker of motor neuron that marks cytoskeleton and axons.

To better characterize the process of differentiation in NSC-34 motor neurons, the expression of some neuronal markers was analyzed in both differentiated and undifferentiated cells. We focused our attention on GAP-43 and nNOS, two well known markers of neuronal differentiation. In particular, in 1992, Cashman *et al*. demonstrated that NSC-34 cells express GAP-43 (growth associated protein 43), a protein found at high levels in neuronal growth cones during development and axonal regeneration. Thus, the expression of GAP-43 and nNOS (neuronal nitric oxide sinthetase) was evaluated in NSC-34 cells before and after treatment in 10 μM retinoic acid (**fig. 20C** and **20D**). Both proteins were expressed in NSC-34 motor neurons, but their levels are significantly higher in differentiated than in undifferentiated cells (**fig. 20C – GAP-43**, and **20D - nNOS**).

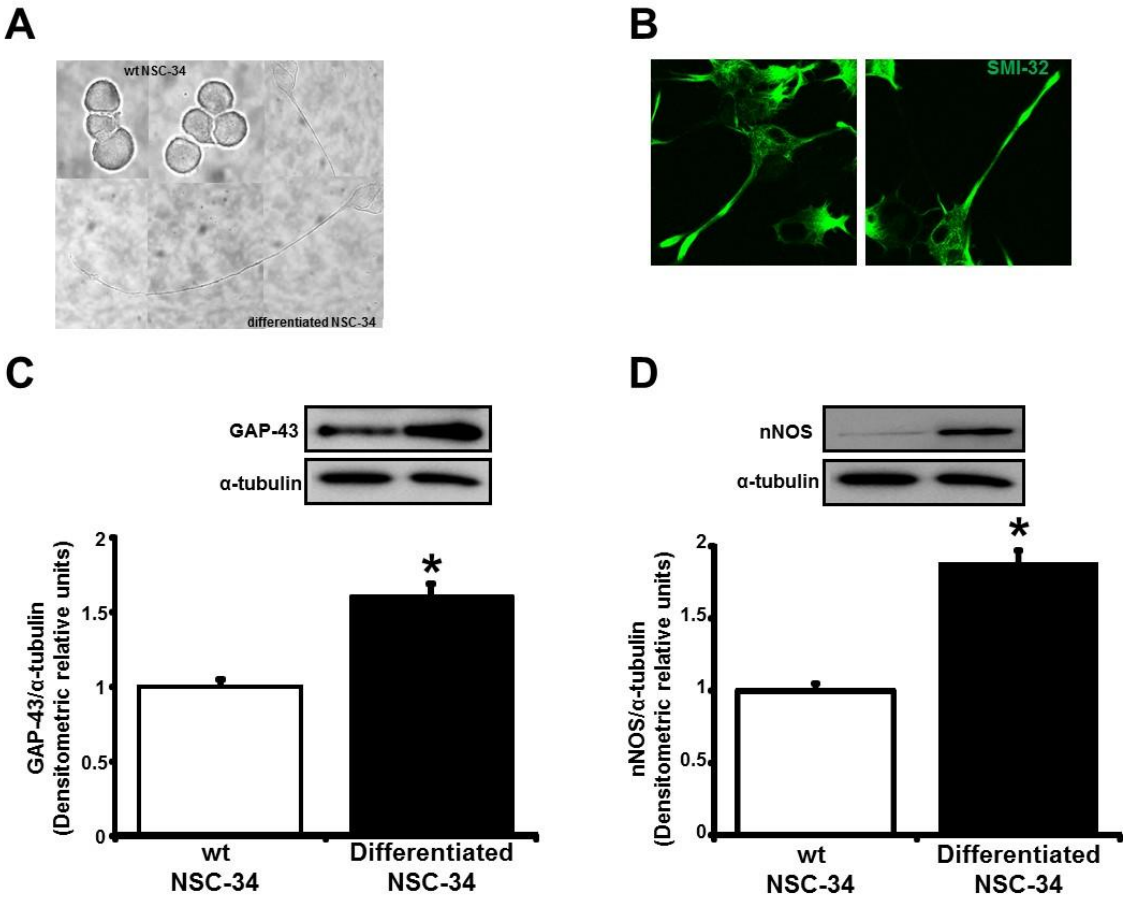

Figure 20. Morphological and biochemical characterization of NSC-34 cells.

A. NSC-34 motor neurons are represented before and after differentiation with 10 µM retinoic acid for 48 h.

B. Immunocytochemical analysis of SMI-32 in NSC-34 motor neurons after differentiation with 10 µM retinoic acid for 48 h.

C/D. Western blotting analysis of GAP-43 (**C**) and nNOS (**D**) expression levels in NSC-34 motor neurons before (in white) and after (in black) differentiation with 10 µM retinoic acid for 48 h.

*$p<0.05$ vs control (wt NSC-34).

IV.2. Characterization of SOD1 release from differentiated NSC-34 motor neurons

SOD1 is secreted in many cellular lines under basal condition and after depolarization mediated by high extracellular K^+ concentration (**322**, **325**). Thus, we verified the ability of motor neurons to release SOD1.

Our results showed that SOD1 was secreted in NSC-34 motor neurons under basal condition and after depolarization. In particular, SOD1 secretion was dependent on extracellular K^+ concentration, since it was secreted when NSC-34 cells were exposed to 9 and 55 mM K^+; indeed, just low K^+ concentration (5.5 mM) induced basal release of SOD1in NSC-34 motor neurons (**Fig. 21A**).

Moreover, the molecular mechanism underlying SOD1 secretion is an ATP-dependent process (**322**) and requires SNARE complex activation (**325**). To verify whether this secretion was Ca^{2+}-dependent, NSC-34 motor neurons were incubated in a solution contained low (5.5 mM) or high (55 mM) K^+ concentration in the presence of 50 nM ω-conotoxin, a well known inhibitor of neuronal voltage-dependent calcium channel, 1 μM L-nimodipine, an inhibitor of long-lasting voltage-dependent calcium channel, and the extracellular calcium chelator EGTA (ethylene glycol tetraacetic acid, 1 mM).

Our results showed that SOD1 extracellular levels strongly decreased after treatment with ω-conotoxin and L-nimodipine compared with SOD1 levels detectable after 5 min in low or high K^+ alone. Furthermore, EGTA induces a strong reduction of SOD1 secretion (**Fig. 21B**).

Then, time-course experiments revealed that SOD1 basal secretion increased gradually in the first 10 min of incubation in a low K^+ solution, while protein levels decreased after 30 min of treatment (**Fig. 21C – 5.5 mM K^+**). Five min exposure to high K^+ induced, instead, an increased secretion of SOD1 that, in turn, gradually decreased (**Fig. 21C – 55 mM K^+**). In this respect, we observed that, while extracellular SOD1 levels decreased after 30 min of depolarization mediated by high K^+ concentration, intracellular SOD1 levels notably increased (**fig. 21D – 55 mM K^+**).

Therefore, our results suggest that the release of SOD1 corresponded to a significant decrease of intracellular protein, and *vicecersa*.

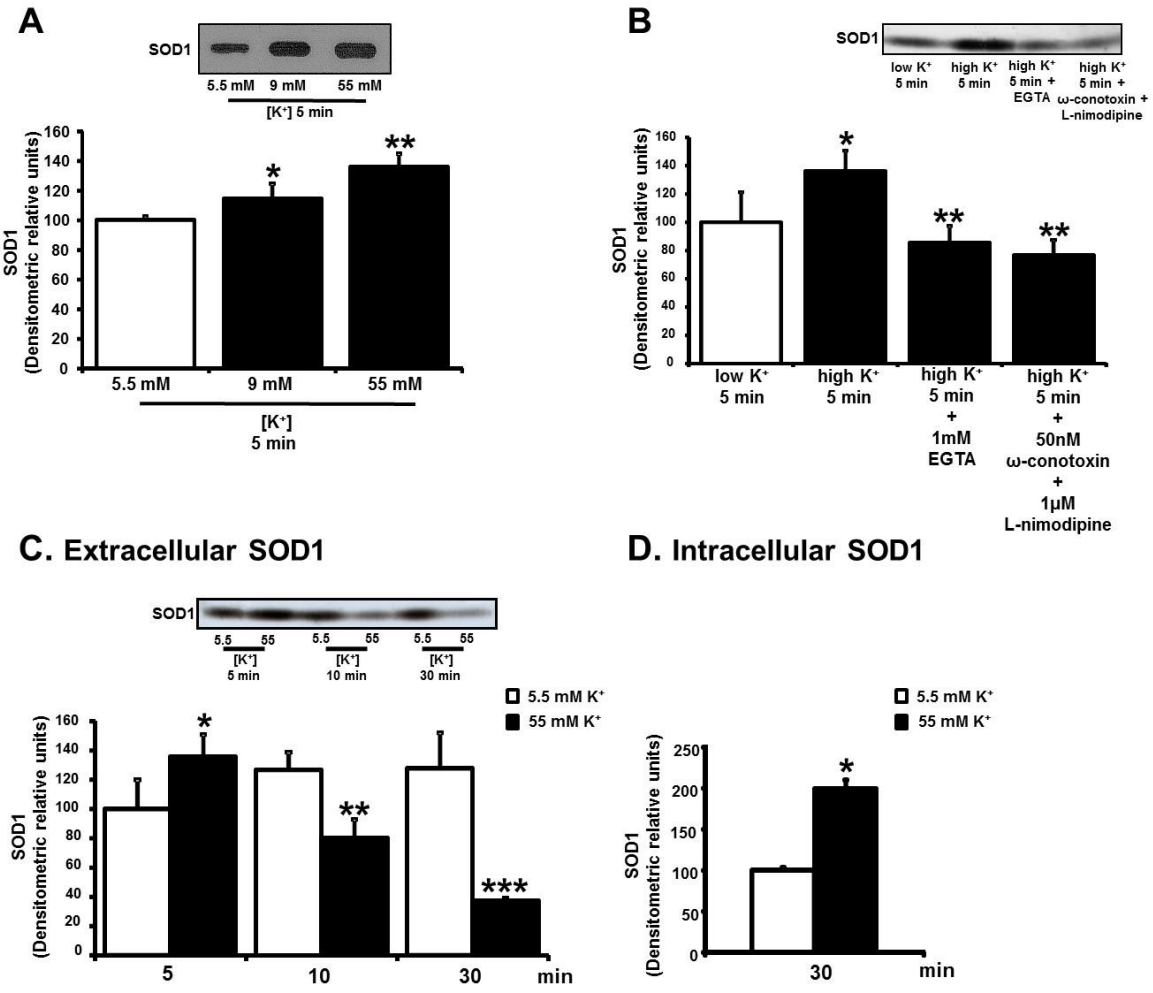

Figure 21. SOD1 release in NSC-34 motor neurons.

A. Western blotting analysis of SOD1 extracellular levels induced by 5.5 mM, 9 mM, and 55 mM K^+.

B. Western blotting analysis of SOD1 extracellular levels induced by 55 mM K^+ in presence of 1 mM EGTA, or 50 nM ω-conotoxin and 1 μM L-nimodipine.

C. Time-course experiment of SOD1 release induced by 5.5 mM (in white) or 55 mM K^+ (in black).

D. Analysis of SOD1 intracellular levels after exposure to 5.5 mM (in white) or 55 mM K^+ (in black).

*p<0.05 vs control (5.5 mM K^+);

**p<0.05 vs control (5.5 mM K^+);

***p<0.05 vs control (5.5 mM K^+).

IV.3. Effect of SOD1 on $[Ca^{2+}]_i$ and phosphorylation of ERK1/2 and Akt in differentiated NSC-34 motor neurons

SOD1 activates the transductional pathway phospholipase C (PLC)/ protein kinase C (PKC), and increases intracellular Ca^{2+} concentration ([Ca2+]i) (**403**). This pathway activates, in turn, downstream kinases, such as ERK1/2 (extracellular signal regulated kinase 1/2) and Akt (**327**).

In order to verify the transductional effect of SOD1 in motor neurons, we first analyzed the effect of SOD1 on $[Ca^{2+}]_i$. Thus, we observed that the administration of 400 ng/mL SOD1 induces a strong and fast increase of intracellular Ca^{2+} concentration (**Fig. 22A**). This effect was observed both in the cellular body (*soma*) and in the dendrites (**Fig. 22B**). In particular, $[Ca^{2+}]$i increase induced by SOD1 involved two different type of voltage-dependent calcium channels: indeed, in the presence of L-nimodipine and ω-conotoxin, SOD1-induced $[Ca^{2+}]$i increase was reduced (**Fig. 22B**).

Then, we verified the ability of SOD1 to induce ERK1/2 and Akt phosphorylation in NSC-34 motor neurons. To this aim, cells were pre-incubated with 400 ng/mL SOD1 for 10 and 30 min and cellular lysates were finally analyzed by Western blotting.

Our results showed that, just after 10 minutes of incubation with SOD1, p-ERK1/2 and p-Akt levels significantly increased (**Fig. 22C - D**).

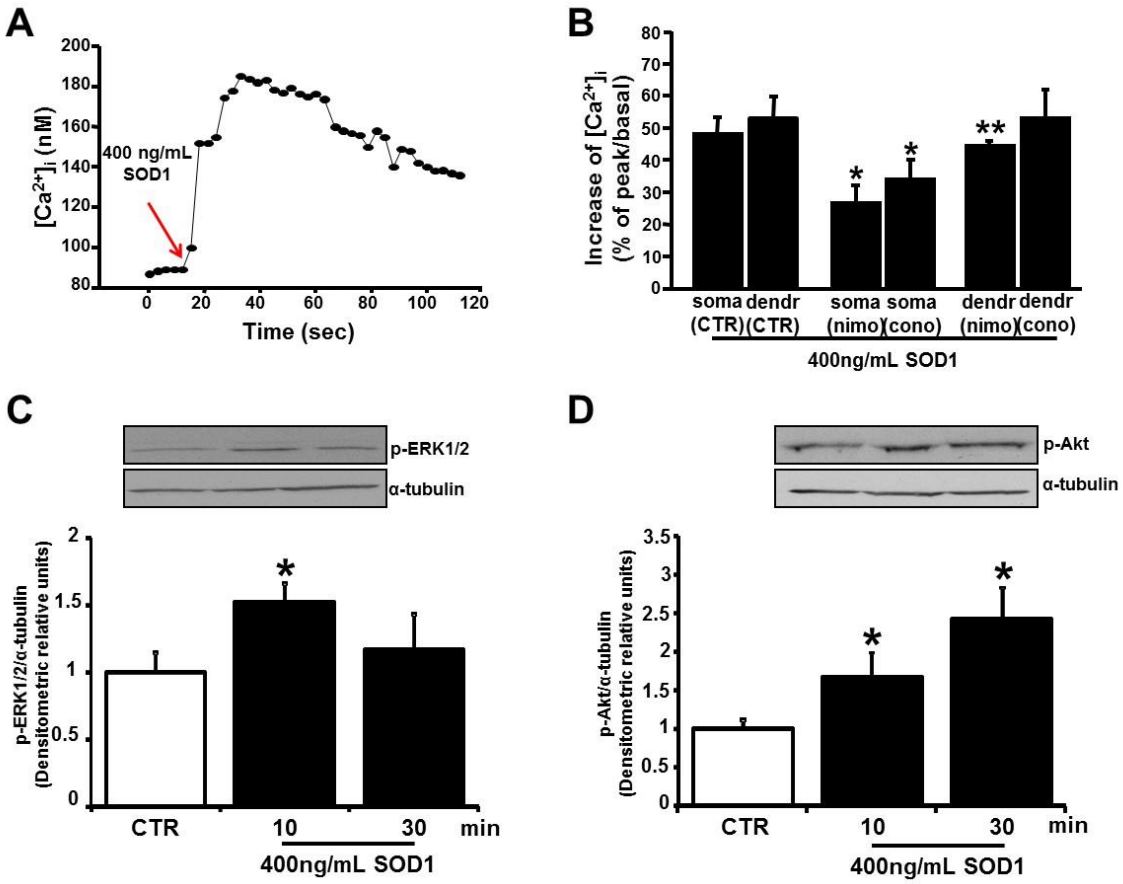

Figure 22. SOD1 transductional effects in NSC-34 motor neurons.

A. Representative trace of the effects on $[Ca^{2+}]_i$ of 400 ng/mL SOD1 on single-cell.
B. Quantification of the effects on $[Ca^{2+}]_i$ of 400 ng/mL SOD1 in presence of 50 nM ω-conotoxin or 1 µM L-nimodipine in both *soma* and dendrites of NSC-34 motor neurons.
C/D. Western blotting analysis of p-ERK1/2 (**C**) and p-Akt (**D**) expression levels induced by 400 ng/mL SOD1, pre-incubated for 10 min or 30 min.
*p<0.05 vs control (CTR);
**p<0.05 vs control (CTR).

IV.4. Effect of SOD1 on cell death induced by L-BMAA in differentiated NSC-34 motor neurons

L-BMAA is a neurotoxic non-proteic amino acid produced by cyanobacteria and associated with amyotrophic lateral sclerosis-Parkinson dementia complex (ALS-PDC), a neurological disease that affects an indigenous population in Guam. Thus, NSC-34 motor neurons exposed to L-BMAA represent an *in vitro* model mimicking ALS, useful, in this study, to verify SOD1-mediated neuroprotection.

Our previous researches indicated that L-BMAA has an EC_{50} of 300 µM and time course experiments revealed that the exposure to L-BMAA for 48 h reduces cell viability of about 50%. Thus, NSC-34 motor neurons were differentiated in 10 µM retinoic acid for 48 h and, then, incubated with 300 µM L-BMAA for 48 h. In order to verify whether SOD1 showed neuroprotective effects against L-BMAA, we performed a dose-response experiments. To this aim, cells were pre-incubated with 40, 400 or 4000 ng/mL SOD1 for 10 min before exposure to the toxin (300 µM). At the end of treatment, cell viability was determined by MTT assay.

Our results showed that SOD1 prevents cell death induced by L-BMAA in a dose-dependent manner (**fig. 23A**); indeed, a strong recovery of cell viability was observed with 400 ng/mL SOD1; thus, we used this concentration in the next experiments. However, also the pre-incubation with the lowest concentration of 40 ng/mL prevented cell death.

Moreover, we verified whether SOD1 could induce neuroprotection of motor neurons through the reduction of reactive oxygen species (ROS). It is known, indeed, that the exposure to L-BMAA is associated with a strong increase in ROS production. ROS amount was detected by fluorescent microscopy, using DCFH-DA (2',7'-dichlorfluorescein-diacetate), a cell membrane-permeable analogue of fluorescein. As shown in **fig. 23B**, SOD1 was able to reduce ROS levels induced by the neurotoxin.

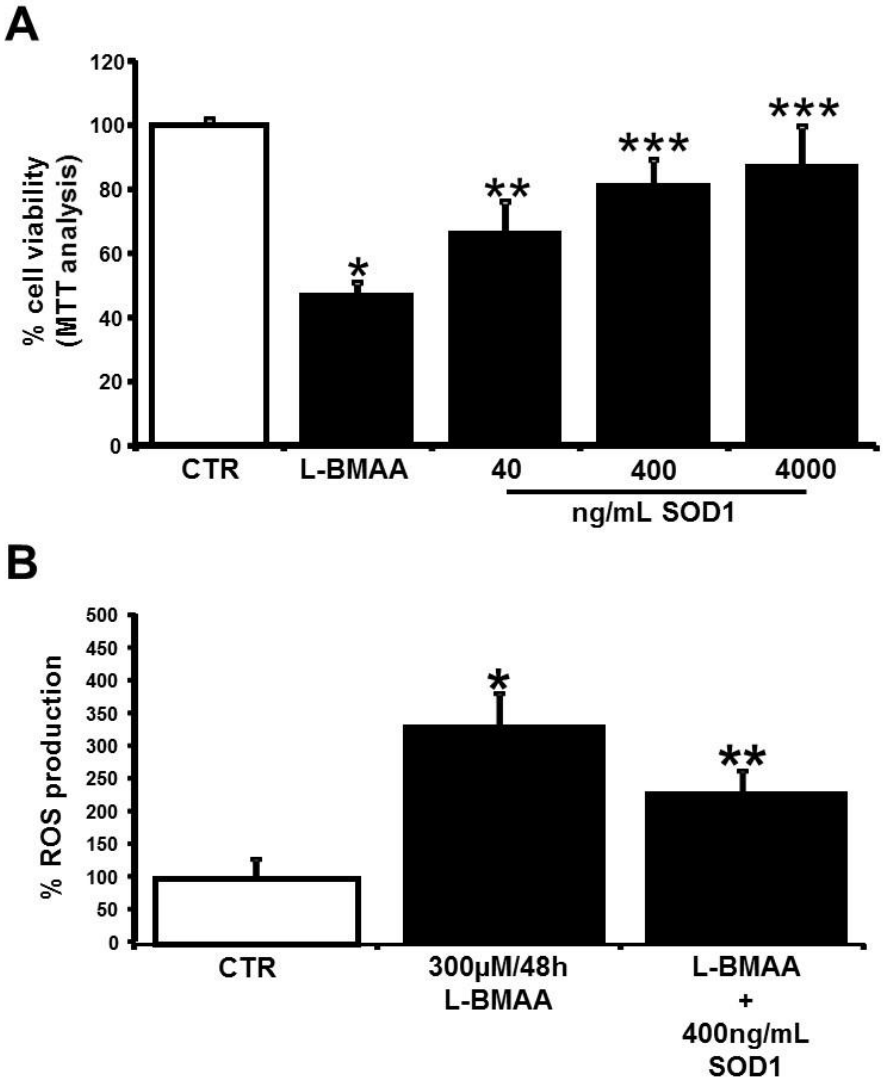

Figure 23. SOD1 prevents cell death induced by L-BMAA.

A. Cell viability rate determined by MTT assay on NSC-34 motor neurons pre-incubated with 40, 400 or 4000 ng/mL SOD1 for 10 min and then exposed to 300 µM L-BMAA for 48 h.

B. Quantification of ROS levels detected by DCF-DA-monitored microscopy induced by 300 µM L-BMAA for 48 h in NSC-34 motor neurons pre-treated with 400 ng/mL SOD1 for 10 min.

*p<0.05 vs control (CTR);
**p<0.05 vs L-BMAA;
***p<0.05 vs L-BMAA + 40 ng/mL SOD1.

IV.5. Effects exerted by recombinant wild type SOD1 and recombinant SOD1^{G93A}

Actually, some recombinant proteins with the most common mutation in the gene coding for SOD1 associated to ALS are commercially available. The use of these proteins could be useful to study ALS *in vitro*. One of the better characterized mutations in SOD1 is the aminoacidic substitution G93A. Thus, we studied the effect exerted by the recombinant wild type SOD1 (rSOD1 wt) and the recombinant SOD1^{G93A} (rSOD1^{G93A}) in NSC-34 motor neurons exposed to L-BMAA.

In order to verify the ability of rSOD1 wt to prevent cell death induced by the neurotoxin, we performed a dose-response experiment. NSC-34 motor neurons were pre-incubated with 40, 400 or 4000 ng/mL rSOD1 wt, and then exposed to 300 µM L-BMAA for 48 h. At the end of treatment, cell viability was measured by MTT assay.

Our results showed that the rSOD1 wt, such as the extractive protein used in the previous described experiments, prevented cell death induced by L-BMAA in a dose-dependent manner (**fig. 24A**).

Successively, we compared the effects mediated by rSOD1^{G93A} with the effects exerted by both extractive SOD1 and rSOD1 wt. To this aim, NSC-34 motor neurons were pre-incubated with the same concentration (400 ng/mL/10 min) of SOD1, rSOD1 wt and rSOD1^{G93A} and then were exposed to L-BMAA (300 µM/48 h).

In **fig. 24B**, it is shown that rSOD1 wt prevented L-BMAA-induced cell death in differentiated NSC-34 motor neurons, as well as extractive SOD1, while rSOD1^{G93A} fails to prevent neurotoxicity.

Collectively, our results demonstrated that the mutated protein is unable to prevent motor neurons death induced by L-BMAA, while the wild type protein has the same effects exerted by extractive SOD1, used in the previous experiments.

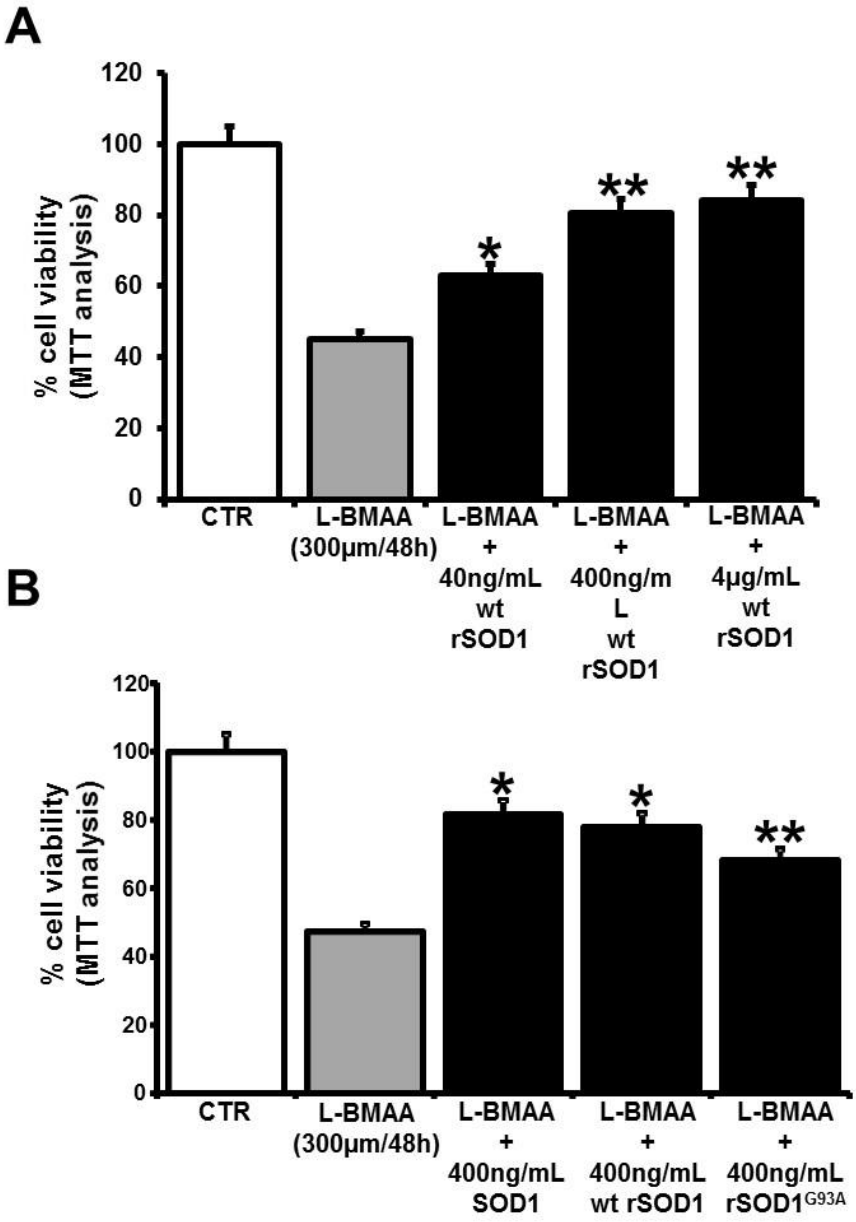

Figure 24. Effects exerted by rSOD1 wt and by rSOD1^{G93A} on cell death induced by L-BMAA.

A. Cell survival rate detected by MTT analysis in NSC-34 motor neurons exposed to 300 µM L-BMAA for 48 h after pre-incubation with 40, 400 or 4000 ng/mL wt rSOD1 for 10 min.

B. Cell survival rate detected by MTT analysis in NSC-34 motor neurons exposed to 300 µM L-BMAA for 48 h after pre-incubation with 400 ng/mL estractive SOD1, rSOD1 wt, or rSOD1^{G93A} for 10 min.

*p<0.05 vs L-BMAA;

**p<0.05 vs L-BMAA + 400 ng/mL SOD1.

IV.6. Role of p-ERK1/2 and p-Akt in SOD1-induced neuroprotection against L-BMAA

In order to verify the involvement of p-ERK1/2 and p-Akt in the neuroprotection exerted by SOD1, we analyzed the effects of SOD1 after the inhibition of both the transductional pathways. To this aim, ERK1/2-mediated pathway was pharmacologically inhibited by PD98059, an inhibitor of p-ERK1/2, and genetically, by using a siRNA against MEK1 (siMEK1), the upstream kinase of ERK1/2. Furthermore, p-Akt pathway was inhibited by using LY294002, a p-Akt inhibitor, and the dominant negative form of Akt (Akt D-) that encodes for an inactive kinase.

Specifically, NSC-34 motor neurons were pre-incubated for 20 min with 50 μM PD98059 in absence or in presence of 400 ng/mL SOD1 for 10 min and then exposed to 300 μM L-BMAA for 48 h. Moreover, NSC-34 cells were transfected with 10 nM siMEK1 for 48 hrs before treatment, and then incubated with 300 μM L-BMAA in absence or in presence of 400 ng/mL SOD1. At the end of treatments, cell viability was measured by MTT assay.

Our results showed that PD98059 counteracted the neuroprotective effects exerted by SOD1 alone (**fig. 25A).** The same result was obtained in silenced cells; indeed, siIMEK1 prevented the neuroprotection induced by SOD1 (**fig. 25A**). In **fig. 25B**, the efficiency of transfection has been reported: in presence of siMEK1, p-ERK1/2 level was reduced and, of course, siMEK1 prevents the transductional effect of SOD1 on ERK1/2 activation.

In another experimental condition, NSC-34 motor neurons were pre-incubated for 30 min with 10 μM LY294002 in absence or in presence of 400 ng/mL SOD1 for 10 min and then exposed to 300 μM L-BMAA for 48h. Furthermore, NSC-34 cells were transfected with 2 μg/μL Akt D-, as previous described, and then incubated with 300 μM L-BMAA in absence or in presence of 400 ng/mL SOD1. At the end of treatments, cell viability was measured by MTT assay.

Our results showed that LY294002 and Akt D- prevented the neuroprotection mediated by SOD1 on L-BMAA-induced cell death (**fig. 25C**). In addition, in **fig. 25D**, the effect of the dominant negative form of Akt on the downstream target ERK1/2 has been reported; we showed that Akt D- reduced p-ERK1/2 in control conditions and prevented SOD1-induced ERK1/2 phosphorylation.

Collectively, these results indicate that SOD1-mediated neuroprotection requires ERK1/2 and Akt activation, suggesting that this effect is possibly linked to the transductional actions of SOD1 rather than its catalytic activity.

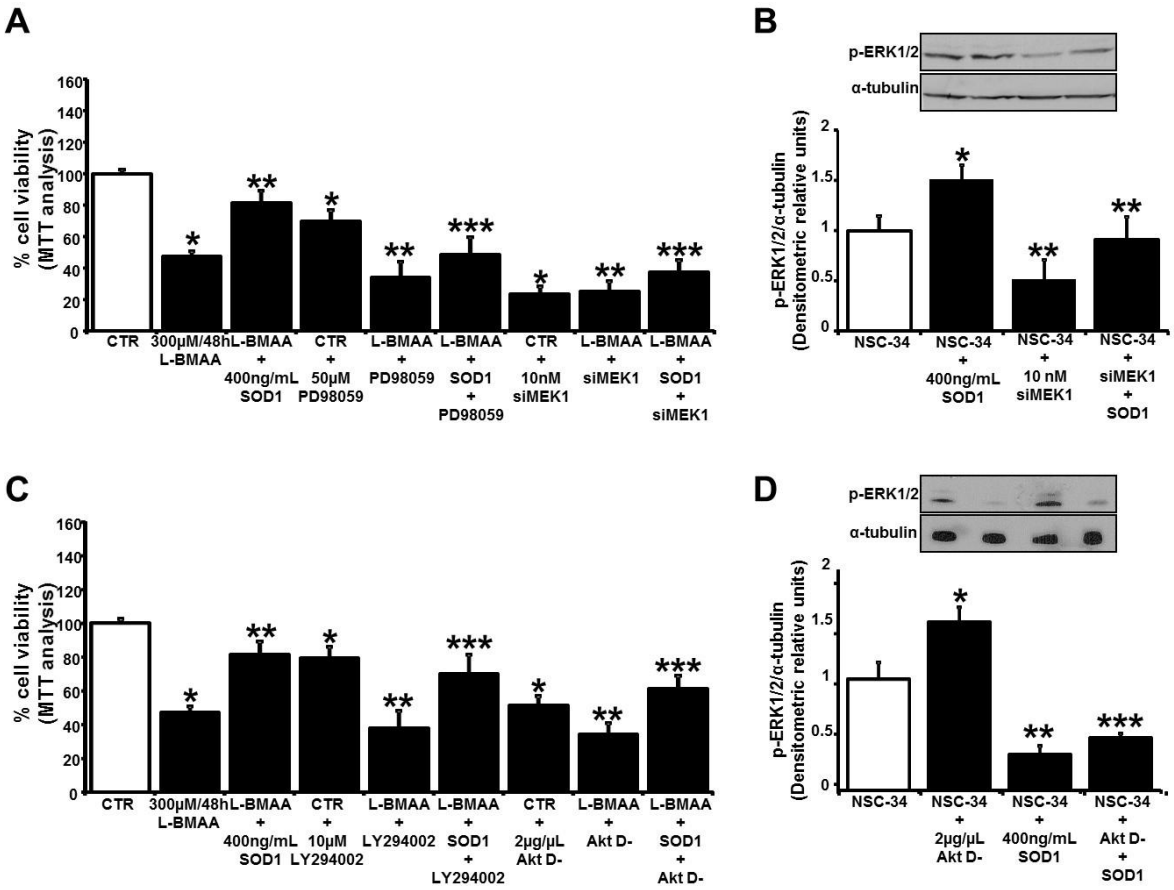

Figure 25. SOD1 prevents cell death induced by L-BMAA in NSC-34 motor neurons through ERK1/2 and Akt phosphorylation.

A. Cell viability rate detected by MTT assay on NSC-34 motor neurons pre-incubated with 50 μM PD98059 for 20 min or transfected with 10 nM siMEK1 for 48 h in absence or in presence of SOD1 (400 ng/mL/10 min) before exposure to 300 μM/48 h L-BMAA.

B. Western blotting analysis on p-ERK1/2 expression levels in NSC-34 cells transfected with 20 nM siMEK1 for 48 h in absence or in presence of 400 ng/mL SOD1 for 10 min and then exposed to L-BMAA.

C. Cell viability rate detected by MTT assay on NSC-34 motor neurons pre-incubated with 10 μM LY294002 for 20 min or transfected with 2μg/μL Akt D- for 48 h in absence or in presence of SOD1 (400 ng/mL/10 min) before exposure to 300 μM/48 h L-BMAA.

D. Western blotting analysis on p-ERK1/2 expression levels in NSC-34 cells transfected with 2 μg/μL Akt D- for 48 h in absence or in presence of 400 ng/mL SOD1 for 10 min and then exposed to L-BMAA.

*p<0.05 vs control (CTR);

**p<0.05 vs L-BMAA;

***p<0.05 L-BMAA + SOD1.

IV.7. Role of the transductional activity of SOD1 in the neuroprotection against L-BMAA: effect of MnTMPyP pentachloride and ApoSOD

Since SOD1 prevents cell death induced by L-BMAA through p-ERK1/2 and p-Akt activation, we study whether this transductional activity could play a relevant role in these neuroprotective effects compared to the catalytic activity of the protein. To this aim, we compared the effects exerted by SOD1 to the effects exerted by MnTMPyP pentachloride, that is a SOD mimetic, and ApoSOD, the enzyme lacking catalytic activity. In particular, MnTMPyP pentachloride has the same catalytic activity of SOD1, but it shows a different molecular structure. In order to inactivate SOD1, we exposed the protein to 200 mM hydrogen peroxide and then we stopped the reaction by adding catalase (1000 U/mL). As previous described in the Method's section, SOD1 activity was measured indirectly by a colorimetric assay.

As shown in **fig. 26A**, our protocol reduced SOD1 activity in a time-dependent manner; indeed, after 2 h exposure to H_2O_2, SOD1 was maximally inactivated, thus showing lowest activity. Furthermore, we verified, by Western blotting analysis, that our protocol did not affect molecular structure of SOD1 protein (**fig. 26B**).

In order to verify our hypothesis, we studied the eventual transductional effects of ApoSOD in NSC-34 motor neurons. Like SOD1, the inactivated enzyme (400 ng/mL) induced a rapid and strong increase of $[Ca^{2+}]_i$ both in the *soma* and in the dendrites of motor neurons (**fig. 26C**). This action depended on different voltage-dependent calcium channels activity, since both ω-conotoxin and L-nimodipine prevented intracellular Ca^{2+} concentration increase induced by ApoSOD (**fig. 26D**). Moreover, ApoSOD was able to activate ERK1/2 and Akt in NSC-34 motor neurons. The pre-incubation with 400 ng/mL ApoSOD for 10 min, indeed, induces an increase of both p-ERK1/2 and p-Akt levels, such as SOD1 (**fig. 26E – F**).

Finally, we analyzed the eventual neuroprotection exerted by ApoSOD and MnTMPyP pentachloride in NSC-34 motor neurons exposed to 300 µM L-BMAA for 48 h. To this aim, differentiated NSC-34 cells were pre-incubated with 400 ng/mL SOD1, 400 ng/mL ApoSOD or 400 ng/mL MnTMPyP pentachloride for 10 min and then exposed to the neurotoxin. At the end of treatments, cell viability was measured by MTT assay.

Our results showed that, like SOD1, ApoSOD that lacks catalytic activity prevented cell death induced by L-BMAA in NSC-34 motor neurons, while MnTMPyP pentachloride fails to have the same effects (**fig. 27A**). This suggests that the neuroprotection mediated by SOD1 was independent on its catalytic activity.

Moreover, we verified the involvement of p-ERK1/2 and p-Akt pathway in the neuroprotection exerted by ApoSOD, by inhibiting both the kinases with the p-ERK1/2 inhibitor PD98059, and the p-Akt inhibitor LY294002. These two drugs counteracted the neuroprotective effects exerted by ApoSOD (**fig. 27B**).

Collectively, our results demonstrated that the neuroprotection exerted by SOD1 depended on its transductional effects rather than its dismutasic activity.

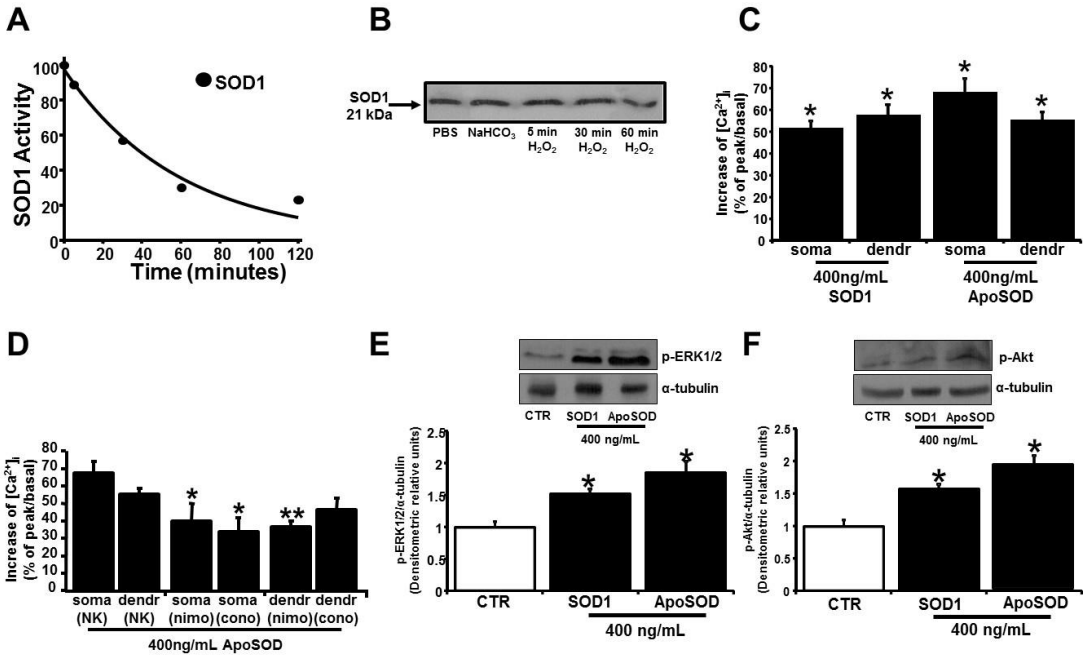

Figure 26. ApoSOD transductional effects in NSC-34 motor neurons.
A. Inactivation of SOD1 with H_2O_2 (200 µM /5, 30, 60, and 120 min)
B. Western blotting analysis of inactivated SOD1 (ApoSOD).
C. Quantification of the effect of 400 ng/mL ApoSOD on $[Ca^{2+}]_i$ in both *soma* and dendrites of NSC-34 motor neurons.
D. Quantification of the effect of 400 ng/mL ApoSOD on $[Ca^{2+}]_i$ in both *soma* and dendrites of NSC-34 motor neurons in presence of 50 nM ω-conotoxin or 1 µM L-nimodipine.
E/F. Western blotting analysis of p-ERK1/2 (**E**) and p-Akt (**F**) expression levels induced by 400 ng/mL ApoSOD, pre-incubated for 10 min or 30 min.
*p<0.05 vs control (CTR);
**p<0.05 vs control (CTR).

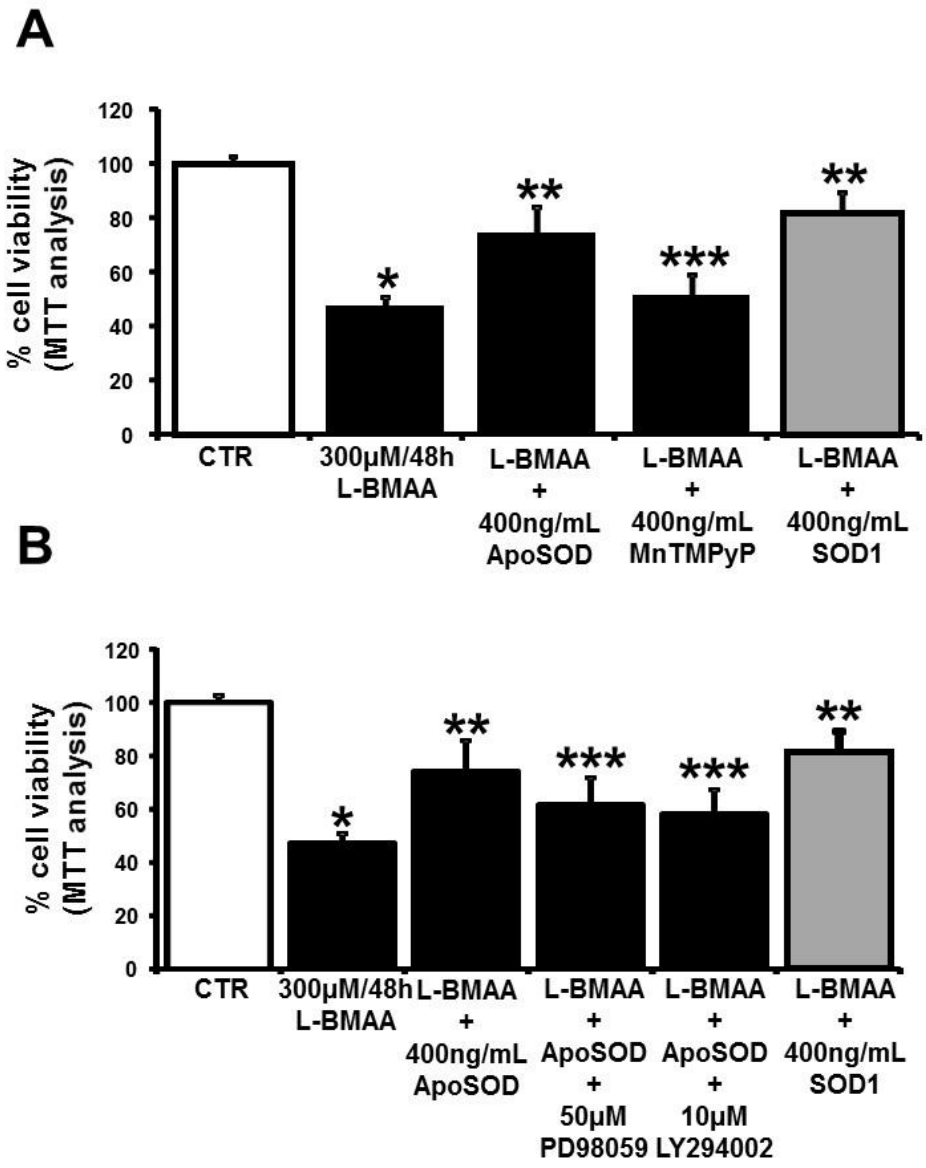

Figure 27. ApoSOD prevents cell death induced by L-BMAA in NSC-34 motor neurons through ERK1/2 and Akt phosphorylation.

A. Cell viability percentage determined by MTT assay on NSC-34 motor neurons pre-treated with 400 ng/mL SOD1, ApoSOD, and MnTMPyP pentachloride for 10 min, and then exposed to L-BMAA (300 µM/48 h).

B. Cell viability percentage determined by MTT assay on NSC-34 motor neurons pre-incubated with 50 µM PD98059 for 20 min or 10 µM LY294002 for 30 min in absence or in presence of 400 ng/mL ApoSOD for 10 min, and then exposed to 300 µM L-BMAA for 48 h.

*$p<0.05$ vs control (CTR);

**$p<0.05$ vs L-BMAA;

***$p<0.05$ vs L-BMAA + ApoSOD.

IV.8. Effects of SOD1 on ER stress induced by L-BMAA in NSC-34 motor neurons

Recent evidence shows that ER stress, usually involved in neurodegenerative disease, plays a key role in motor neuron degeneration typical of ALS (**374**). Thus, we studied whether the neuroprotective effects of SOD1 was due to the modulation of ER stress occurring after L-BMAA incubation. To this aim, ER stress markers, such as GRP78 (or BiP), a molecular chaperone that activates the unfolded protein response (UPR) in presence of misfolded proteins, CHOP (or GADD153), a transcriptional factor activated in presence of mitochondrial damage, and caspase-3 and caspase-12, two enzymes involved in apoptosis, have been studied.

In order to verify ER stress involvement in SOD1-mediated neuroprotection, NSC-34 motor neurons were pre-incubated with 400 ng/mL SOD1 for 10 min and then exposed to 300 µM L-BMAA for 24 h or 48 h. At the end of treatments, cells were collected and analyzed by Western blotting in order to evaluate GRP78 expression. Our results showed an increase of GRP78 occurred after 24 h exposure to L-BMAA, and this increase was more evident after 48 h exposure. The pre-incubation with SOD1 prevented the increase of GRP78 induced by the neurotoxin, suggesting that SOD1 protects neurons from ER stress induced by L-BMAA (**fig. 28A**).

Moreover, since our previous results indicate that the neuroprotection exerted by SOD1 is independent on its catalytic activity, in order to verify the ability of ApoSOD to prevent GRP78 increase induced by L-BMAA, NSC-34 motor neurons were pre-incubated with 400 ng/mL SOD1 or 400 ng/mL ApoSOD and then exposed to the neurotoxin (300 µM/48 h). Then, cells were collected and analyzed by Western blotting. In **fig. 28B**, it is shown that ApoSOD, like SOD1, prevented GRP78 increase induced by 300 µM L-BMAA after 48 h exposure.

Collectively, our results indicate that SOD1 protected neurons from ER stress by ALS-mediated neurodegeneration, and this prevention seems to be independent on its dismutasic activity.

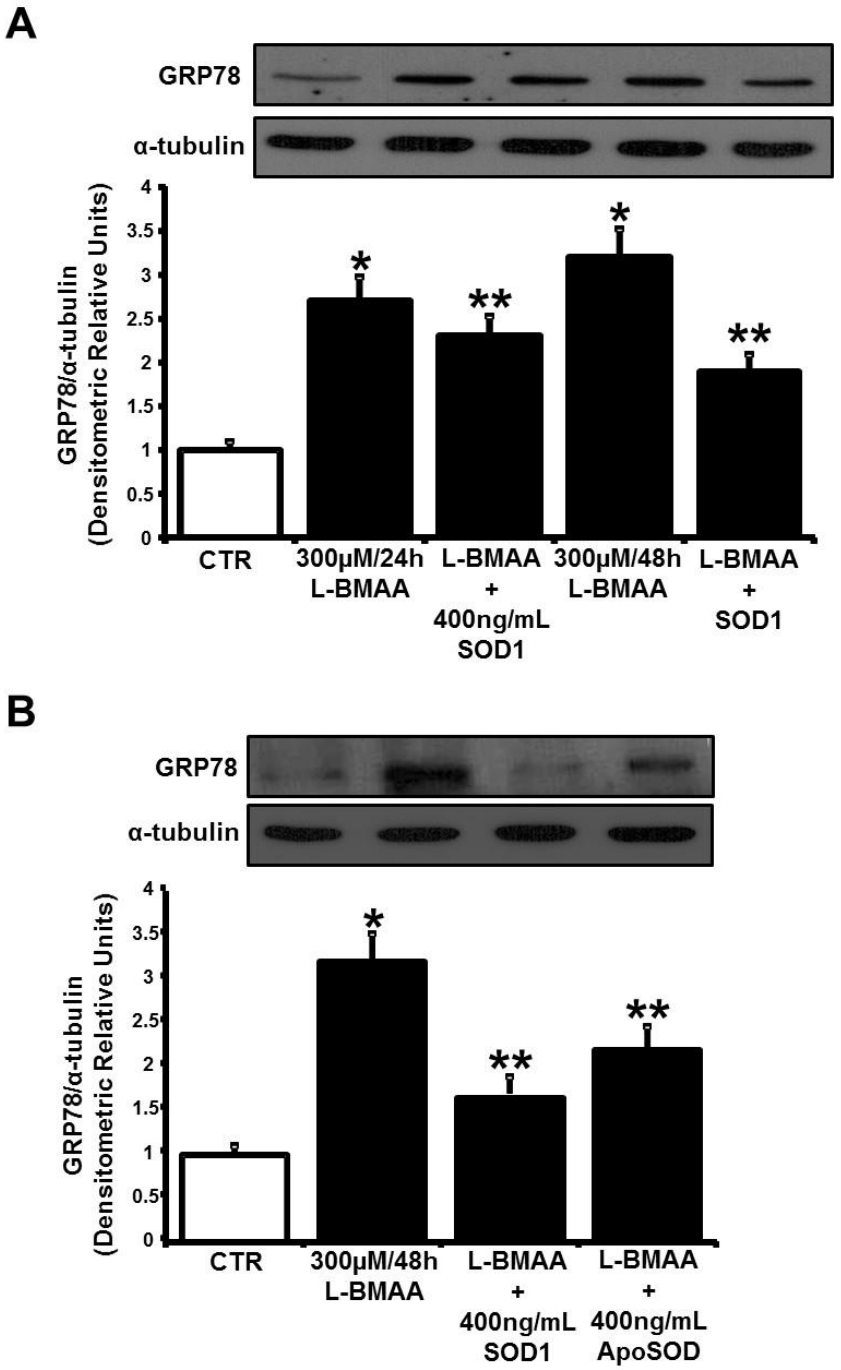

Figure 28. SOD1 and ApoSOD prevent ER stress induced by L-BMAA in NSC-34 motor neurons.

A. Western blotting analysis of GRP78 expression levels in NSC-34 motor neurons pre-incubated with 400 ng/mL SOD1 for 10 min and the exposed to 300 μM L-BMAA for 48 h.

B. Western blotting analysis of GRP78 expression levels in NSC-34 motor neurons pre-incubated with 400 ng/mL SOD1 or 400 ng/mL ApoSOD for 10 min and then exposed to L-BMAA (300 μM/48 h).

*p<0.05 vs control (CTR);

**p<0.05 vs L-BMAA.

IV.9. Effects of SOD1 on cell death induced by L-BMAA in primary cultures of motor neurons

Primary cultures of motor neurons were obtained from spinal cord of E12/E14 embryo mice, as described in Material and Methods.

These cultures were characterized by immunocytochemical analysis, evaluating SMI-32 (a typical motor neuron marker) expression. By confocal microscopy, we showed a strong expression of SMI-32, that marks the not-phosphorylated H neurofilament, both in the *soma* and in the dendrites of primary cells (**fig. 29A - B**).

In order to verify the susceptibility of primary motor neurons to L-BMAA, we performed a time-course experiment, by exposing cells to 300 µM L-BMAA for 24 h or 48 h.

Our results showed that cell viability was reduced just after 24 h exposure to the neurotoxin, but a strong reduction (about 50%) was observed only after 48 h exposure (**fig. 29C - D**)

Then, we evaluated the neuroprotective effects exerted by SOD1 and their molecular mechanisms. To this aim, primary motor neurons were pre-treated with 400 ng/mL SOD1, 400 ng/mL ApoSOD, 400 ng/mL rSOD1wt, and 400 ng/mL $rSOD1^{G93A}$, and then exposed to L-BMAA (300 µM/48 h). At the end of treatments, cell viability was detected by measuring the ratio between dead and living cells.

Our results demonstrated that SOD1, rSOD wt and ApoSOD prevented in the same extent cell death induced by L-BMAA in primary cultures of motor neurons. On the contrary, the $rSOD1^{G93A}$ fails to have the same neuroprotective effects (**fig. 29**).

Collectively, these results indicated that SOD1 prevents cell death also in primary motor neurons independently from its catalytic activity.

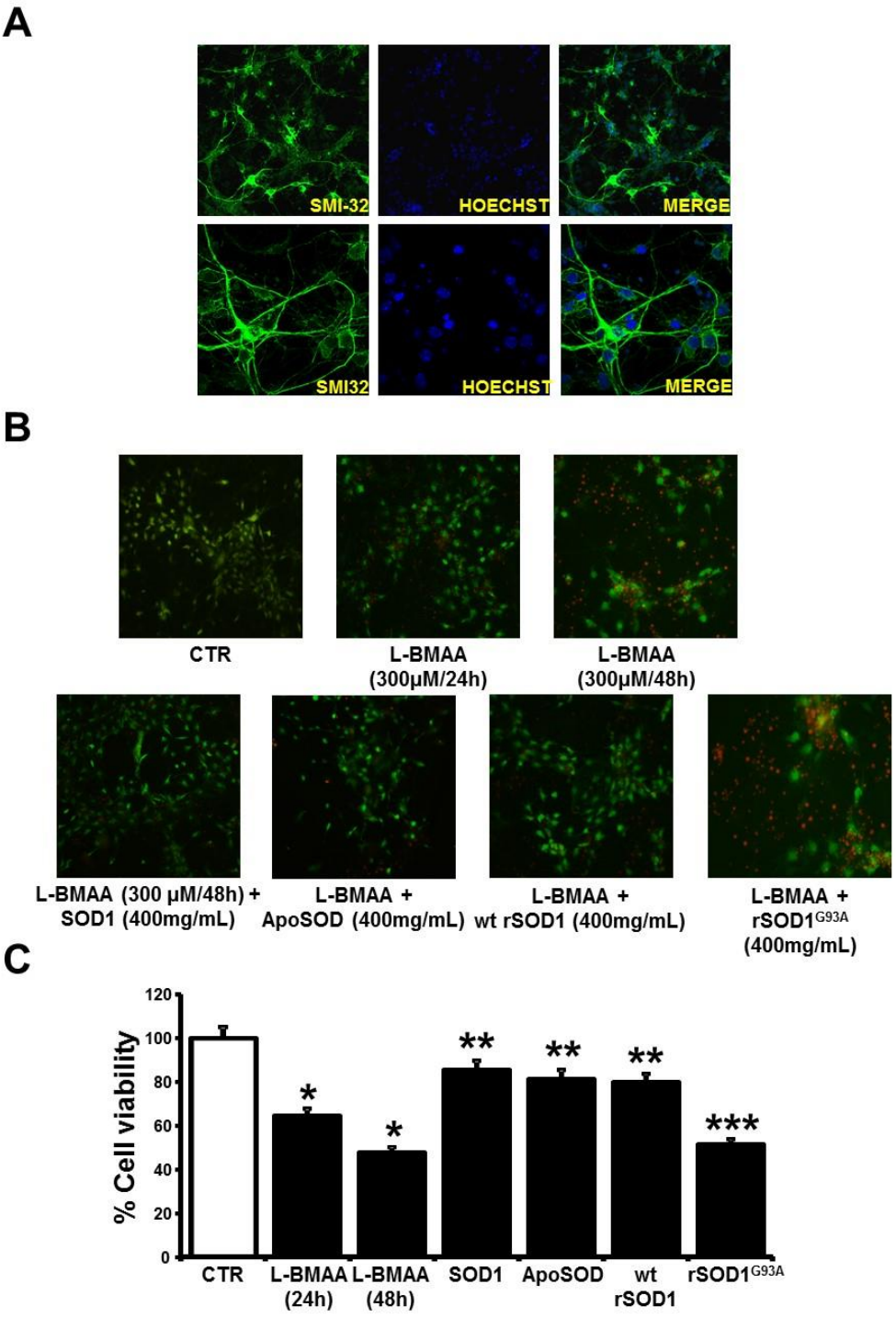

Figure 29. SOD1 prevents cell death induced by L-BMAA in primary motor neurons.

A. Sequence of immunocytochemical confocal images of SMI-32 expression in primary cultures of motor neurons. Cells are represented at two different magnification.

B/C. Percentage of cell viability of primary motor neurons treated with L-BMAA (300 µM) for 24 h or 48 h in absence or in presence of 400 ng/mL SOD1, ApoSOD, wt rSOD1, and $rSOD1^{G93A}$ for 10 min.

*p<0.05 vs control (CTR);

**p<0.05 vs L-BMAA;

***p<0.05 vs L-BMAA + SOD1.

IV.10. Effects of SOD1 on cell death induced by chemical hypoxia in NSC-34-SOD1^{G93A} motor neurons

To better characterize the neuroprotective effects exerted by SOD1, we tested the ability of SOD1 to prevent cell death induced by chemical hypoxia in stably transfected NSC-34 motor neurons, that express SOD1^{G93A} after treatment with 1 μg⁄mL doxycycline for 48 h.

NSC-34-SOD1^{G93A} motor neurons were pre-incubated with 400 ng/mL SOD1 for 10 min and then exposed to chemical hypoxia for 45 min. At the end of treatment, cell viability was evaluated by MTT assay.

Our results showed that the exposure to chemical hypoxia for 45 min strongly reduce cell viability of NSC-34-SOD1^{G93A} motor neurons and SOD1 was able to prevent cell death induced by hypoxic *stimulus* in these stably transfected motor neurons (**fig. 30A**).

Since SOD1 prevented cell death induced by chemical hypoxia in NSC-34-SOD1^{G93A}, we studied the effects exerted by two different recombinant protein, recombinant wt SOD1 and recombinant SOD1^{G93A}, on NSC-34 motor neurons exposed to chemical hypoxia. To this aim, cells were first pre-incubated with 400 ng/mL rSOD1 wt or with 400 ng/mL rSOD1^{G93A} and then exposed to chemical hypoxia for 45 min.

As shown in **fig. 30B**, rSOD1 wt prevented cell death induced by chemical hypoxia as well as SOD1. On the contrary, rSOD1^{G93A} fails to induce neuroprotection on motor neuron against hypoxia.

Collectively, our results showed that SOD1 exerted neuroprotective effects against chemical hypoxia, a Ca^{2+}- and ROS-dependent mechanism both in wild type NSC-34 and NSC-34-SOD1^{G93A} motor neurons.

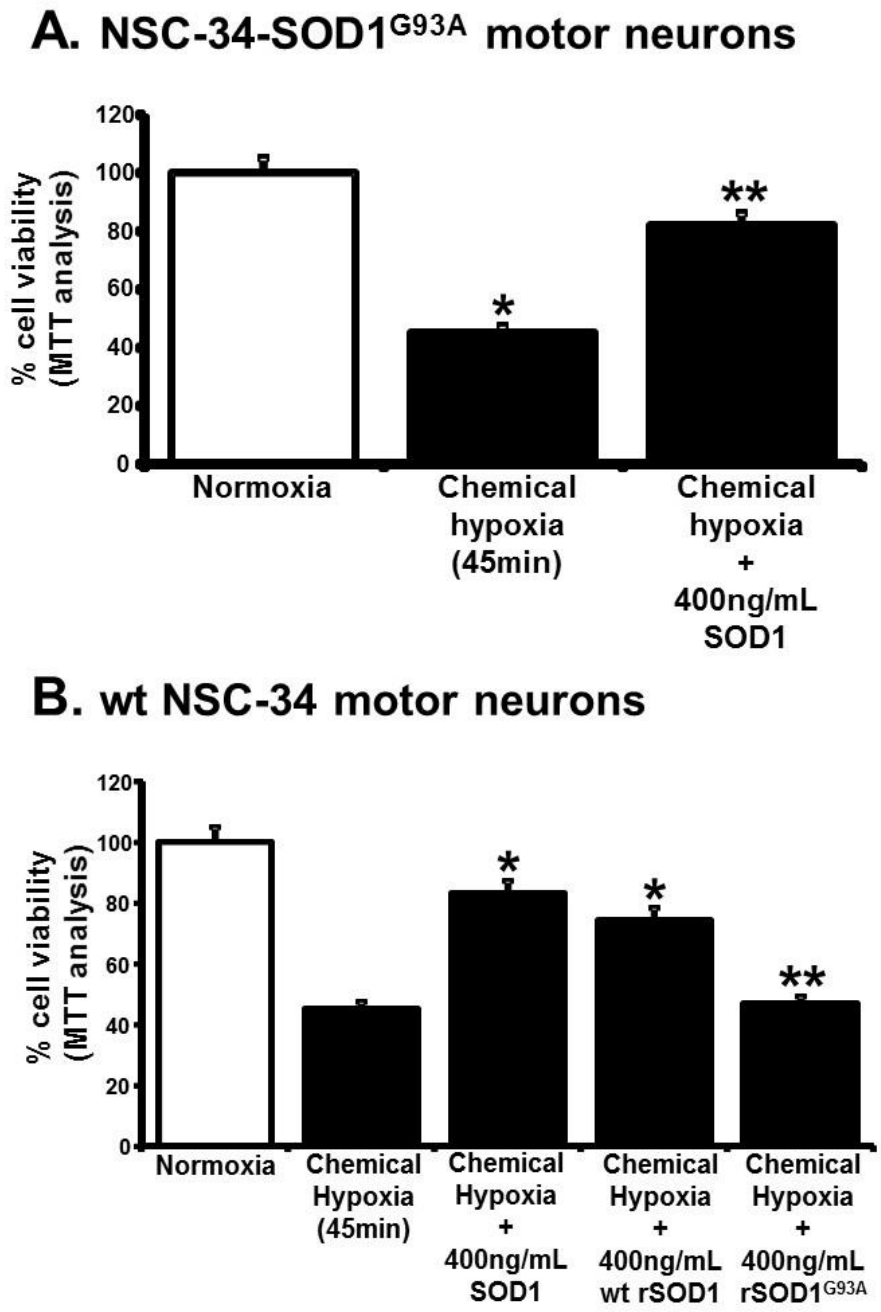

Figure 30. SOD1 prevents cell death induced by chemical hypoxia in NSC-34-SOD1^{G93A} motor neurons.

A. Cell survival rate determined by MTT analysis on stably transfected NSC-34-SOD1^{G93A} motor neurons exposed to 45 min chemical hypoxia.

B. Cells survival rate determined by MTT analysis on NSC-34 motor neurons pre-incubated eith 400 ng/mL SOD1, wt rSOD1, and rSOD1^{G93A} for 10 min.

*p<0.05 vs control (CTR);

**p<0.05 vs Chemical Hypoxia.

IV.11. Effects of transductional activity of SOD1 on chemical hypoxia-induced cell death in NSC-34 motor neurons

Since SOD1 prevents cell death induced by chemical hypoxia in stably transfected NSC-34-SOD1^{G93A} motor neuron, we studied the molecular mechanisms underlying this neuroprotection. Thus, we exposed NSC-34 motor neurons to chemical hypoxia, which represents an *in vitro* model of Ca^{2+}- and ROS-dependent neurodegeneration.

NSC-34 motor neurons were first incubated with a Krebs glucose-free solution containing 2 mM 2-deoxyglucose, an inhibitor of glycolisis, and 5 mg/mL olygomycin, an oxidative phosphorylation inhibitor, for 45, 90 and 180 min. At the end of treatments, cell viability was detected by MTT assay.

Our results demonstrated that chemical hypoxia strongly reduced cell viability in motor neurons; indeed, just after 45 min exposure, cell viability was reduced of about 50% (**fig. 31A**).

Successfully, we performed a dose-response experiment to verify SOD1-mediated neuroprotection. To this aim, NSC-34 motor neurons were pre-incubated with 40, 400 and 4000 ng/mL SOD1 for 10 min and then exposed to 45 min chemical hypoxia. Cell viability, finally, was measured by MTT assay.

In **fig. 31B**, it is shown that SOD1 prevented cell death in a dose-dependent manner. A strong recovery of cell viability was detected with 400 ng/mL SOD1, as revealed in motor neurons exposed to L-BMAA.

Since chemical hypoxia is associated to a great amount of ROS, we verified the ability of SOD1 to prevent ROS increase in NSC-34 motor neurons exposed to chemical hypoxia for 45 min.

ROS amount was detected by fluorescent microscopy, using DCFH-DA (2',7'-dichlorfluorescein-diacetate), a cell membrane-permeable fluorescein analogue, and revealed that SOD1 was able to reduce ROS levels induced by chemical hypoxia (**fig. 31C**).

Then, we analyzed the neuroprotective effects exerted by SOD1 in motor neurons exposed to chemical hypoxia and reoxygenation. To this aim, NSC-34 motor neurons were pre-treated with SOD1 for 10 min, exposed to 45 or 90 min chemical hypoxia and finally to reoxygenation, by the substitution of Krebs glucose-free solution with a complete culture medium for 3 or 5 h.

As shown in **fig. 31D**, SOD1 prevented cell death induced also by reoxygenation. In particular, after 5 h of reoxygenation, we observed a strong reduction of cell viability (about 30% cell viability). However, SOD1 reduced cell death induced by hypoxia.

Then, in order to verify the involvement of p-ERK1/2 and p-Akt in SOD1-mediated neuroprotection in this model, we studied the effects exerted by the enzyme by inhibiting these two kinases.

As previous described in NSC-34 motor neurons exposed to L-BMAA, we analyzed the effects of SOD1 in presence of PD98059 (a p-ERK1/2 inhibitor), siMEK1 (that silences MEK1, the upstream kinase of ERK1/2), LY294002 (a p-Akt inhibitor), and Akt D- (that codes for an inactivated kinase). Thus, for the pharmacological treatments, NSC-34 cells were pre-treated with 10 µM PD98059 for 10 min or 50 µM LY294002 for 20 min in absence or in presence of 400 ng/mL SOD1. Furthermore, cells were transfected with 10 nM siMEK1 or 2 µg/µL Akt D-. At the end of both the pharmacological and the molecular treatments, cell viability was detected by MTT assay.

In **fig. 32A**, it is shown that the pharmacological and molecular inhibition of p-ERK1/2 prevent the neuroprotective effects exerted by SOD1. Analogously, in **fig. 32B**, it is demonstrated that also the pharmacological and molecular inhibition of p-Akt counteract SOD1-mediated neuroprotection under hypoxic conditions.

Finally, we studied the involvement of catalytic and transductional actions of SOD1 in the neuroprotection in this model.

As previous described, NSC-34 motor neurons were pre-incubated with 400 ng/mL SOD1, 400 ng/mL ApoSODor 400 ng/mL MnTMPyP pentachloride (a SOD1 mimetic drug) and then exposed to chemical hypoxia for 45 min. At the end of treatments, cell viability was detected by MTT assay.

Our results showed that ApoSOD, lacking catalytic activity, exerted the same neuroprotective effects mediated by SOD1, while MnTMPyP pentachloride failed to have these actions (**fig. 33A**).

Moreover, it seems that the neuroprotection exerted by ApoSOD required ERK1/2 and Akt activation. Indeed, we studied the effects of ApoSOD also in NSC-34 motor neurons pre-treated with 10 µM PD98059 for 10 min or 50 µM LY294002 for 20 min and then exposed to chemical hypoxia for 45 min.

As shown in **fig. 33B**, both the pharmacological inhibition of p-ERK1/2 and p-Akt counteract the neuroprotective effects exerted by ApoSOD.

Collectively, our results demonstrated that SOD1 prevents cell death induced by chemical hypoxia in a dose-dependent manner through ERK1/2 and Akt activation. Moreover, this neuroprotection seems to be independent on SOD1 dismutasic activity, because also ApoSOD, that lacked catalytic site, prevented cell death induced by chemical hypoxia through ERK1/2 and Akt activation.

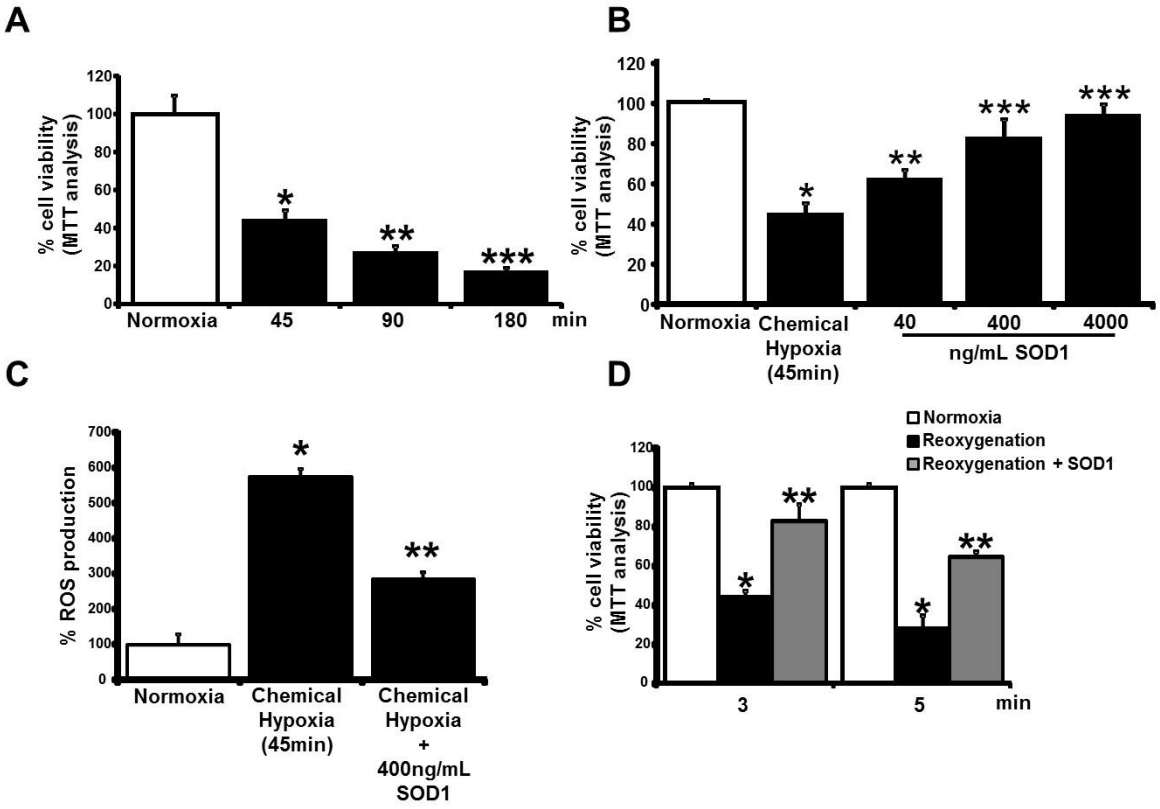

Figure 31. SOD1 prevents cell death induced by chemical hypoxia in NSC-34 motor neurons.

A. Cell viability rate of NSC-34 motor neurons exposed to chemical hypoxia for 45, 90 and 180 min

B. Cell viability rate of NSC-34 motor neurons exposed to 45 min chemical hypoxia after preincubation with SOD1 (40, 400 and 4000 ng/mL) for 10 min.

C. Determination of ROS levels in NSC-34 motor neurons exposed to chemical hypoxia for 45 min in absence or in presence of 400 ng/mL SOD1 for 10 min.

D. Cell viability rate of NSC-34 motor neurons exposed to chemical hypoxia for 45 min and, then, to 3 h or 5 h reoxygenation in absence or in presence of 400 ng/mL SOD1 for 10 min.

*p<0.05 vs control (Normoxia);

**p<0.05 vs 45 min Chemical Hypoxia;

***p<0.05 vs 40 ng/mL SOD1

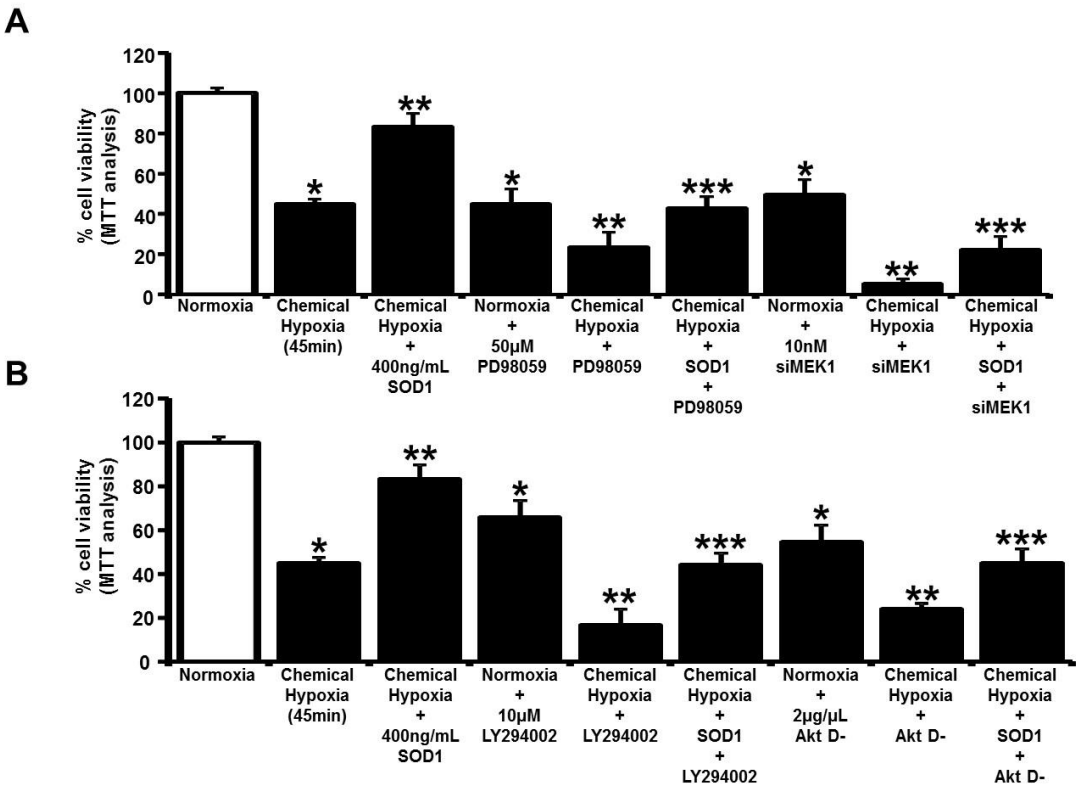

Figure 32. SOD1 prevents cell death induced by chemical hypoxia in NSC-34 motor neurons through ERK1/2 and Akt phosphorylation.

A. Cell viability percentage of NSC-34 motor neurons pre-incubated with 50 µM PD98059 for 20 min or transfected with 10 nM siMEK1 for 48 h in absence or in presence of SOD1 (400 ng/mL/10 min) before exposure to 45 min chemical hypoxia.

B. Cell survival rate determined by MTT assay on NSC-34 motor neurons pre-incubated with 10 µM LY294002 for 20 min or transfected with 2µg/µL Akt D- for 48 h in absence or in presence of SOD1 (400 ng/mL/10 min) before exposure to 45 min chemical hypoxia.

*p<0.05 vs control (Normoxia);

**p<0.05 vs Chemical Hypoxia;

***p<0.05 vs Chemical Hypoxia + SOD1.

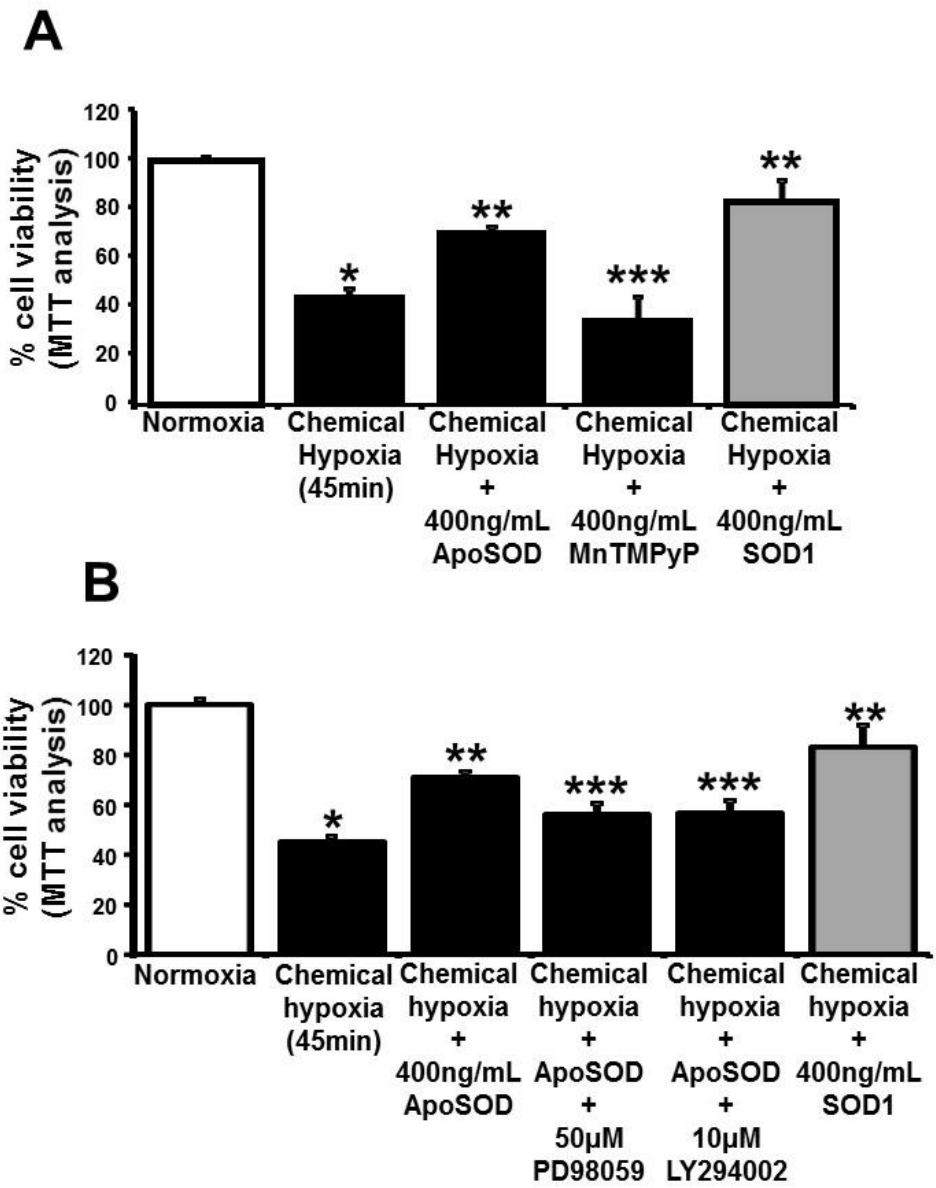

Figure 33. ApoSOD prevents cell death induced by L-BMAA in NSC-34 motor neurons through ERK1/2 and Akt phosphorylation.

A. Cell survival percentage of NSC-34 motor neurons exposed to 400 ng/mL/10 min SOD1, ApoSOD, SOD1, and MnTMPyP pentachloride and then to 45 min chemical hypoxia.

B. Cell survival percentage of NSC-34 motor neurons pre-incubated with 50 µM PD98059 for 20 min or 10 µM LY294002 for 30 min in absence or in presence of ApoSOD (400 ng/mL/10 min) before exposure to chemical hypoxia for 45 min.

*p<0.05 vs control (Normoxia);

**p<0.05 vs Chemical Hypoxia;

***p<0.05 Chemical Hypoxia + SOD1.

IV.12. Effects of SOD1 on ER stress induced by chemical hypoxia in NSC-34 motor neurons

In order to verify in the adopted models of neurodegeneration the role of ER stress, a type of apoptosis documented in several neurodegenerative diseases, and to test the effects of SOD1 on ER stress, NSC-34 motor neurons were exposed to 45 or 180 min chemical hypoxia after pre-incubation with 400 ng/mL SOD1. At the end of treatments, cells were collected and analyzed by Western blotting to evaluate the expression of GRP78 (a molecular chaperone), CHOP (a transcription factor), caspase-3, and caspase-12 (two mediators of apoptosis).

Our results showed that 45 min exposure to chemical hypoxia increased GRP78 and CHOP protein expression levels, and this increase was more evident after 180 min exposure. Analogously to the results obtained in motor neurons exposed to L-BMAA, pre-treatment with 400 ng/mL SOD1 prevented GRP78 and CHOP increase induced by 45 min exposure to chemical hypoxia (**fig. 34A - B**). Furthermore, we observed the activation of both caspase-3 and caspase-12 in motor neurons exposed to 45 or 180 min chemical hypoxia. Interestingly, SOD1 prevented the activation of both the protein expression of caspases (**fig. 34C -D**).

Because the neuroprotective effects exerted by SOD1 are independent on catalytic activity, we verified also the ability of ApoSOD to prevent the increase in protein expression of GRP78, CHOP, caspase-3 and 12. To this aim, NSC-34 motor neurons were pre-incubated with 400 ng/mL SOD1 or 400 ng/mL ApoSOD and then exposed to chemical hypoxia for 45 min. At the end of treatment, cells were collected and analyzed by Western blotting.

As shown in **fig. 35**, ApoSOD protects ER from stress induced by chemical hypoxia. Indeed, the pre-incubation with ApoSOD prevented GRP78 (**fig. 35A**) and CHOP (**fig. 35B**) increase induced by hypoxia. Similarly to the effects observed for SOD1, ApoSOD also prevented caspase-12 (**fig. 35C**) induced by chemical hypoxia.

Collectively, our results showed that SOD1 is able to protect motor neurons from ER stress induced by chemical hypoxia independently on its catalytic activity.

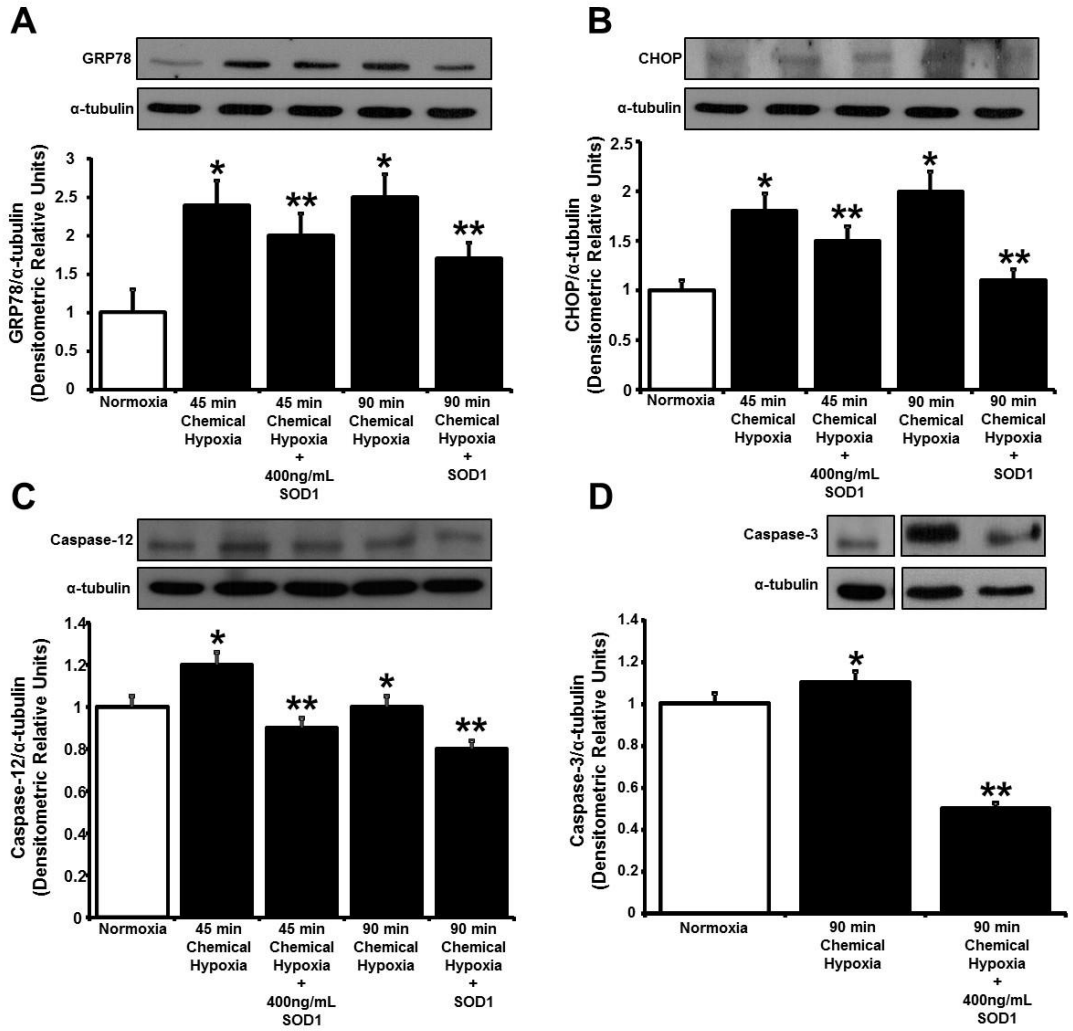

Figure 35. ApoSOD prevents cell death induced by L-BMAA in NSC-34 motor neurons through ERK1/2 and Akt phosphorylation.

A-C. Western blotting analysis of GRP78 (**A**), CHOP (**B**), and caspase-12 (**C**) expression levels in NSC-34 motor neurons pre-incubated with 400 ng/mL SOD1 for 10 min and then exposed to chemical hypoxia for 45 or 90 min.

D. Western blotting analysis of caspase-3 expression levels in NSC-34 motor neurons pre-incubated with 400 ng/mL SOD1 for 10 min and then exposed to chemical hypoxia for 90 min.

*p<0.05 vs control (Normoxia);

**p<0.05 vs Chemical Hypoxia.

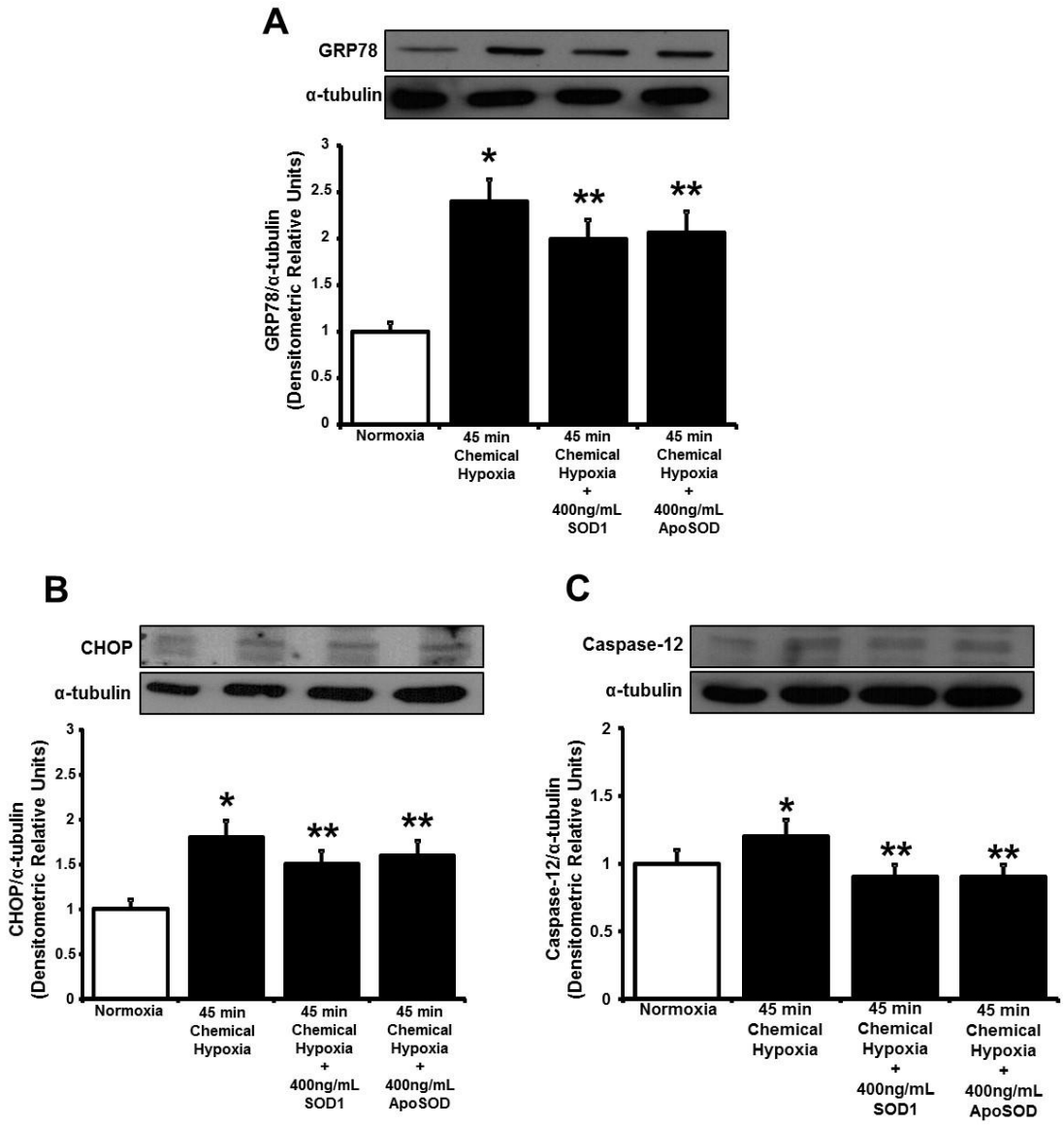

Figure 36. ApoSOD prevents cell death induced by L-BMAA in NSC-34 motor neurons through ERK1/2 and Akt phosphorylation.

Western blotting analysis of GRP78 (**A**), CHOP (**B**), and caspase-12 (**C**) expression levels in NSC-34 motor neurons pre-incubated with 400 ng/mL SOD1 or 400 ng/mL ApoSOD for 10 min and then exposed to chemical hypoxia for 45 min.

*p<0.05 vs control (Normoxia);

**p<0.05 vs Chemical Hypoxia.

Conclusions

CONCLUSIONS

The first link between SOD1 and ALS was found in 1993 and led to the hypothesis that the disease resulted from loss of function mutations in the gene encoding for SOD1 (**268**). However, most of the mutated SOD1 preserve catalityc activity and thus the loss of function hypothesis was rapidly refuted.

In addition, many evidence showed that SOD1 could be secreted in many cellular lines (**322**, **325**, **326**) and SOD1 secretion is impaired in ALS (**366**). Moreover, SOD1 can induce PLC/PKC pathway activation through an increase of intracellular calcium concentration (**325**), independently on its dismutasic activity.

The idea that SOD1 could exert neuroprotective effects at neuronal levels is due to several evidence:

- SOD1 administration in $SOD1^{G93A}$ transgenic mice could modify motor symptom of disease (**366**);
- SOD1 is released by microglial cells and could protect against the toxicity mediated by 6-hydroxy-dopamine (6OHDA) (**404**);
- and, at last, drugs, as the insulin-like growth factor 1 (IGF-1), partially prevent motor neuron loss in $SOD1^{G93A}$ transgenic mice through ERK1/2 activation (**405**).

However, the molecular mechanisms underlie the neuroprotective effects exerted by SOD1 are still unknown.

Thus, the present study highlighted the neuroprotective profile of SOD1 in two different models of neurodegeneration at motor neuronal level: L-BMAA-induced neurotoxicity, mimicking amyotrophic lateral sclerosis (ALS) *in vitro,* and chemical hypoxia, a Ca^{2+}- and ROS-dependent cell death.

The present data showed that SOD1 prevents cell death induced by L-BMAA and by chemical hypoxia in a dose-dependent way. Furthermore, our results demonstrated that the neuroprotection exerted by the enzyme was not correlated to its dismutase activity, but depended on the transductional actions exerted at the level of Ca^{2+}/ERK1/2/Akt pathway. Indeed, ApoSod, the enzyme lacking catalytic activity, at molecular level, induced intracellular Ca^{2+} concentration increase, and ERK1/2 and Akt phosphorylation in the same extent of SOD1. Moreover, ApoSOD mimicked the neuroprotective effects played by SOD1 on motor neurons exposed to L-BMAA or to

chemical hypoxia. At last, pharmacological and biochemical inhibition of ERK1/2 and Akt phosphorylation prevented SOD1- and ApoSOD-induced neuroprotection.

ALS and hypoxia are associated with endoplasmic reticulum (ER) stress, a pathological condition resulting to the accumulation of misfolded proteins in the ER lumen. Thus, both ALS and hypoxia increase the expressions of several ER stress markers, such as GRP78, a molecular chaperon that activates the unfolded protein response (UPR), CHOP, a transcriptional factor involved in UPR and able to induce apoptosis, and caspasis, the main apoptosis mediator.

Molecularly, the neuroprotective effect of SOD1 and ApoSod on Ca^{2+}/ERK1/2/Akt pathway determined the prevention of GRP78, CHOP, and caspases, suggesting that SOD1 neuroprotection passed through the prevention of ER stress. Interestingly, this effect was due to Ca^{2+}/ERK1/2/Akt pathway activation.

Considering that SOD1 was continuously secreted from NSC-34 motor neurons in a Ca^{2+}-dependent manner, and that the impairment of SOD1 secretion is present in ALS models (**366**), it is possible to speculate that the reduction of SOD1 release from motor neurons may contribute to cell death and neuronal loss occurring in ALS through the reduction of Ca^{2+}/ERK1/2/Akt pathway activation that, in turn, culminated in ER stress, or leaded to ER stress.

The results of the present study contributed to identify a new biochemical feature of SOD1 that eventually may be considered useful to draw new therapeutical strategy in ALS. Furthermore, the identification of the transductional pathway underlying the neuroprotective effect of the enzyme may contribute to the acquisition of new knowledge potentially useful for the field.

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