INTRACELLULAR CALCIUM HOMEOSTASIS OF MOTONEURONS IN ANIMAL MODELS OF AMYOTROPHIC LATERAL SCLEROSIS

Ph. D. Thesis

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List of publications related to the Ph.D. thesis

Manuscripts in peer reviewed journals

I. Siklós L, Engelhardt JI, **Adalbert R,** Appel SH:
Calcium-containing endosomes at oculomotor terminals in animal models of ALS.
NeuroReport, **10:**2539-2545(1999)

II. Siklós L, Engelhardt JI, Reaume AG, Scott RW, **Adalbert R,** Obál I, Appel SH:
Altered Ca-homeostasis in spinal motoneuorons but not in oculomotor neurons of SOD-1 knock-out mice.
Acta Neuropathologica, **99:**517-524(2000)

III. **Adalbert R,** Engelhardt JI, Siklós L:
DL-homocysteic acid application disrupts calcium homeostasis and induces degeneration of spinal motoneurons in vivo
Acta Neuropathologica, in press.

Presentations at international and Hungarian scientific conferences

Siklós L, Engelhardt JI, **Adalbert R,** Appel SH:

Intracellular calcium distribution of motoneurons with missing- or genetically altered superoxide dismutase.

Brain Pathol., **10:**726-727(2000)

(XIV. International Congress of Neuropatholgy, Birmingham, September 3-6, 2000.)

Adalbert R, Engelhardt JI, Siklós L:

Ultrastructure and calcium distribution of spinal motoneurons of rats exposed to excitotoxins in vivo.

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Adalbert R, Siklós L, Engelhardt JI:

Ultrastructural alterations and changes in the homeostasis of calcium in spinal motor neurons in acute excitotoxic injury.

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Adalbert R, Engelhardt JI, Siklós L:

Structural changes and specificities of the calcium homeostasis of motoneurons during excitotoxic lesions.

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Siklós L, **Adalbert R,** Obál I, Engelhardt JI:

Modifications of the neuronal calcium homeostasis in relation to the Degenerative alterations of motoneurons.

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Contents

1. Introduction

1.1 Historical overview

Progressive muscular wasting was a clinical syndrome well known to physicians already in the early 19th century. The term progressive muscular atrophy (PMA) was introduced by Aran, who believed that this syndrome was a muscular disorder [Aran, 1850]. In the same time, however, Cruveilhier, who noted the degeneration of the anterior spinal roots, regarded PMA as a myelopathic disorder [Cruveilhier, 1853]. Progressive bulbar palsy (PBP) was first described by Duchenne [Duchenne, 1860] who, similar to Aran, also favoured a muscular cause of the disease. It was Charcot and Joffroy who studied just the same bulbar motor nuclei, and recognizing loss of motoneurons concluded that the disease was of neurogenic origin. The diagnostic criteria they used remained valid to the present days [Charcot and Joffroy, 1869]. Five years after the original description of two cases, the term for amyotrophic lateral sclerosis, in brief ALS, was introduced by Charcot when he extended his first observation by systematic characterization of 20 other patients [Charcot, 1874].

1.2 Terminology and definition

The term motoneuron disease (MND) was introduced by Brain, in recognition of the relation between the syndromes of ALS, PMA and PBP [Brain, 1962]. This term has become commonly used in the specialized English literature, although Charcot's designation of ALS was prefered in the continental Europe. Nowadays, the general term MND is used to describe a family of degenerative lower motoneuron (LMN) and the upper motoneuron (UMN) disorders, as shown in *Table 1* [Swash, 2000].

Because the non-ALS patients account for not more than 10-15 % of cases of MND, their impact on the statistics data of differential diagnosis, mortality, frequency etc. is practically negligible [Eisen and Krieger, 1998]. In the USA, as a memory for the famous baseball player, Lou Gehrig's disease is more often used as the name for ALS.

According to the present definition, ALS is a relentlessly progressive, invariably fatal disease of the central nervous system. It is characterized by the degeneration of the upper motoneurons (UMNs) and lower motoneurons (LMNs) in the brain and spinal cord, associated with the neurogenic atrophy of muscles. At the begining, the disorder is often asymmetric and it may be quite localized. The varying extent and localization of the involvement of the motor system results in differing clinical features, but ultimately, as the disorder progresses, the clinical expression of the disease is rather uniform, with extreme muscular wasting and spasticity. Usually, death results from respiratory failure in 2 to 4 years after diagnosis.

Table 1. Idiopathic motoneuron diseases
(According to Swash [2000])

Amyotrophic lateral sclerosis (ALS)
Progressive bulbar palsy (PBP)
Progressive muscular atrophy (PMA)
Primary lateral sclerosis (PLS)
Familial amyotrophic lateral sclerosis
Juvenile amyotrophic lateral sclerosis
Madras motoneuron disease

1.3 Epidemiology

ALS, which afflicts primarily middle-aged adults, occurs in about 1.6-7.0 people per 100,000 population (mean value 4.1 / 100,000) [Kondo, 1996]. Previous data have shown an unusually high incidence foci of motor neuron disease in the Kii Peninsula, Guam- and Mariana Island and West New Guinea. Since 1970, however, a dramatic fall in the incidence of ALS has been observed in these area, resulting in a rather homogeneous worldwide distribution [Yanagihara *et al.*, 1996]. More recent data shows a prevalence of about 0.8-2.6 people / 100,000 population per year [Belsh, 1996]. Statistical analysis revealed an increasing occurrence of the disease in the last few years, which can be attributed to an increased life expectancy rather than a true increase in the occurrence [Kondo, 1996]. There is a sexual prevalence, males are 1.5-2 times more affected than females which makes ALS unique among neurodegenerative diseases. Therefore, besides age, sex is the second most important risk factor in ALS. The importance of physical trauma as a risk factor, although repeatedly emphasized in the litearature, is still controversial [Belsh, 1996]. ALS exists also in familial form, which constitutes 5-10% of the total ALS cases.

1.4 Clinical and pathological features of ALS

Diagnosis of ALS

Currently, there is no specific marker or unique test for the diagnosis of ALS. Therefore, the diagnosis of ALS is based on an array of clinical, electrophysiological and neuroradiological tests. The goal is to identify the local or general involvement of the motor system and to exclude other conditions, which may mimic ALS. Clinical and pathological criteria for diagnosis have been defined at conferences held in El Escorial in 1994 and revised at Airlie House, Virginia [Brooks *et al.*, 2000]. The Airlie House criteria currently used for the diagnosis of ALS are shown in *Table 2.*

Table 2. Airlie House Criteria for the diagnosis of ALS

To be proved:	To be excluded:
Degeneration of lower motoneurons (clinical, electrophysiological, or neuropathological examinations)	Other disease processes that might explain the signs of LMN or UMN degeneration (electrophysiological, or pathological examinations)
Degeneration of upper motoneurons (clinical examination)	Other disease processes that might explain the observed clinical and electrophysiological signs (neuroimaging examinations)
Progression of the motor syndrome within a region or to other regions, as determined by case history or repeated examinations	

Pathology of ALS

The major pathological features of ALS are the degeneration of lower motoneurons (anterior horn cells), myelin pallor in the corticospinal projection pathway and the loss of large calibre axons from peripheral nerves [King and Mitsumoto, 1996]. The onset of symptoms seen in ALS patients is assumed to occur when approximately a 50-80% loss of motoneurons has been achieved [Brooks *et al.*,

2000]. These, almost dogmatic statements, however, are not completely supported by the most recent quantitative morphometric investigations [Ince, 2000]. This will be exemplified by the results of attempts to assay the number of upper and lower motoneurons.

Upper motoneurons: The most severely affected region is cortical layer 5, where an obvious absence of the giant pyramidal (Betz) cells can be noted, accompanied by reactive gliosis (astrocytosis) and diffuse microgliosis. Due to the lack of specific markers for Betz cells, these findings were based on conventional Nissl-stained material. Thus, shrinkage of giant pyramidal cells to the extent, which makes them indistinguishable from neighbouring pyramidal cells, could also explain the virtual absence of the Betz cells. Therefore, the previous findings might be at least partially confusing [Gredal et al, 2000].

Lower motoneurons: Studies based on 3-dimension morphometric probes demonstrated that the mode of neuronal degeneration in the case of lower motoneurons appears to be predominantely shrinkage, similar to upper motoneurons. Further evidence for the occurrence of neuronal shrinkage, prior to neuronal loss, comes from studies of peripheral nerves where quantitative analysis has shown a systematic reduction of axonal diameters which is more prevalent than an overall decrease in absolute numbers of axons [Dyck *et al.*, 1975]. Although, aspects of such morphological alterations might help in identifying the actual mechanism of motoneuron degeneration, in the case of ALS, the picture is rather complicated. Several recent studies revealed that in ALS motoneuron death follows neither apoptotic nor necrotic pathways but most probably takes place in a necrosis-apoptosis continuum, exhibiting intermediate forms with features of both modes of cell death [Ince, 2000].

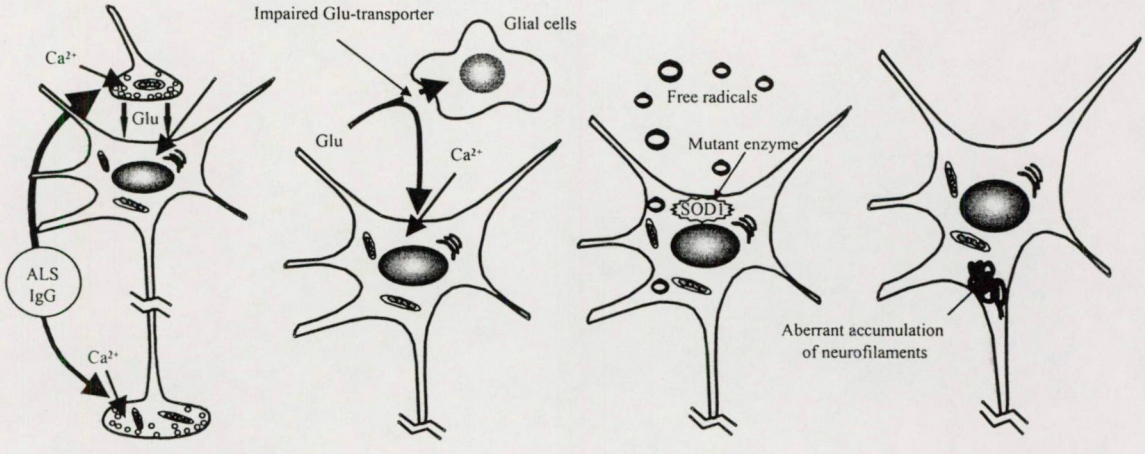
In skeletal muscles the typical changes have features of denervation atrophy with clusters of angular atrophic fibres. This clustering, fibre–type grouping is a non-specific consequence of a series of denervation and compensatory reinervation. The reinervation arises from the collateral sprouting of intramuscular axons [Wohlfart, 1957].

At present, besides classical pathological and histochemistry techniques, intracellular lesions of motoneurons are increasingly investigated by modern molecular techniques, which are based on specific intracellular markers. Such studies revealed proximal axonal enlargements in motoneurons [Carpenter, 1968],

fragmentation of the Golgi apparatus detected with organelle specific antibodies [Gonatas et al, 1992], and a range of intracellular inclusion bodies. The major type of inclusion seen in motoneurons are Bunina bodies (small eosinophilic bodies present in approximately 70% of autopsy cases of ALS), ubiquitinated inclusions (compared with other neurodegenerative diseases, those in ALS do not contain neurofilaments), and hyaline inclusions. The hyaline inclusions which are associated with the lesion of neurofilaments may occur as globules or in diffuse forms and are weakly immunoreactive with ubiquitin. In comparision with other neurodegenerative diseases, the above mentioned pathological changes are less characterized, they are less specific for ALS, furthemore, little is known about their evolution and their role in the disease.

1.5 Basic theories of the pathomechanism of ALS

ALS has an unknown etiology and there is a limited understanding of the mechanism of motoneuron injury and loss. Therefore, only hypotheses have been put forward so far to explain for the selective degeneration of motoneurons in ALS. The present hypotheses rely on isolated causative factors or idividual features of the pathological mechanism of the disease, since there is no generally valid concept available, which explains all the aspects of ALS. The basic mechanisms of the four most popular theories, based on phenomena involving autoimmunity, excitotoxicity, oxidative stress and cytoskeletal abnormalities are illustrated in Fig. 1, below.



Autoimmunity

Any hypothesis for the patomechanism of ALS must account for the specificity, i.e. why only motoneurons are involved in the disease, and for the selectivity, namely why some motoneurons are affected while others are not. The fact that there is a specific motoneuronal vulnerability favours the idea that autoimmune mechanisms may be involved in the pathogenesis of ALS. A support for this hypothesis first came from studies which indicated the presence of antibodies (IgG) for L-type voltage gated Ca^{2+} channels in 75% of sporadic ALS patients [Smith el al., 1992]. Other studies have shown that ALS IgG binds the ionophore containing the $\alpha 1$ subunit of the voltage-gated Ca^{2+} channels [Kimura et al., 1994]. ALS IgG can interact and alter the function of muscular L type calcium channels [Magnelli *et al.*, 1993], and neuronal N-/ P-/ Q-channels, as well [Llinas *et al.*, 1993]. The interaction of ALS IgG with these calcium channels enhances inward calcium current because of the prolonged channel open time [Mosier *et al.*, 1995] and increases intracellular calcium as was demonstrated in a motoneuron cell line. These results are in agreement with *in vivo* studies where IgG reactivity [Engelhardt and Appel, 1990] and limphocytic infiltrations in spinal cords of ALS patients were seen [Engelhardt, et al., 1993]. The release of inflamatory cytokines may account also for the progression of ALS: first the limbs are affected on one side and then the disease progresses to the opposite side and finally attacks the muscles of the head.

Despite such observations, at present we cannot say for certain that ALS is an autoimmune disease. The failure of immunosupression in ALS [Brown et al., 1986; Drachman et al., 1994], and the lack of success of inducing motoneuron degeneration in animals by IgG inoculation, are the main obstacles against proposing an immune-mediated etiology.

Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system, which acts through ionotropic or metabotropic receptors. Excessive glutamate accumulation in the synaptic cleft or a functional alteration of the glutamate receptors may lead to an overactivation of the synapse, which finally results in an

excitotoxic neuronal death [Choi, 1992]. The excitotoxicity hypothesis is supported by observations, which suggest that the excitotoxic lesions play an important role in motoneuron degeneration. It has been shown that glutamate levels in the plasma of ALS patients were two fold higher compared to controls (Plaitakis and Caroscio, 1987) and high levels of glutamate and aspartate have been detected in cerebrospinal fluid from ALS patients (Rothstein et al., 1991). A rapid uptake of the released glutamate is accomplished by the EAAT1 and EAAT2 glutamate transporters present in astrocytes [Mitsumoto et al., 1997]. Thus, a loss or a decreased activity of these glutamate transporters could lead to an increased extracellular concentration of glutamate. A support for this idea came from the study of Rothstein and colleagues [1995] who found that majority of sporadic ALS patients (about 65%) have a reduced level of the EAAT2 astroglial glutamate transporter in the motor cortex and spinal cord.

In other studies it was documented that some ALS patients have alterations of the AMPA glutamate receptor structure, which were sufficient to induce excitotoxicity. Under normal physiological conditions AMPA receptors are impermeable to calcium. However, if one of its subunits is missing (GluR2), the AMPA receptors become permeable to calcium ions [Sommer et al., 1991]. During neurotransmission this alteration induces an increased calcium influx into the postsynaptic motoneurons, which may trigger motoneuron injury. The low expression of the GluR2 protein subunit [Williams et al., 1997], or of the mRNA for the GluR2 [Takuma et al., 1999] seen in ALS patients, can indeed induce the above-mentioned pathological processes.

Like the autoimmunity hypothesis, the excitotoxicity hypothesis does not provide an explanation for the familiar form of ALS. Moreover, there is no specificity for the reduced expression of the glutamate transporters, since alterations could be also found outside of the motor system, and in other neurodegenerative diseases, as well. Finally clinical experience suggest that neither Gabapentin (which inhibits the syntesis of glutamate) nor Riluzole (which inhibits the glutamate release), could influence the outcome of the disease [Rowland, 2000].

Oxidative stress

It is well documented that cellular injury can be induced by overproduction of free radicals, which exposes the tissue to oxidative stress [McCord, 1985]. The

phenomenon regained the attention of scientists investigating the patomechanism of ALS after discovering that missense mutations in the SOD1 (superoxide dismutase 1, or Cu-Zn SOD) gene are present in some 1-2% of ALS cases [Rosen et al., 1993]. SOD1 catalyses the neutralization of superoxide anions in physiologic and pathologic conditions [Halliwell, 1992]. It is not yet known whether a loss of function or a toxic gain of function of mutant SOD1, or both, are responsible for motor neuron injury in ALS. There is, however, compelling evidence favouring the gain of function hypothesis. When testing the disease mechanism, the elimination or elevation of wild-type SOD1 was found to have no effect on mutant-mediated disease [Bruijn et al., 1998]. Many of the missense mutations in SOD1 have the potential to alter the active site of the SOD1 enzyme. X-ray crystallographic studies have demonstrated that the active channel of the mutant SOD1 protein is larger than that of the wild-type enzyme, enabling greater accessibility or unshielding of the cooper active site [Deng et al., 1993]. Such changes could allow mutant SOD1 to react with additional substrates such as hydrogen peroxide and peroxynitrite [Beckman et al., 1993; Wiedau-Pazos et al., 1996]. These aberrant reactions could lead to an increased cellular production of highly reactive and damaging free radical species such as hydroxyl radicals and nitronium ions, and nitration of tyrosine residues of susceptible proteins. It is also feasible that not the aberrant oxidative activity of SOD1 which is responsible for the neurotoxicity, but rather, the increased aggregation of this protein may contribute to neuronal degeneration [Cleveland and Liu, 2000]. Such protein aggregates can be commonly seen not only in ALS but in other neurodegenerative diseases, as well.

Many transgenic mouse models expressing human mutant SOD1 have been generated, but these mice develop clinical and neuropathological alterations, which only partly resemble changes observed in human ALS. These animals die at the age of 5-10 months, depending on the quality and quantity (copy number) of the transgene employed [Gurney et al., 1994]. Although transgenic animals are the most commonly used models in ALS, there are some problems regarding the extrapolation of the results to the human case. Despite the positive benefits of Riluzole and vitamin E in transgenic mice [Gurney et al., 1996], for example, these agents proved ineffective in clinical trials with ALS patients [Rowland and Shneider, 2001]. Moreover, the oxidative stress hypothesis offers explanation only for a minor, not more than 1-2% of diagnosed ALS cases.

Neurofilament inclusions and altered axonal transport

The idea that aberrant accumulation of neurofilaments may play a role in ALS was first suggested by the discovery that accumulation and abnormal assembly of neurofilaments are common pathological hallmarks in the majority of ALS patients [Hirano, 1991]. However, it is not yet known whether the abnormal accumulation of neurofilaments is a consequence of axonal transport blockade or whether the accumulation of these proteins leads to secondary impairment of axonal transport [Shaw and Egget, 2000].

The most abundant structural proteins in neurons are the intermediate neurofilaments (IF) which are assembled from three polypeptide subunits of differing molecular weights: NF-light (NF-L), NF-medium (NF-M) and NF-heavy (NF-H). The first evidence that neurofilament accumulations can cause motoneuron disease emerged from the expression in transgenic mice of three or four times the normal level of NF-L [Xu et al., 1993]. Despite the fact that the morphological changes were similar to those seen in humans (perikaryonal neurofilament accumulation) these mice did not exhibit motoneuron death. Next, to examine whether aberrant neurofilament subunits could be a direct cause of motoneuron degeneration, several lines of transgenic mice were constructed to express mutations of NF-L. In this experiment motoneuron death could finally be induced [Lee et al., 1994]. Based on such results, large-scale investigations were initiated, and mutation of the NF-H subunit gene could be found in approximately 1% of ALS population [Bruijn et al., 2000].

The presence of aberrant neurofilament accumulations in familial ALS suggests that neurofilaments may be a target for free radicals and therefore, may act as toxic intermediates in the disease. The results of the studies, which tried to clarify this assumption are still controversial. In one study, in which mice deleted for the NF-L gene were bred with mice that express the mutant SOD1 gene, the absence of neurofilaments delayed disease onset and produced a significant increase in life span [Bruijn et al., 2000]. Since in these animals only perikaryonal inclusions could be seen, it was concluded that neurofilament accumulation attenuates the damaging effect of SOD1 mutant enzyme, likely by means of buffering the calcium load at the perikaryon by the accumulated proteins [Julien, 2001]. In another study the motoneuron degeneration induced by periferin (IF protein) overexpression was dramatically accelerated by the absence of NF-L [Julien, 2001].

In summary, it can be concluded that cytoskeletal abnormalities may be viewed as important risk factors in ALS, however, different types of IF inclusions may have unique effects on motoneuron function.

1.6 Hypothesis and aims of the study

Working hypothesis

Despite the accumulating data that autoimmunity, excitotoxicity and the aberrant accumulation of neurofilaments are involved in sporadic ALS, as well as, mutant SOD1 in familial ALS, none of these mechanisms explains sufficiently the observed selective vulnerability in motoneuron populations. Although animal models developed on the basis of each theory could be effectively used to study some individual molecular pathways, the development of the disease was always attributed to a single etiological factor. Each model showed numerous pathological characteristics similar to those found in the human disease, yet it is not possible, from these studies, to conclude that ALS is purely an autoimmune, excitotoxic, free radical-mediated or neurofilamentary disease. Instead, based upon our present knowledge, it is more likely that ALS is a multifactorial disease, in which the already known, or as yet unknown, pathogenic processes contribute cooperatively to the development of the disease [Eisen, 1995].

Our hypothesis is that in the development of a uniform clinico-pathologic profile of ALS the crucial step is the irreversible damage of the calcium homeostasis of motoneurons. Changes in the levels of intracellular calcium could link, synchronise and amplify the various processes, regardless of whether the initial trigger to the lesion of motoneurons was associated with oxidative stress, immune mechanisms, excitotoxic activity, or other genetic or environmental factors, yet to be defined.

Prior studies supporting our hypothesis

The neurofilament accumulation, which is typical of ALS autopsy samples, is also observed in motoneurons from the SOD1 transgenic animals used as model systems of the free radical lesion. Through genetic manipulation, neurofilament overexpressing and knock-out (-/-) mice have been developed [Collard *et al*, 1995].

Crosses of SOD1 transgenic mice with NF-L -/- mice results in a significant retardation of the experimental disease [Williamson *et al*, 1998], which indicates that neurofilament accumulation plays an active role in neuronal damage caused by free radicals. The passive transport of ALS IgG into mice, can induce the phosphorylation of neurofilament H [Engelhardt *et al*, 1995], which further strengthens the postulate that these differentiated processes are linked to one another.

Examination of SOD1 transgenic mice shows that the astrocytes are also damaged. In the final stages of the modelled disease, it is possible to detect a 50% decrease in the amount of glial glutamate transporter (EAAT2) [Bruijn *et al*, 1997], indicating that the SOD1 mediated free radical damage may result in secondary excitotoxic lesions. The relationship of both excitotoxicity and oxidative stress, with the calcium regulation, is well known in the literature. The binding of glutamate to excitatory amino acid receptors may induce substantial calcium influx [Choi, 1988], or, vice versa, an increase in transmitter release may be evoked by an increase in the influx of calcium. Furthermore, increase in calcium levels may result in elevated free radical production through the interactions of calcium-activated enzymes (phospholipase A_2, calpain I, II, xanthin-oxidase) [Mitsumoto *et al*, 1997]. Recent studies of intracellular calcium homeostasis and the levels of free radicals suggest more complex and synergistic relations [Mattson, 1998].

If intracellular calcium levels are, in fact, the key factors in ALS-type motoneuron injury, is it probable that the stability of calcium regulation, for example through calcium-buffering by calcium-binding proteins, and the vulnerability of the motoneurons are interconnected variables. On the basis of immunocytochemical studies of ALS autopsy samples it seems that the decrease or absence of calbindin-D_{28K} and parvalbumin, in populations of motoneurons parallels their vulnerability during the course of the disease. In the early stages of the disease, the affected cells (spinal motoneurons, XII nucleus) do not contain these proteins, while those cells which are only affected later (III, IV, VI nuclei, Onufrowitz nucleus) may be immuno-labelled for these proteins [Alexianu *et al*, 1994, Elliott and Snider, 1995]. Experiments carried out on an *in vitro* motoneuron cell line exhibited compatibible results: undifferentiated VSC 4.1 motoneurons remained resistant to the effects of IgG, purified from an ALS patient, while they contained calbindin-D_{28K} and parvalbumin. Yet, after their differentiation, once they had lost these proteins, the same IgG resulted in calcium influx and caused cell death [Ho *et al*, 1996]. If these

cells were transfected with calbindin-D_{28K} cDNA, the level of this calcium-binding protein remained high, even in differentiated cells and the cell remained viable when treated with ALS IgG [Ho *et al*, 1996]. The precise physiological functions of calbindin-D_{28K} and parvalbumin are not yet known. However, based on these observations and other data from the literature, they appear to play a role in the stabilisation of calcium homeostasis of the cell, either through the regulation of calcium influx, sequestration of intracellular calcium, or calcium efflux [Lledo *et al*, 1992].

Aims of the study

As we have seen, several attempts have been made on diverse theoretical backgrounds for the interpretation of the pathomechanism of ALS. These theories, while successfully explain certain cascades of molecular events, cannot account for the uniform clinico-pathological picture of the disease, particularly, when the apparently distantly related sporadic and familial forms are considered. Our approach is based on the assumption that the initial injury may be triggered by a variety of causes, then the primary pathology initiates a cooperative process during which the sensitivity of neurons to further damaging insults is continuously increasing. In this positive-feedback loop, calcium-dependence may be a common factor in all of the stages. Our previous experiments have shown that in autoimmune- or purely oxidative stress induced lesion, the calcium regulation of motoneurons is disturbed, degenerative changes are induced which may lead to the destruction of the cells. In our present experiments, we are extending our studies to other ALS models, with the final aim to prove that the observed components of the pathomechanism of ALS could be linked to each other through their ability to increase the calcium level of motoneurons. Furthermore, we attempt to study the function and relationship of some cellular mechanisms (calcium buffering, antioxidant defense) supposed to play a major role in the pathomechanism of ALS, according to the present theories. Our specific aims are:

- Ultrastructural characterization of motoneuronal calcium distribution in excitotoxic-stress induced lesions *in vivo*.

- Investigation of the similarities of the disturbed calcium homeostasis of motoneurons with different Ca-binding protein content in the autoimmune- and oxidative stress model.

- Investigation of the role of SOD1 in the stabilization of calcium homeostasis of parvalbumin-containing and parvalbumin-deficient motoneuron populations using SOD1 knock-out animals.

2. Material and Methods

2.1 Animal strains

To study the alterations of calcium distribution of motoneurons in animal models of sporadic and familial ALS, experimentally treated or genetically modified animals were used, respectively. All animal experiments were performed according to the appropriate institutional guidelines and govermental laws for animal protection.

Animals for the sALS models

Passive transfer of ALS IgG: To mimic the chronic conditions present in the patients Wistar rats were inoculated regularly with intraperitoneal injection of IgG from ALS patients. IgG purified from the blood of a patient with demyelinating polyneuropathy was used as control. Injections were started on the day of birth of animals to reduce the response of their immune system against the foreign IgG. The dose of the injected IgG, with the initial amount of 10 mg protein/day, was adjusted continuously to the weight of animals. Rats were killed at the age of 30 days.

Excitatory amino acid application (EAA): Male, 21 days old CFY rats were used for the experiments, which were sorted to control- and EAA-treated groups. Rats in the EAA-treated group were allowed to survive for 1, 3, 6, and 24 h following treatment with D,L-homocysteic acid (DL-HCA), a non-specific agonist of AMPA/kainate and NMDA receptors (730 μg DL-HCA injected in 10 μl of 400 mM solution, pH 7.4). Rats were first deeply anesthetized with intraperitoneal injection of xylazine (12 mg/kg) and ketamine (108 mg/kg), then their spinal cords were exposed, by removing the posterior arches of the lumbar vertebrae over 2-3 segments, finally, DL-HCA was injected subdurally. Control rats were treated similarly, but injected with vehicle only (10 μl distilled water with adjusted pH to 7.4). After the surgery the skin over the spine was sutured and the animals were allowed to recover.

Animals for the fALS models

SOD1 mutant transgenic animals: The human SOD1 transgene, containing the $G^{93}\rightarrow A$ mutation [Rosen et al, 1993], including the promoter and enhancer sequences for transcription, were injected into the fertilized eggs of C57BL6 × SJL F1 hybrid mice [Gurney et al, 1994]. Founder animals were bred with C56BL6 mice. In their progeny ($G1H^+$ line) the transgene was identified by polymerase chain reaction amplification of tail DNA. The transgene copy number (n=20) was determined by Southern DNA hybridization. A $N28^+$ line was developed as a control strain, in which the wild type human SOD1 gene was expressed with similar copy number. Mice were obtained from Jackson Laboratory (Bar Harbor, Maine) for our experiments.

SOD1 knock out animals: SOD1 knock out animals were developed on the basis of CD-1 mouse strain. Using homologous recombination in embryonic stem cells, the entire coding sequence of the mouse SOD1 gene has been deleted. These mutant cells were used to create mice homozygous for the SOD1 deletion ($SOD1^{-/-}$) [Reaume et al, 1996]. Blood samples were used to confirm that the targeted deletion of SOD1 gene resulted in no detectable amount of Cu/Zn SOD protein and only a minor level of enzyme activity. CD-1 mice were used as controls. Mice were obtained from Cephalon, Inc. (West Chester, Pennsylvania).

2.2 Specimen preparation for eletron microscopic histochemistry

The aim of our specimen preparation is to preserve both the organization of the tissue and the distribution/amount of calcium at the ultrastructural level. This dual goal requires a fixative of special composition, which should be ideally applied by perfusion through the blood circulation. Thus, all the animals were first deeply anaesthetized, then transcardially perfused with the fixative, containing 3% glutaraldehyde and 90 mM potassium oxalate (pH 7.4, adjusted with KOH) [Borgers et al, 1979, 1981]. In some experiments, prior to the application of the fixative, the blood was flushed out with a plain 90 mM potassium oxalate solution (pH 7.4), but the two procedure yielded equivalent structural preservation and calcium retention. Following perfusion, depending on the actual experimental paradigm, samples were dissected out from the (a) hindlimb interosseus muscle, (b) external eye (superior

rectus) muscle, (c) lumbar segment of the spinal cord, and (d) a region from the brainstem containing the oculomotor nucleus. After dissection, specimens were placed in the primary fixative for 24h at 4^0C, then rinsed for 15 min in 90 mM potassium oxalate, containing 7.5 % sucrose, and postfixed for 2 hours in a solution of 1 % osmic acid and 2% potassium pyroantimonate (pH adjusted to 7.4 with acetic acid). Following postfixation tissue samples were rinsed in distilled water (pH 10.0 with KOH), dehydrated in graded series of ethanol, processed through propylene oxide and embedded in Durcupan ACM. Blockes were polymerized for 2 days at 56^0C. After polymerization 0.5 μm thick sections were cut on a RMC MT-7, or on a Reichert UM II ultramicrotome and stained according to Richardson [1960]. Sections were screened in a light microscope for the presence of preterminal branches of the motor nerves, or large motoneurons in muscle samples or nerve tissue, respectively. For an unbiased selection of the fields for microscopic examinations random numbers were generated ($1 < n < 20$) and "n" number of 0.5 μm thick sections were cut and discarded. Then, from this new starting point, ultrathin sections were obtained at regular, 20 μm intervals [Cruz-Orive, 1988; Gundersen et al, 1987]. Ultrathin sections were mounted on formvar-coated single-hole copper grids, stained with uranyl acetate [Hayat, 1970] and lead citrate [Reynolds, 1963], then examined in a Philips CM 12 or Zeiss CEM 902 electron microscope.

2.3 Transmission electron microscopic examinations

When only qualitative description of the calcium distribution and the structural alteration of the tissue was aimed, sections (containing neuromuscular synapses or motoneurons from different brain regions) were studied carefully at a low-to-medium instrumental magnification (3,000 – 10,000 ×) to determine the general features of the objects to be characterized. Conventional bright field imaging with instrumental parameters of 80 kV accelerating voltage, and 30 μm objective aperture was used for this purpose. Conclusions with regard to the changes compared to appropriate controls were regularly established on the basis of inspecting of 30-40 randomly selected features. Then, sample photographs were made in suitable primary magnifications, regularly at 10,000 – 30,000 ×, documenting the noticed changes in the best way. Micrographs were further enlarged photographically by 2.7 ×.

2.4 Quantitative characterization of calcium distribution

In some instances, minute changes in the calcium distribution and/or content of neurons, or axon terminals could not be determined by a plain visualization of the sections. In these cases quantitative characterization of the amount and distribtution of electron dense deposits (EDDs), assumed to correspond to those of tissue calcium, was decided. Since simple grain counts would have consituted an imprecise measure of the amount of granules, because EDDs differ in shape and size, their volume fraction was determined in relation to appropriately selected reference (cytoplasmic-, axoplasmic-, or mitochondrial-) volumes. Volume fractions (volume-to-volume ratios) were determined on systematic-randomly made electron microscopic prints, using point counting procedures [Mayhew, 1992]. To determine mitochondrial calcium volume fractions, mitochondrial profiles were first marked then a sampling grid was superimposed on the micrographs. Grid points lying over EDDs (P_{Ca}) and the mitochondrial volume (P_{mit}) were counted, then pooled to each animal/muscle, or neuron type. (ΣP_{Ca}, ΣP_{mit}). The $\Sigma P_{Ca}/\Sigma P_{mit}$ ratio was introduced to characterize the mitochondrial calcium content of the particular presynaptic terminal and motoneuron/animal. Similar calculations were applied to characterize non-mitochondrial (axoplasmic- and cytoplasmic-) calcium volume fractions at the neuromuscular synapses and perikaryonal regions.

2.5 Analytical microscopy

Since the conclusions with regard to the calcium distribution of the tissue, made on the basis of point counting procedures, depended on the specificity of the histochemical reaction, the calcium content of the EDDs was time-to-time controlled, using the electron spectroscopic imaging (ESI) mode of the microscope [Bauer, 1988]. For ESI, 20-30 nm thin unstained sections were prepapred, the significant calcium distribution was determined in randomly selected regions of the sections, then correlated with the distribution of the EDDs. To perform the ESI analysis individual images were recorded at 20,000-30,000× primary magnification by 80 kV accelerating voltage of the microscope, using an objective aperture of 90 μm and a

spectrometer entrance aperture of 650 μm with a slit width of 8-10 eV. Element specific- and background image pairs were recorded at energy loss values of 355 eV and 310 eV, respectively. Between 500 and 600 video images were recorded and averaged for each energy loss value. Next, the net calcium distribution was determined by subtracting the background image from the „edge" image. The significant calcium distribution pattern was visualized by color-coding the difference image according to the „mean" + 2.5×s.d. → „mean" + 6×s.d. rule. This way pixels identifying significant signal were colored and retained in the analytical image, while the non-significant signal was eliminated. To check the correlation of the true calcium distribution with the pattern of the EDDs, this color-coded image was superimposed onto the black-and-white fine-structural digital image of the same area, recorded at the carbon absorption edge (dE=250 eV) (Figure 2.).

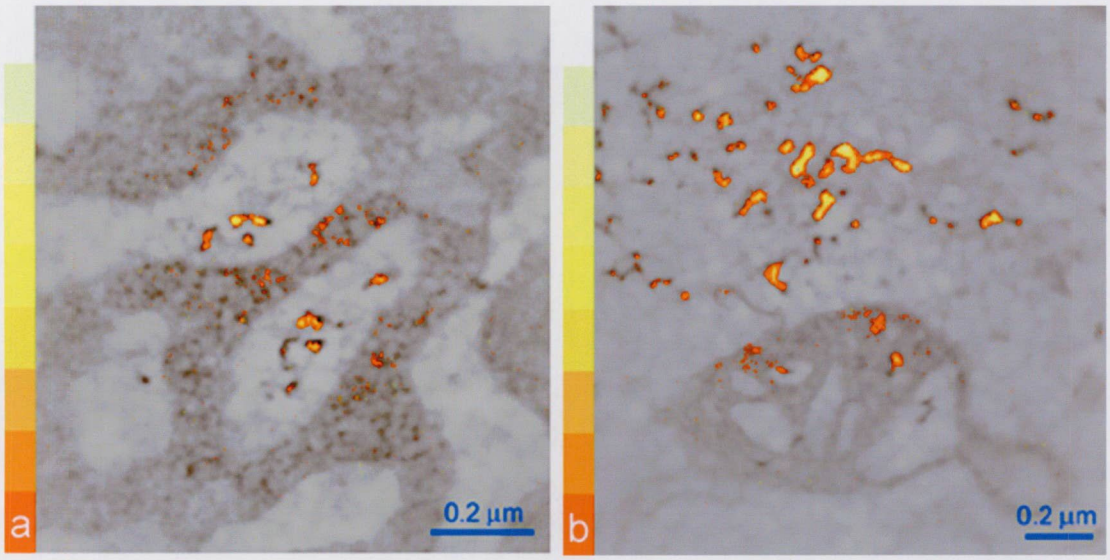


Fig. 2. Calcium distribution is exemplified in a: dilated cisterns of the endoplasmic reticulum with internal calcium precipitates, and b: partially swollen mitochondrion surrounded by scattered cytoplasmic calcium deposits. The true (colored) calcium signal can be adequately correlated with the EDDs.

3. Results

3.1 Ultrastructure and calcium distribution of spinal motoneurons in excitotoxicity

The specific aim of these experiments was to document if *in vivo* exposure of motoneurons to excitotoxins could result in their degeneration and, particularly, if destabilization of their calcium homeostasis could be induced similarly to other examined animal models of ALS. Thus, ultrastructural alterations of spinal motoneurons of rats were analyzed and corresponding changes in the intracellular calcium distribution were characterized in a 24 hours interval following subdural injection of DL-HCA. Qualitative description of the evolution of alterations in the morphology and calcium distribution of motoneurons was performed. At the earliest time point, immediately after DL-HCA injection (0 h survival time), neither structural changes, nor shifts from the normal calcium levels could be documented (Fig 3).

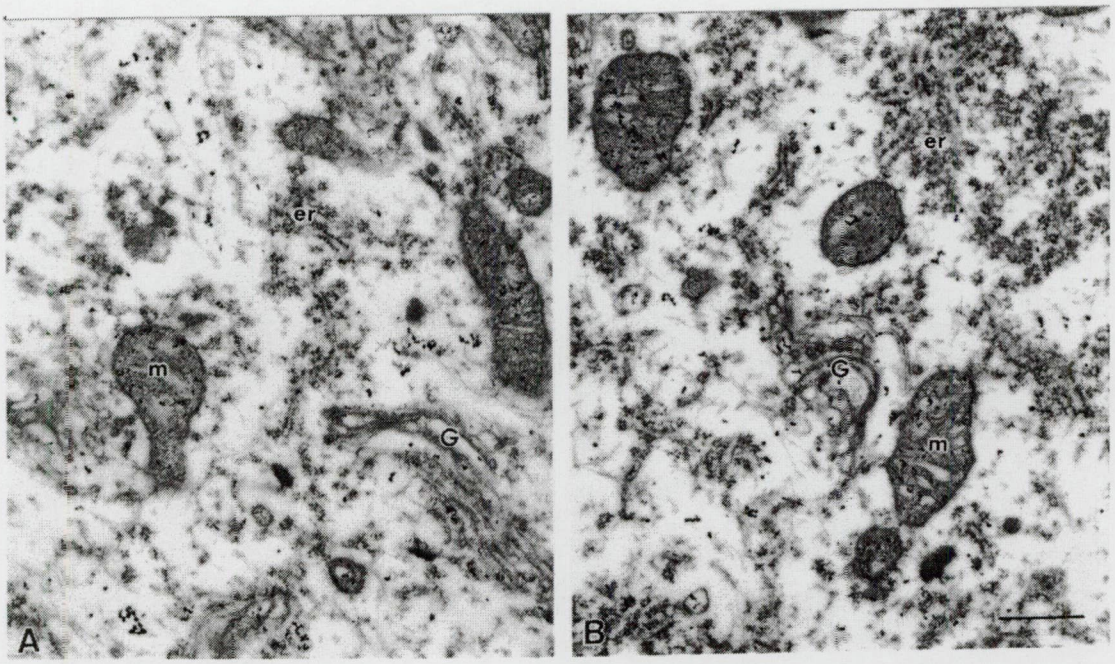


Fig.3. (A) Electron micrograph of a motoneuron from the lumbar section of the spinal cord of a 21-day-old untreated rat illustrates the normal ultrastructure and physiologic calcium distribution. (B) At 0 h survival time neither morphological changes nor elevated calcium can be seen. Oxalate-pyroantimonate fixation. m: mitochondrion, er: endoplasmic reticulum, G: Golgi apparatus, arrowhead: calcium deposits; bar: 0.5 μm.

By 1 hour following DL-HCA injection generally no structural changes and, at most, an increase in the cytoplasmic calcium level could be seen (Fig 4A). Some initial signs of degeneration only occasionally could be noticed, such as dilatation of cisterns of the endoplasmic reticulum (ER) and Golgi complex paralleled with calcium accumulation (Fig 4B).

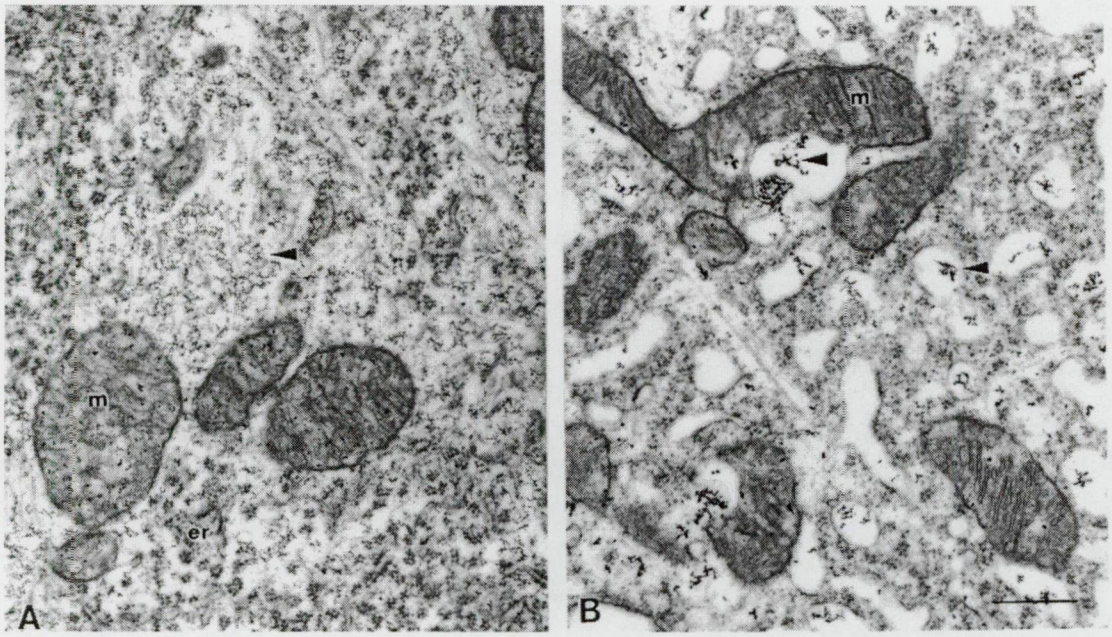


Fig.4. Electron micrographs of motoneurons from the lumbar segment of the spinal cord of a DL-HCA injected rat at 1 hour survival time. (A) Regularly, no structural alterations are present, and only an elevated level of cytoplasmic calcium can be seen. (B) Exceptionally, slight degeneration of the endoplasmic reticulum, accompanied by calcium accumulation, can be observed. The majority of mitochondria are uninfluenced with only restricted calcium accumulation. m: mitochondrion, G: Golgi apparatus, arrowhead: calcium deposits bar: 0.5 μm.

Considerably larger heterogeneity of the reactions of motoneurons to the EAA application could be noticed at 3 h survival time. The changes, as illustrated in Fig 5, ranged from a brief increase of cytoplasmic calcium level to a substantial calcium accumulation by organelles, accompanied by serious degeneration of mitochondria, or the ER/Golgi system, or both.

Six hours after EAA injection strong fragmentation and vacuolization of the ER and the Golgi apparatus could be seen (Fig 6A). Clusters of calcium precipitates were regularly present in the swollen cisternae of the organelles. At this stage mitochondria were usually shrunken, with no visible calcium deposits. By 24 h following DL-HCA injection motoneurons typically lost their structural integrity with

only few cytoplasmic organelles remaining recognizable (Fig 6B). Very low calcium level was regularly present.

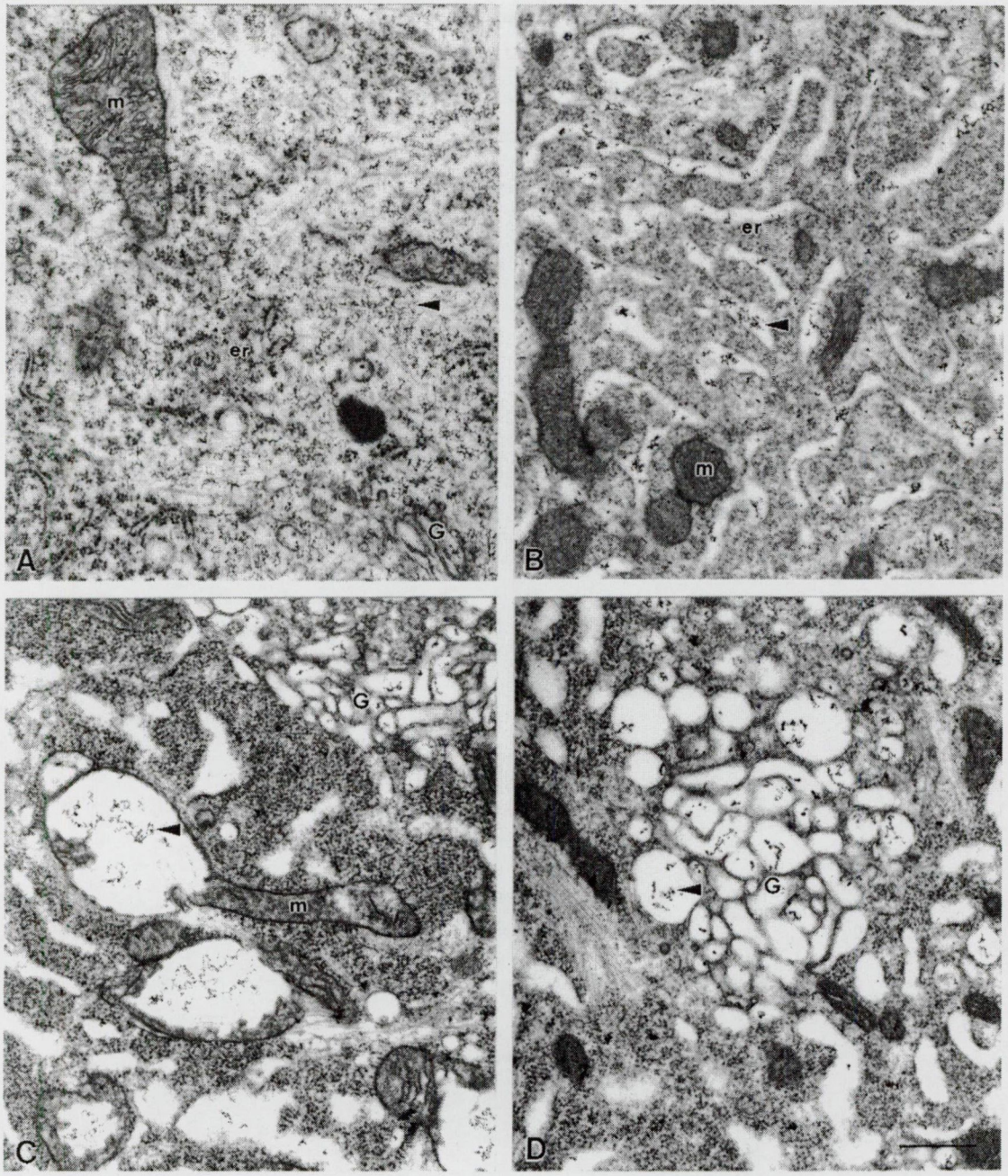


Fig. 5. Electron micrographs of spinal motoneurons from DL-HCA injected rats in different stages of degeneration. (A) Plain cytoplasmic calcium increase with no structural damage. (B) Slight degeneration of the ER with a moderate calcium accumulation. (C) Localized mitochondrial swelling with calcium accumulation. The neighboring organelles are devoid of calcium. (D) Advanced vacuolization of the Golgi complex and ER with calcium accumulation. Mitochondria are shrunken and deficient of calcium. m: mitochondrion, er: endoplasmic reticulum, G: Golgi apparatus, arrowhead: calcium deposits; bar: 0.5 μm.

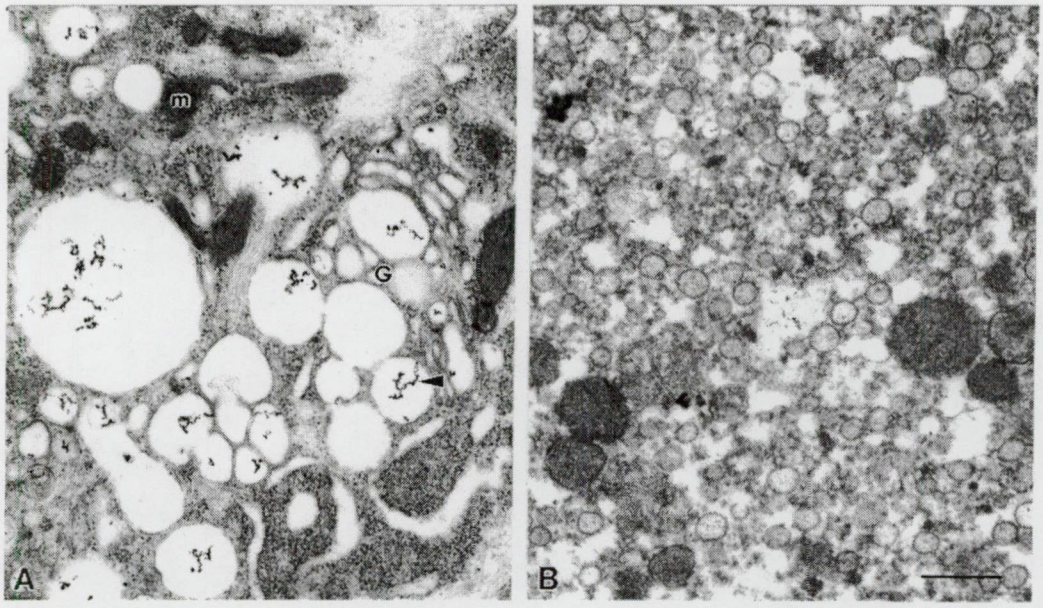


Fig. 6. Motoneurons from the lumbar section of the spinal cord from DL-HCA injected rats. (A) At 6 h following injection advanced vacuolization of the Golgi complex and the endoplasmic reticulum could be observed, which was paralleled by calcium accumulation. The neighboring mitochondria were regularly shrunken with no visible calcium. (B) At 24 h survival time complete disintegration of the cytoplasmic structure could be seen which was accompanied by depletion of the calcium from the organelles. G: Golgi apparatus, arrowhead: calcium deposits; bar: 0.5 μm.

3.2 Similar alterations of the motoneuronal calcium induced in the autoimmune- and oxidative stress models of ALS

The availability of animal models of both the sporadic- and familial forms of ALS provides the opportunity of the direct comparison of the similarities of degeneration of motoneurons and the corresponding changes in their intracellular calcium. The particular aim of this study was to prove that such animal models, developed on different etiological backgrounds, could manifest the same pattern of the altered calcium distribution of motoneurons. If this aim is achieved, the results could provide additional support for the idea that impaired calcium homeostasis might be responsible for the uniform clinico-pathological picture of ALS. Furthermore, these models afford the possibility of a more detailed analysis of the alteration of motoneuron populations, differently affected in the course of the disease, which could provide more details about the mechanism of the motoneuronal lesion during ALS.

For these experiments, to approach the chronic conditions exist in the patients as well as in the transgenic animals (modelling familial ALS) a slight modification of the passive transfer paradigm was applied: newborn rats were used, and daily IgG injections were made. Neuromuscular synapses in the hindlimb interosseus muscle and in the external eye muscle (superior rectus) were qualitatively characterized, representing vulnerable and viable motor axon terminals, respectively. In the interosseus muscles, obtained from both models, increased calcium could be seen in the mitochondria and synaptic vesicles (Figure 7), which was in good aggreement with the findings obtained by analyzing muscle biopsies from ALS patients.

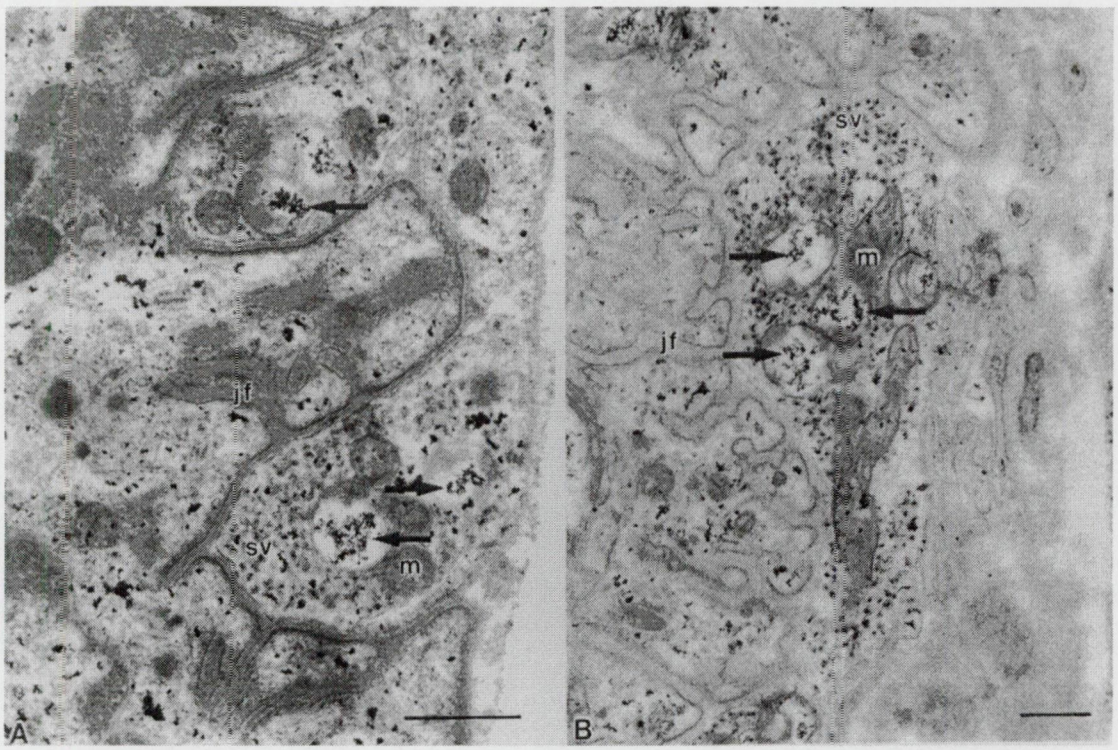


Fig. 7. Calcium accumulation and degeneration of motor axon terminals in the interosseus muscle of SOD1 mutant mice (A) and rats injected with ALS IgG (B). Qualitatively, the type of generation (rupture of the mitochondrial structure) and the degree of calcium accumulation are comparable to each other. m: mitochondrion, jf: junctional folds, sv: sypatic vesicles; bar: 0.5 μm.

In contrast to the motor axon terminals of the interosseus muscles, in the oculomotor nerve terminals no mitochondrial degeneration/calcium accumulation could be seen. However, vacuolar structures, called as endosomes, with characteristic size of 2-3 μm, could be often seen (Figure 8). The majority of intraterminal calcium – which increased compared to the corresponding controls, like in the interosseus

muscles – was mainly concentrated in these structures. Though in most of the sections endosomes appeared intracellularly, serial sectioning of the blocks containing the superior rectus muscles from both the SOD1 transgenic mice and ALS IgG injected rats revealed that they were in contact with the extracellular space (Figure 8C, 8D). Since these connections were usually present in a single section, we concluded that they were confined to a narrow area, representing a fine tunnel to the extracellular space. Since these structures developed only in the terminals of the resistant type of motoneurons, we hypothesized that they may represent a component of the cellular defense system against the high calcium load in these models.

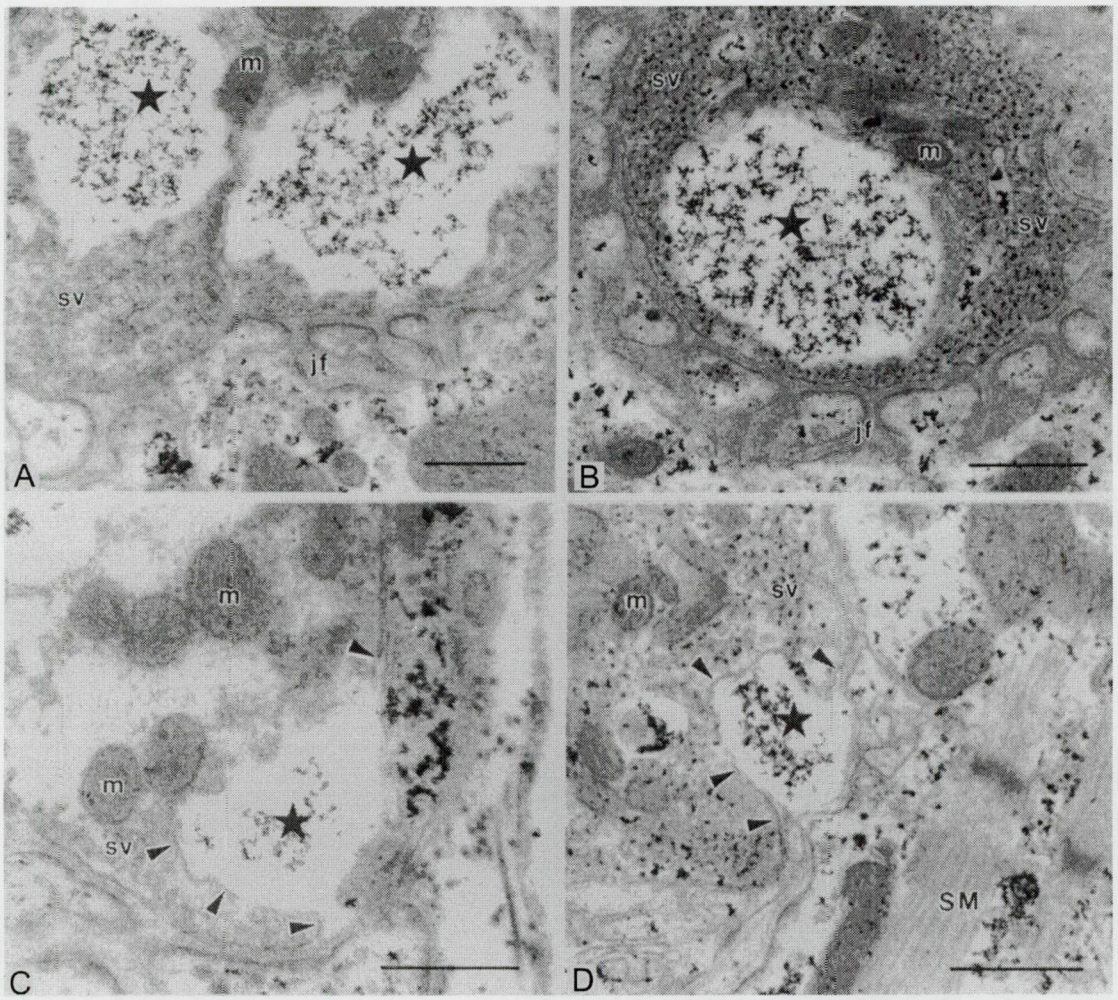


Fig. 8. Oculomotor nerve terminals from SOD1 mutant mice (A, B) and from rats injected with ALS IgG (B, D). Regularly, large vacuolar structures could be seen intracellularly, which concentrated the majority of intracellular calcium (★), however, their connection with the extracellular space could be proved by serial sectioning (C, D). m: mitochondrion, jf: junctional folds, SM: striated muscle, sv: synaptic vesicles, arrowheads: connection of the endosomes to the extracellular space. Bar: 0.5 μm.

3.3 Ultrastructure and calcium distribution in spinal- and oculomotor neurons of SOD1 knock-out mice

Similarities of the profiles of the altered calcium distribution of motoneurons in the models of sporadic- and familial ALS suggested, that impaired handling of the intracellular calcium level might be indeed responsible for their degeneration. Since the exact function of the mutant SOD1 enzyme was not known, it was desired to rule out the contribution of a deficient cytoplasmic antioxidant system to the observed collapse of the calcium homeostasis of the vulnerable motoneurons. The development of mice deficient of the SOD1 enzyme provided an ideal tool for this examination.

A detailed characterization of the calcium distribution of a prototype of vulnerable (spinal) and resistant (oculomotor) neurons was performed at early (3 months) and advanced adult age (11 months) of SOD1 knock-out mice, and the results were compared to age-matched controls. Besides qualitative description, cytoplasmic and mitochondrial calcium levels were characterized quantitatively, too, by expressing the relative volume (volume fraction) of electron dense deposits.

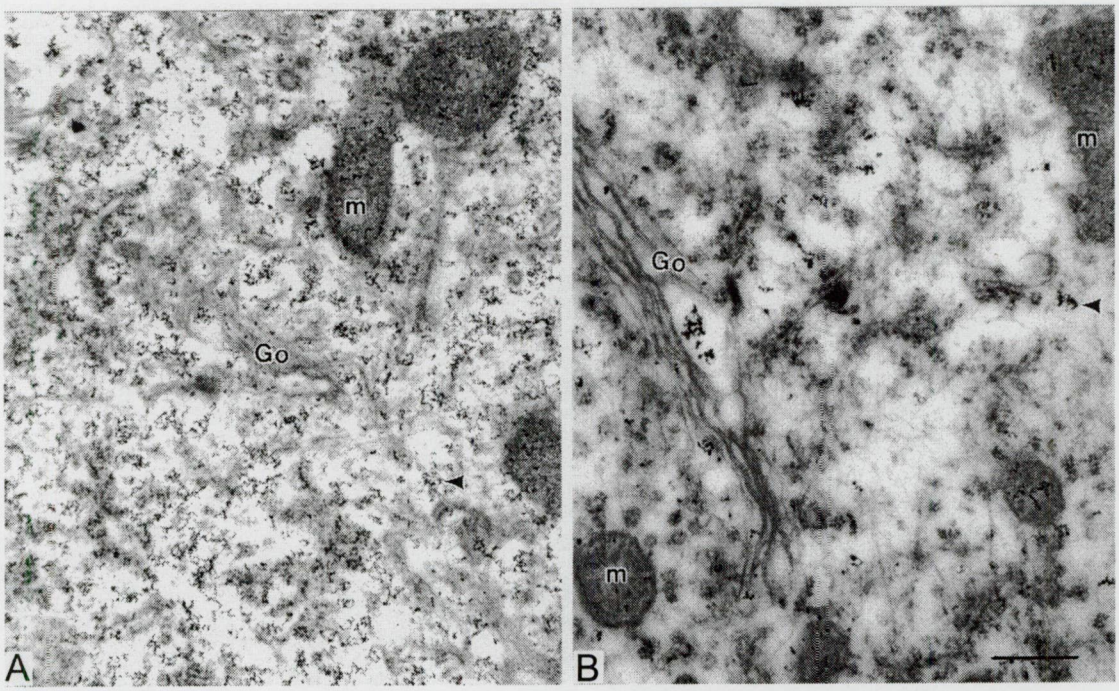


Fig. 9. Increased calcium could be demonstrated in spinal motoneurons of 11 months old wild-type mice (A). In SOD1 knock-out animals, even at this age, low level of intracellular calcium was present in the motoneurons of the spinal cord (B). m: mitochondrion, Go: Golgi apparatus, arrowhead: calcium deposit; bar: 0.5 μm.

In the wild-type animals no age-dependent changes were found in the intracellular calcium level of the oculomotor neurons. In contrast to neurons innervating external eye muscles, in spinal motoneurons, a marked increase of intracellular calcium could be noted as a function of age of control animals. No such age-dependent change in the calcium level could be observed either in spinal- or oculomotor neurons of the SOD1 knock-out mice. Consequently, qualitatively, differences only between the spinal motoneurons of wild-type and SOD1 knock-out animals clould be established, as the intracellular calcium level is considered (Figure 9, 10). No degenerative changes could be seen at any time point of any cell type of either animals. (Figure 9, 10).

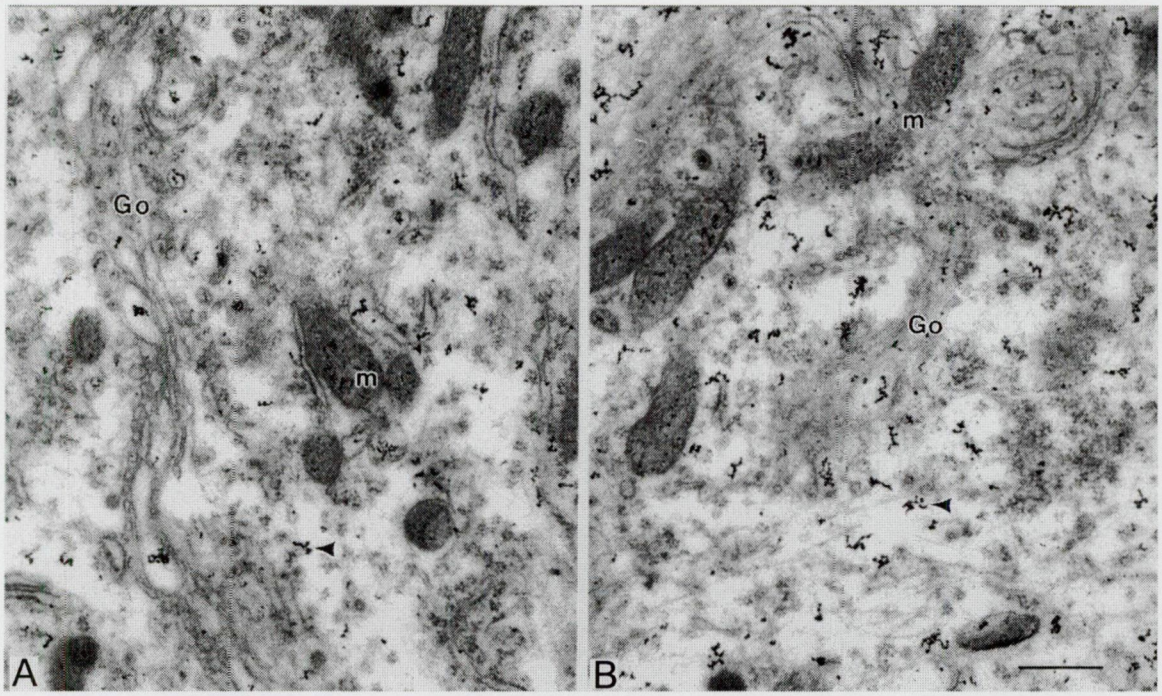


Fig.10. In the oculomotor neurons of both wild-type (A) and SOD1 knock-out animals (B) low levels of intracellular calcium could be seen, even at the demonstrated age of 11 months. m: mitochondrion, Go: Golgi apparatus, arrowhead: electron-dense deposit; bar: 0.5 μm.

In our quantitative characterization of the calcium level of motoneurons we considered the mitochondrial and cytoplasmic changes separatedly. As summarized in *Table 3*, no significant changes could be demonstrated in the mitochondrial calcium content between the wild-type and control animals in any age-matched comparison. The calculations were extended to the corresponding motor axon terminals, too.

Tissue	SOD1 knock-out		Wild-type	
	3 months	11 months	3 months	11 months
Spinal cord	36.55 ± 4.37	77.34 ± 31.75	30.15 ± 9.65	98.79 ± 47.09
Interosseus muscle	18.80 ± 9.81	52.75 ± 20.82	18.94 ± 6.57	64.40 ± 6.76
Oculomotor nucleus	38.42 ± 17.00	31.90 ± 8.29	23.72 ± 6.57	60.04 (n=1)
External eye muscle	13.63 ± 1.05	66.39 ± 3.91	12.22 ± 5.60	23.15 ± 12.18

Table 3. Calcium volume fraction ($\times 10^{-3}$) in mitochondria of motoneurons as a function of age of SOD1 knock-out and wild-type animals. Data are expressed as mean ± SEM. None of the differences between the corresponding age-matched controls and SOD1 knock-out groups were statistically significant.

The non-mitochondrial calcium volume fractions were determined using a reference space of the corresponding perikaryonal, or axoplasmic volume, excluding mitochondrial profiles. Thus, these data contained contributions from the cytoplasm, endoplasmic reticulum, Golgi apparatus, and synaptic vesicles, as well. The calcium volume fraction numbers were usually smaller in the motor axon terminals than in the cell bodies, and exhibited an increase with age in the wild type animals (Figure 11A, 11C). With these data, as a physiological baseline, the correspondig numbers of the spinal motoneurons from the SOD1 knock-out animals were always smaller. This difference increased with age, and by the age of 11 months reached a significant level both in the perikaya and axon terminals of spinal motoneurons (Figure 11A, 11C) but not in any region of the oculomotor neurons (Figure 11B, 11D). The age-dependent increase of non-mitochondrial calcium was always smaller, or negligible in the oculomotor system, compared to the spinal motoneurons of wild-type animals (Figure 11A-D).

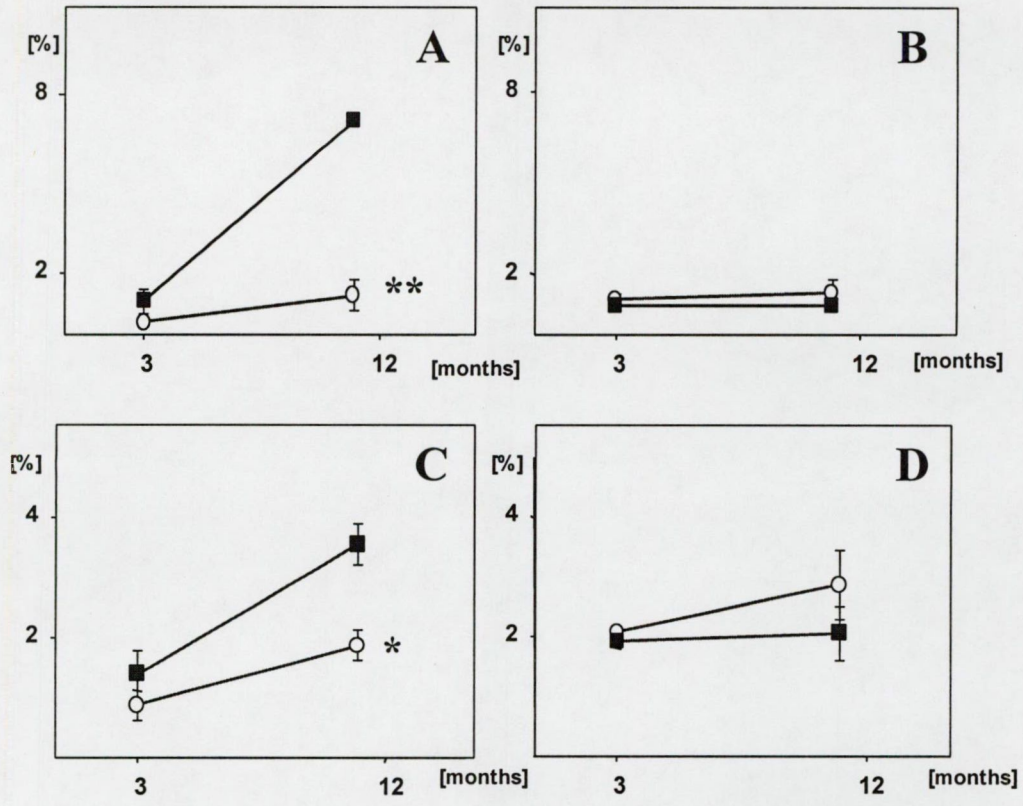


Fig. 11. Non-mitochondrial calcium volume fractions (% of the volume of calcium precipitates in relation to the cytoplasmic volume) in perikarya of spinal motoneurons (A), in motoneurons of the oculomotor nucleus (B), as well as in motor axon terminals of the interosseus muscle (C) and external eye muscles (D). Data are shown at 3 and 11 months of age of SOD1 knock-out mice (○) and wild-type animals (■). The corresponding values were significantly different at 11 month of age of animals in spinal motoneurons ($p < 0.05$) and motor axon terminals of the interosseus muscle ($p < 0.01$).

4. Discussion

The aim of our study was to investigate the general validity of the calcium hypothesis in animal models of amyotrophic lateral sclerosis. On the basis of our previous experiments [Engelhardt et al, 1995; Siklós et al, 1998], as well as based on observations made in human biopsy material [Siklós et al, 1996], we assumed that – regardless of the actual trigger of the lesion – the development of the uniform clinico-pathological profile of the disease can be attributed to the impaired calcium homeostasis of motoneurons. To test this hypothesis we selected different animal models, based on different etiological backgrounds, and investigated if similar changes in the motoneuronal calcium distribution (or level) could be demonstrated. In these and related studies we also attempted to reveal individual steps, processes or cellular characteristics influencing the degree of injury of calcium homeostasis in motoneuron populations.

4.1 Excitotoxic lesion of motoneurons *in vivo*

With regard to the excitotoxicty hypothesis, we did not want to investigate the justification of the concept, which, despite of firm supporting data, could be still debated. Particularly, it has been shown that Ca^{2+}-permeable AMPA receptors are equally expressed in resistant- and vulnerable brain stem nuclei [Laslo et al, 2001], or alternatively, the Ca^{2+}-non-permeable variant of the receptor is strongly expressed in vulnerable hypoglossal neurons [Paarmann et al, 2000]. Furthermore, splice variants of mRNA of the glial glutamate transporter, responsible for the reduced amount of EAAT2, are equally expressed in ALS patients and in normal individuals [Meyer et al, 1999], or, can be demonstrated in Alzheimer's disease-, and Lewy body dementia patients, as well [Honig et al, 2000]. Instead of planning additional (clarifying) experiments on the basis of the above arguments, assuming that excitotoxic lesion plays at least a partial role in the pathomechanism of ALS, we intended to demonstrate that impairment of motoneuronal calcium homeostasis and degeneration of motoneurons could be evoked by pure excitotoxic stress.

In our excitotoxicity experiments DL-HCA, which is a nonspecific agonist of NMDA- and non-NMDA glutamate receptors, was directly applied to the spinal cord

of rats. As the main finding, we could document that acute EAA application could indeed induce calcium increase and degeneration in spinal motoneurons. The pattern of intracellular calcium increase exhibited a characteristic sequence in the observed period: a plain cytoplasmic increase was noted first, which was followed by sequestration of calcium by organelles, with predominant calcium accumulation either in the endoplasmic reticulum, or in mitochondria, or both. Finally, the calcium content was diminished in the disintegrating, degenerating cells. The documented intracellular calcium increase, followed by redistribution, always preceded the degeneration of the cell and its organelles, which suggests, that actually a calcium-induced/mediated destruction of the motoneurons could be evoked in the model.

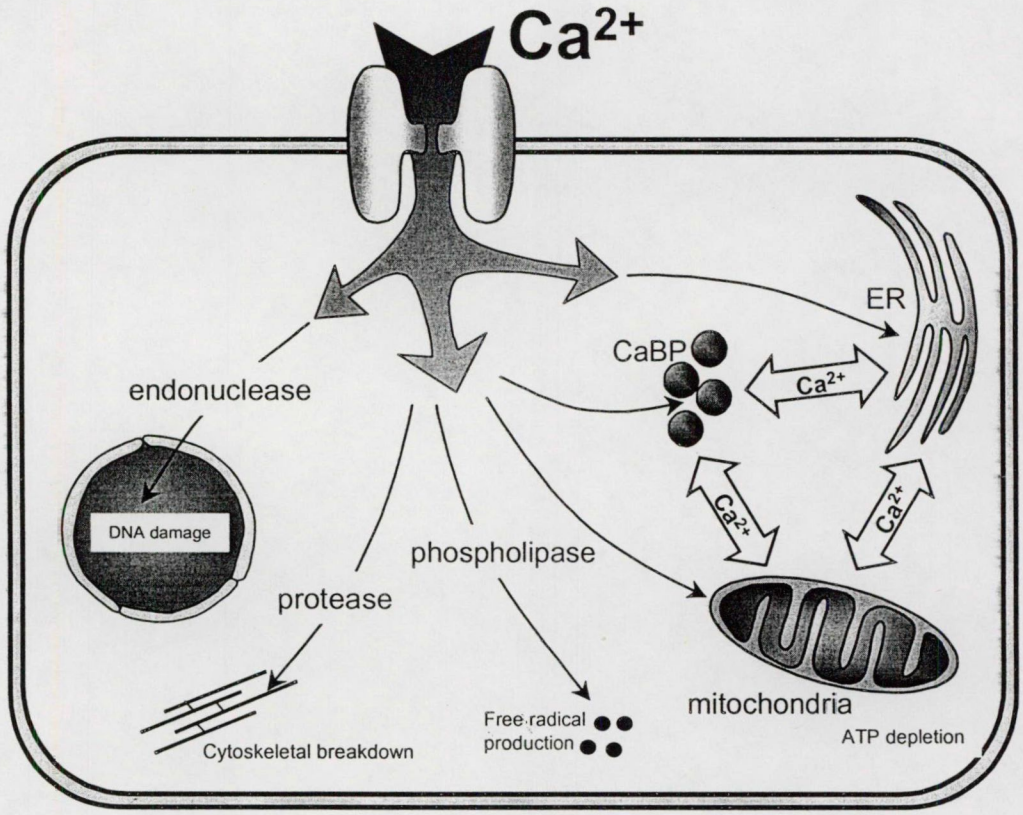


Fig. 12. Some elements of the Ca^{2+}-dependent cell destruction mechanism. Certain enzymatic processes (endonucleases, proteases), through their calcium-dependent activity, are able to exert direct injury, while others (e.g. phospholipases) induce the production of toxic intermediates. If calcium influx exceeds the efflux capacity (not shown) the intracellular buffers (CaBP, calcium-binding proteins; ER, endoplasmic reticulum; mitochondria) would be overwhelmed, which finally results in mitochondrial dysfunction, energy depletion, and apoptotic, or necrotic cell death, depending on the actually available energy.

The calcium-mediated cytotoxicity, particularly excitotoxicity is a well know phenomenon since decades [Olney, 1969]. Despite of intensive investigations in this field, however, the precise mechanism of the Ca^{2+}-dependendent cell death is still poorly understood [for a recent review see e.g. Sattler & Tymianski, 2000]. Although numerous calcium-dependent enzymatic processes are known to participate in cellular destruction (illustrated in Figure 12.), it seems that not the calcium load itself, but the route of entry and the way of intracellular handling are the decisive factors in the final outcome of the initial injury [Sattler & Tymianski, 2000].

Although the exact sequence of molecular events cannot be reconstructed on the basis of our data obtained by electron microscopy, mainly due to the low time-resolution of the technique, the results are in good agreement with the current concept of handling of intracellular calcium by calcium sequestering organelles. As the appropriate EAA receptors are activated, calcium influx and release from the internal stores (due to the activation of the IP_3 receptors of the ER) could lead to an increase of intracellular calcium (Fig 4A) [Siesjö et al, 1999]. Next, a mitochondrial calcium uptake takes place, even at physiologic conditions [Werth & Thayer, 1994], preferably at those microdomains, where the ER and mitochondria are at intimate proximity (Fig 4B). If the calium load is high enough, however, a biphasic mitochondrial calcium uptake can be induced [Ichas & Mazat, 1998], before the final collapse of the mitochondrial homeostasis occurs. In the first phase this uptake is not paralleled by detectable mitochondrial swelling, which might correspond to those mitochondria in our material where only local calcium accumulation could be seen (Fig 4B). The second phase, accompanied by gradual swelling of organelles (Fig 5) could evetually lead to opening of mitochondrial permeability transition pore in its high conductance state, release of matrix calcium and disintegration of the organelles. Although parallel to these mitochondrial events an ATP-dependent reload of the ER-stores could take place, explaining the heterogeneity of the observed calcium distribution at appr. 3 h survival time (Fig 5), the collapse of mitochondrial ATP synthesis should induce a secondary depletion of such stores, too. Finally, the mitochondrial permeability transition leads to the release of apoptosis inducing factors which results in apoptotic or necrotic cell death, depending on the available energy (ATP) of the actual cell [Kroemer et al, 1998; Nicotera & Lipton, 1999]. In accord with our results, similar sequence of calcium redistribution could be documented in *in*

vitro experiments, regardless if apoptotic or necrotic type of cell death was induced [Kruman & Mattson, 1999].

4.2 Correlation of disturbed calcium homeostasis of vulnerable and resistant motor axon terminals in models of familial- and sporadic ALS

The availability of SOD1 mutant transgenic animals and the modified passive transfer model, based on regular IgG inoculation, made possible the extension of the above investigations not only to additional models, but also to study the consequences of chronic stress conditions, imitating better the true pathomechanisms of the human disease. In this comparative analysis of disturbed calcium homeostasis – in ALS models with entirely different etiologic backgrounds – it was also possible to correlate the changes in distinct motoneuron populations differently affected in the disease. Because we aimed to reveal differences in their calcium handling properties, for this comparison of calcium distribution, we choosed spinal motoneurons and oculomotor neurons, since their calcium buffer composition was already proved to be different [Alexianu et al, 1994; Siklós et al, 1998]. In light of the autoimmune theory and earlier data from passive transfer experiments [Engelhardt et al, 1995] we expected the major changes at the neuromuscular junctions, thus, we focused our study to the corresponding motor axon terminals in the interosseus- and external eye muscles.

The analysis of motor axon terminals of the interosseus muscle of both animal models revealed mitochondrial degeneration and and extensive calcium accumulation mainly in the mitochondria (Figure 7). These results were in agreement with previous findings obtained by examination of the same muscle after acute IgG treatment of animals [Engelhardt et al, 1995], or by analysis of muscle biopsy samples of sporadic ALS patients [Siklós et al, 1996].

The microscopic examination of the neuromuscular junctions in the external eye muscles also revealed similar alterations in the two models (Figure 8A, 8B), i.e. appearance of large vacuolar structures (endosomes) accumulating the majority of intraterminal calcium. The development of such structures was not unexpected on the basis of detailed analysis of motor nerve terminals of the superior rectus muscle of SOD1 transgenic mice [Siklós et al, 1998]. However, the present results proved that similar alterations of the calcium homeostasis could be equally induced in the models of the sporadic- and familial ALS. A further result of the present study is the

demonstration that the endosomes are in fact in contact with the extracellular space via a narrow, tunnel-like channel (Figure 8C, 8D). These findings may suggest a mechanism how could the oculomotor neurons resist the applied stress conditions, likely by specialized management of intracellular calcium, or through stabilization of their calcium homeostasis.

The exact origin or identity of endosomes could not be determined on the basis of the present experiments, however, their development was probably connected to the peculiar modification of synaptic transmission in the oculomotor synapses. Stimulation of nerve terminals has been reported to lead to a transient increase of endosome-like organelles, because of the different kinetics of exo- and endocytosis [Takei et al, 1996]. These vacuoles could posess very narow channels communicating with the extracellular space [Gad et al, 1998], similar to the endosomes documented in our study. Since increased intracellular calcium has been demonstrated to hinder vesicle membrane retrieval in a graded fashion [von Gersdorff & Matthews, 1994; Brodin et al, 1997], differential formation of endosomes in vulnerable and resistant motoneurons could result from different ways of handling of intracellular calcium possibly influenced by calcium binding protein content. Indeed, in a present study Burrone and colleagues [2002] demonstrated, that intracellular (endogenous) calcium buffers could decrease the release of vesicles towards the active zone, thus decrease the size of the rapidly releasable pool of vesicles. If we assume that, in such a way, a saturation of the reserve pool of vesicles could take place, this may favour a decreased rate of endocytosis and a formation of a non-pinched off membrane-enclosed structure, i.e. endosome (Figure 13).

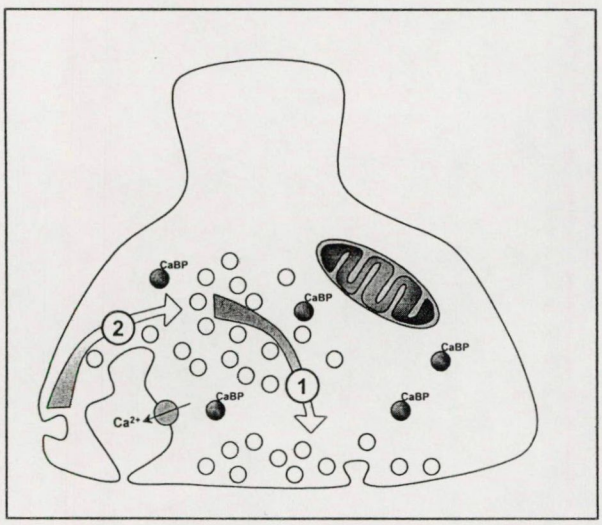


Fig. 13. Endogenous calcium buffers (CaBP) in axon terminals can decrease the release of vesicles towards the periphery of the active zone (1). Thus, probaly the saturation of the reserve pool of vesicles might develop, which, in turn, might decrease the rate of retrieval of vesicles (2), favouring the formation of endosomes. The endosome membrane, largely being constituted of vesicle membrane, possessing calcium pumps, would help to keep cytoplasmic calcium at low level.

As an alternative explanation for the development of endosomes we should keep in mind that endosome-like structures usually develop after prolonged stimulation [Leenders et al, 2002], offering an alternative vesicle recycling route, probably to compensate for the exhaustion of the releasable pool of vesicles. Since, if calcium binding proteins (i.e. endogenous calcium buffers) are present, the releasable pool is exhausted faster [Burrone et al, 2002], thus endosomes may develop preferentially at those axon terminals, where the cytoplasmic calcium buffering capacity is larger. In our proposed model, regardless of the actual driving force for their development, endosomes would specifically build up in axon terminals with larger cytosolic calcium binding capacity. Since the membrane of endosomes would be largely constituted from synaptic vesicle membrane, possessing ATP-dependent calcium pumps directed to the luminal domain [Michaelson et al, 1980], these structures would facilitate the removal of calcium from the intracellular space, compensating for the calcium load invoked by the presence of ALS IgG, or the mutant SOD1 enzyme.

4.3 Cooperation of cytosolic free-radical- and calcium homeostasis in SOD1 knock-out animals

The availability of SOD1 knock-out animals made possible the refined analysis of the role of the cytosolic antioxidant system in the pathomechanism of models of ALS. Since these animals – although were more prone to injury than the wild-type controls [Reaume at al, 1996] – developed normally, which suggests that they could compensate for the loss of a component of the antioxidant defense system. Since the synergetic effect of the neuronal free-radical- and calcium homeostasis was suggested [Mattson, 1998], we decided to study the differential changes of the calcium distribution in motoneuron populations with different calcium binding protein composition (i.e. spinal-, versus oculomotor neurons), known to be affected in the disease in a different way. During these studies we documented that *(i)* the mitochondrial calcium distribution was not affected in either of the neuron populations, and *(ii)* the cytoplasmic calcium distribution was different from age-matched controls only in the spinal motor neurons.

With regard to the mitochondrial calcium level in SOD1 knock-out animals, we should keep in mind that the main source of superoxide radicals ($O^{\bullet -}$), the target of

the SOD1 enzyme, is the mitochondria where SOD2 (Mn-SOD) is present, and functioning as the main defense system [Michiels et al, 1994]. Thus, it is likely that under unchallenged (physiological) conditions no remarkable $O^{\bullet -}$ stress is put on mitochondria.

Concerning the cytoplasmic calcium, we noted a selective age-dependent increase of the calcium level of the spinal motoneurons in relation to the oculomotor neurons in wild-type animals, which supports that intrinsic cellular properties affect calcium levels during aging [Kirischuk & Verkhratsky, 1996; Verkhratsky & Toescu, 1998]. Knowing that, at least certain calcium binding proteins are present in oculomotor neurons, which are missing in the spinal motoneurons, this observation suggest that stabilization of calcium homeostasis during aging is associated with the presence of certain calcium binding proteins, which thus afford neuroprotection [Clementi et al, 1996].

An unexpected finding of the present experiments was the relative decrease of cytoplasmic calcium from the spinal motoneurons of SOD1 knock-out animals compared to the age-matched controls. No such changes were noted either in the axon terminals or perikarya of the oculomotor neurons (Figure 11). These data suggest that decrements in the free-radical defense system could selectively impair calcium homeostasis in neurons with presumably lower calcium buffer capacity. Indeed, recent physiological and anatomical characterization of SOD1 knock-out mice revealed a slow development of mild, chronic peripheral hindlimb axonopathy and a moderate decline in motor unit number, suggesting impaired sprouting. [D. Flood & S. Shefner, personal communication]. This finding could be explained on the basis of the observed reduction of calcium in spinal motoneurons, since suitable intracellular calcium levels and intact intracellular stores are known to be necessary for synapse remodeling and neurite elongation [Kocsis et al, 1993; Mattson & Kater, 1987; Suares-Isla et al, 1984)].

Although brain tissue level of lipid peroxidation and protein carbonyl content demonstrated no oxidative damage in SOD1 knock-out mice compared to wild-type controls [Reaume et al, 1996], a mechanism could be proposed how the lack of SOD1 enzyme could lead to minute changes in the calcium homeostasis. Changes could be primarily expected at the organellar store, the ER, due to its intimate geometrical proximity to mitochondria, the main source of $O^{\bullet -}$. {The lack of SOD1 activity in the

cytosol and the resultant primary breakdown route of $O^{\bullet -}$ radicals through the reaction $O^{\bullet -} + NO^{\bullet} \rightarrow ONOO^{-}$ [Warner, 1994], would imply that *(1)* the $O^{\bullet -}$ level in the cytosol is slightly increased, *(2)* the cytosolic $ONOO^{-}$ concentration is proportionally increased, and *(3)* the cytosolic $NO^{\bullet}$ concentration is decreased (since the competition for $O^{\bullet -}$ would be now dominated by $NO^{\bullet}$ alone)}. (i) Effects of $O^{\bullet -}$ on intracellular calcium homeostasis were directly studied on isolated sarcoplasmic reticulum (SR) preparations, and $O^{\bullet -}$ produced less calcium accumulation in SR due to increased calcium permeability [Okabe et al, 1988]. (ii) In calf pulmonary artery endothelial cells, $ONOO^{-}$ donors selectively decreased agonist-stimulated influx of external Ca^{2+}, and depleted internal stores of releasable Ca^{2+} [Elliott, 1996]. (iii) Finally, $NO^{\bullet}$ has been documented to modulate Ca^{2+} mobilization from internal stores in human platelets [Sang et al, 1996], and function as a sensor for $[Ca^{2+}]_i$ [Suarez-Isla et al, 1984]. Thus, if decreasing concentrations of $NO^{\bullet}$ have any effect, they should facilitate the release from the calcium stores and deplete such stores. The above processes, acting either individually, or together, could reduce the intracellular storage capacity of membrane enclosed organelles, making them functionally "leaky".

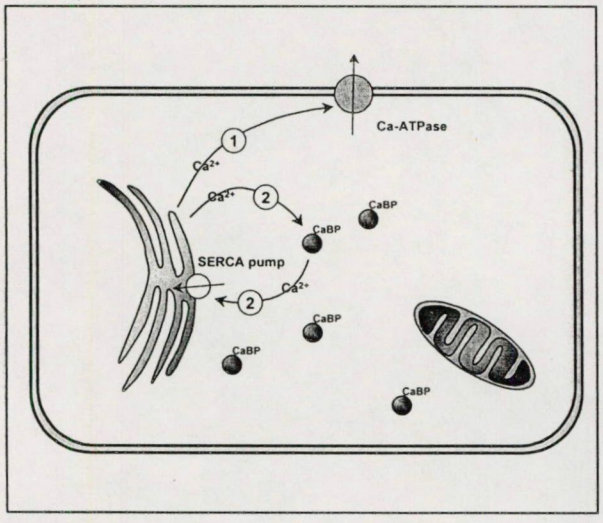


Fig. 14. The effect of cytosolic calcium buffers on the intracellular calcium content, on the basis of Hofer et al [1998]. With low cytosolic buffer capacity calcium ions leaking from the organelles, reach the Ca-ATPase pumps on the plasma membrane and will be extruded (1). With effective cytosolic buffering (CaBP), however, calcium ions leaving the ER would be captured (2) and recycled (2) by the SERCA pumps of the ER.

The protection of oculomotor neurons from such a decrease of intracellular calcium could be explained on the basis of their higher calcium binding protein content, as proposed by Hofer at al [1998] (Figure 14). Accordingly, in oculomotor neurons, assuming the same effects of the $O^{\bullet -}$ breakdown components, calcium ions released from the "leaky" internal stores can be immediately captured by calcium binding protein molecules and recycled into the stores.

4.4 A synthetic model of the calcium-mediated degeneration of motoneurons in amyotrophic lateral sclerosis

On the basis of our experiments we propose that impaired management of intracellular calcium is the key factor in coupling different pathways leading to cell destruction in models of ALS, consequently, stabilization of motoneuronal calcium homeostasis could be a potential tool to increase their resistance in stress conditions. The summary of our present concept is illustrated in Figure 15, below.

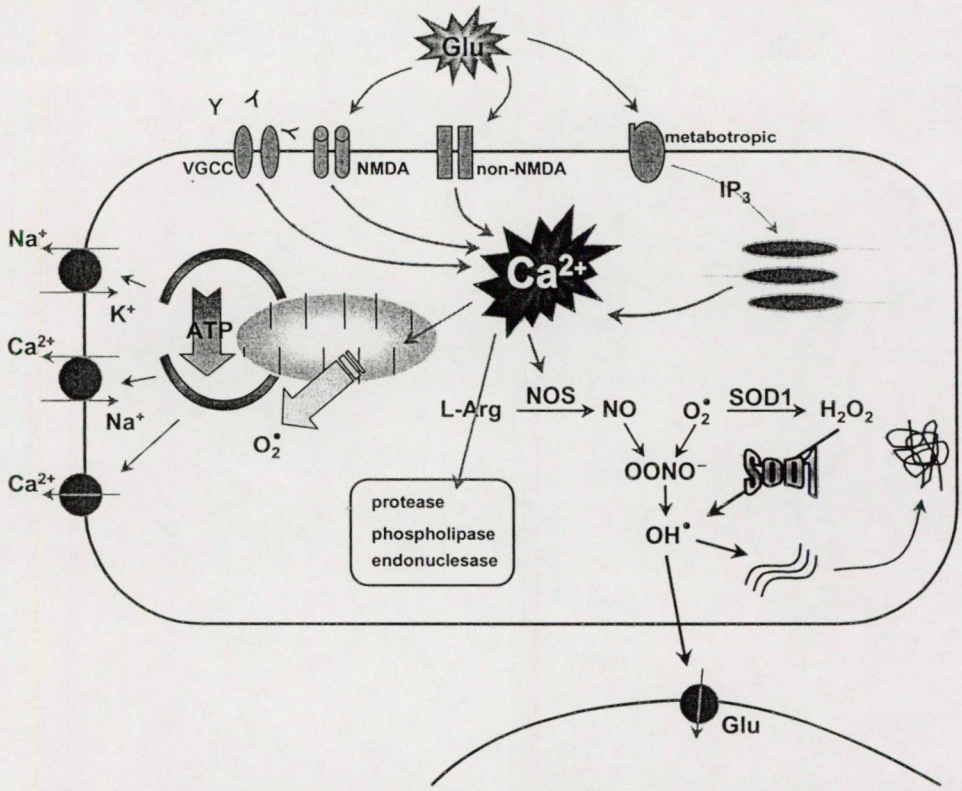


Fig.15. Interaction of calcium-dependent mechanisms of motoneuronal degeneration on the basis of models of ALS. If intracellular calcium is increased, several direct (proteases, phopholipases, endonucleases) and indirect destructive mechanisms (production of free radicals) are initiated. Normally, free radicals are neutralized by appropriate components of the cellular defense system, however, if a mutant enzyme is present (SOD1), the reaction is shifted towards the production of more harmful species. The highly reactive (and diffusibile) hydroxyl radicals may damage cytoskeletal components, as well as pumps/transport molecules (e.g. glutamate transporters) on the neighbouring cells. Impaired glutamate transport could induce overexcitation of appropriate receptors leading to further increase of intracellular calcium. The mechanism might be paralleled by calcium influx through voltage-gated calcium channels, due to IgG binding, and release of calcium from intracellular stores as a consequence of activation of metabotropic receptors. If the calcium increase endures, it might overwhelm extrusion pumps and intracellular-, particularly mitochondrial storing capacity, thus might impair mitochondrial function, leading to overproduction of free radicals, depletion of ATP, and, finally, the process culminates in the opening of the mitochondrial megachannel leading to cell death.

On the basis of the above scheme we anticipate that the stabilization of motoneuronal calcium homeostasis might improve their viability in different stress conditions. Indeed, in prior *in vitro* experiments transfection of motoneurons with calbindin D_{28K} prevented ALS IgG-mediated cytotoxicity [Ho et al, 1996]. A further support of the concept was obtained recently by developing transgenic mice overexpressing parvalbumin in their motoneurons [Beers et al, 2001]. Introducing these animals into experimental paradigms modelling sporadic- and familial ALS, by inoculating ALS IgG, and crossing to SOD1 mutant mice, respectively, significant improvement in the viability of the (normally) vulnerable spinal motoneurons could be demonstrated [Beers et al, 2001].

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7. Appendix