

INTRODUCTION

General context of the study

Our study is aimed at understanding the mechanisms underlying the selectivity of degeneration of motor neurons during amyotrophic lateral sclerosis (ALS): why do certain groups of motor neurons succumb to the disease while others are spared. Our hypothesis is that the impaired calcium homeostasis (i.e. increased calcium level) plays a pivotal role in the degeneration of motor neurons, and by its stabilization an increased resistance could be provided. Indeed, the increased level of intracellular calcium in the affected motor neurons was documented in ALS patients, which study was paralleled by the demonstration of a correlation between the resistance of such cells against injury and their calcium binding protein (CaBP) content. This observation was subsequently confirmed by a series of *in vitro*, *ex vivo* and *in vivo* experiments.

ALS is a progressive degenerative disease destroying upper and lower motor neurons though lesions outside of the motor system also occur. Although the degeneration of motor neurons is extensive in ALS the spinal motor neurons innervating the pelvic floor and associated sphincters, and the brainstem motor neurons projecting to external eye muscles are conspicuously spared. Pathological processes proposed to explain degeneration of motor neurons in ALS include: *excitotoxicity, oxidative damage, mitochondrial dysfunction, protein misfolding and aggregation, defects in axonal transport, immune-inflammatory dysfunction, impairment of the blood-brain/spinal cord barrier and calcium-mediated toxicity*. It is assumed that rises in intracellular calcium ions represent the final common pathway in the degeneration of neurons irrespective of the primary etiological factors.

Proof of concept experiment: protection by calcium binding in ALS

Our studies were a continuation of previous experiments in our laboratories, which addressed the role of immune/inflammatory mechanisms and rises in intracellular calcium in model of ALS. Although several approaches may be feasible how to stabilize intracellular calcium homeostasis, clinical observations and harmonizing experimental data pointed to the possibility that by means of increasing the intracellular buffering capacity through overexpressing CaBPs this goal could be achieved. The acid test of the protective role of calcium buffering capacity concept in ALS is the production and characterization of a model, in which ALS-like stress and increased CaBP level simultaneously exist. In an effort along this line, double transgenic mice possessing mSOD1 transgene, and

concurrently exhibiting increased PV levels have been developed. Although these animals exhibited improved parameters with regard to morphological- and physiological characteristics, ultimately could not be cured, furthermore, the gain in the survival data was just above the level, to be accepted as significant.

This unexpected low efficacy of protection by means of upregulated calcium buffering capacity, in view of the pivotal role of calcium in cell destruction was surprising. Obviously, next, the reason(s) of the unexpectedly low benefit of this intervention was decided to be investigated, with the hope of finding ways to improve neuroprotection on the same principles, i.e. to stabilize the intracellular calcium homeostasis, if feasible. Our aims in this direction were: setup of a lesion → detection assay based on a uniform intervention, and a standardized characterization of the intracellular calcium changes of the same group of cells, as well as, knowing their involvement in the injury, setup of an assay to quantify the degree of the participation of the non-motoneuronal cells in the injury.

Previous experiments relevant to the present study

Most importantly, an acute injury, i.e. axotomy/target deprivation experimental paradigm has been established, to quantify motoneuronal calcium increase in pools of motor neurons (oculomotor-, hypoglossal motor neurons) with documented different vulnerability to injury in ALS. These experiments, using electron microscopic histochemical methods and light microscopic immunohistochemistry, quantitatively documented a correlation between the resistance of motor neurons against injury and their CaBP level contrasted by an enduring increase in intracellular calcium content in CaBP-low cell types. Next, more importantly, these experiments provided a reference for comparisons whether an artificial increase in CaBPs could alter the profile of calcium response in these cells.

AIMS OF THE STUDY

- To demonstrate, whether the scale/profile of intracellular calcium increase after injury in a naturally vulnerably cell type (motor neurons in the hypoglossal nucleus) could be transformed to that of resistant motor neurons (oculomotor neurons) by PV upregulation
- To develop methods to quantify perineuronal (micro)glial reaction after injury
- To analyze the consequence of the reduced motoneuronal calcium increase on the local glial reaction after injury in PV upregulated mice

Paizs M, Engelhardt JI, Katarova Z, Siklós L: Parvalbumin upregulation reduces calcium increase and decreases the duration of inflammatory reaction in spinal motor neurons after acute lesion.

Magyar Idegtudományi Társaság XII. Konferenciája, Budapest, 2009. Január 22-24.

Paizs M, Tortarolo M, Bendotti C, Siklós L: Effect of an AMPA-receptor antagonist, talampanel, on the calcium level of the spinal motor neurons of G93A SOD1 mutant mice.

International IBRO Workshop, Pécs, 2010. Január 21-23.

Immunohistochemical characterization of inflammatory markers in the spinal cord

After transection of the sciatic nerve, chemokine (MCP1) upregulation in the motor neurons, and the microglia reaction (CD11b) in the lumbar region were characterized by immunocytochemistry at postoperative day 1, 4, 7, 14, and 21.

Evaluation of the functional recovery after sciatic nerve cut

Additional, twelve week old, male PV+/, B6/SJL and Balb/C mice were allocated for the study. The function of the operated hindlimb of animals was characterized daily at the same time. An arbitrary, but systematically applied scale was used to assess the recovery of the hindlimb function

Statistics

Results of experiment were analyzed by appropriate statistics using the Statistica for Windows program package (StatSoft).

RESULTS

Calcium increase is reduced after injury in hypoglossal motor neurons of PV+ mice

Qualitatively a gradual elevation of the intracellular calcium content in the axotomized hypoglossal motor neurons of the B6/SJL mice was seen with increasing postoperative time relative to the non-operated side, with a tendency to return to the control level by postoperative day 21. In the hypoglossal neurons of the PV+ animals, the calcium content was observed not to be elevated relative to the control side on any postoperative day. In the oculomotor neurons of the PV+ mice, similarly as in the hypoglossal neurons in these animals, no calcium increase was detected in the injured neurons as compared with the intact cells at any time point after the surgery. In both motor nuclei, the volume fraction of the EDDs, characterizing the distribution of calcium in the tissue, was expressed quantitatively. Statistically, the time dependences of the calcium levels in the two nuclei in the PV+ strain did not differ. However, the postoperative calcium increase in the motor neurons from the B6/SJL mice was significantly different from that of the PV+ animals.

PUBLICATIONS RELATED TO THE THESIS

In extenso publications in peer reviewed journals

Melinda Paizs, József I. Engelhardt, László Siklós:

Quantitative assessment of relative changes of immunohistochemical staining by light microscopy in specified anatomical regions.

Journal of Microscopy, **234**:103-12(2009)

Melinda Paizs, József I. Engelhardt, Zója Katarova, László Siklós:

Hypoglossal motor neurons display a reduced calcium increase after axotomy in mice with upregulated parvalbumin.

Journal of Comparative Neurology, **518**:1946-1961(2010)

Conference presentations

Oral presentations

Paizs M, Siklós L: In vivo neuroprotection assay: study the role of parvalbumin in motoneuronal injury.

Straub-Napok, Szeged, 2006. November 15-17.

Siklós L, Párducz Á, Engelhardt JI, Gyenes A, **Paizs M**: A mozgató idegsejtek degenerációjának nem sejt-autonóm aspektusai és ezekre alapozott protekciós kísérletek.

MITT Konferencia, Szeged, 2007. Január 24-27.

Paizs M, Engelhardt JI, Katarova Z, Gyenes A, Siklós L: Reduced calcium increase after axotomy in hypoglossal motor neurons of mice with increased parvalbumin level.

Ist International Student Medical Congress, Kassa, Szlovákia, 2009. Június 23-25.

Paizs M: Egy kalcium-kötő fehérje (parvalbumin) védő szerepének vizsgálata a mozgató idegrendszer akut sérülése során in vivo.

Immunhisztokémia Továbbképző Tanfolyam, Pécs, 2009. Április 2-3.

Paizs M: A mozgató idegsejtek degenerációját befolyásoló intracelluláris és extracelluláris folyamatok.

Szegedi Tudományegyetem Sófi József a Szegedi Tehetségekért Alapítvány Ösztöndíj Konferencia, Szeged, 2009. Március 25.

Paizs M, Engelhardt JI, Katarova Z, Siklós L: Egy kalcium-kötő fehérje (parvalbumin) védő szerepének vizsgálata a mozgató idegrendszer akut sérülése során in vivo.

Kisfaludy Lajos Alapítvány Előadói napja (Richter Gedeon), Budapest, 2010. Február 22.

Poster presentations

Paizs M, Engelhardt JI, Siklós L: Immune/inflammatory reactions in the spinal cord after axotomy.

International IBRO Workshop, Budapest, 2006. Január 26-28.

Siklós L, Párducz Á, Engelhardt JI, Gyenes A, **Paizs M:** Non-cell-autonomous aspects of the degeneration of motor neurons – new basis of attempts of neuroprotection.

Straub- Napok, Szeged, 2007. November 28-30.

Paizs M, Siklós L: Neuroprotective effect of parvalbumin in acute lesion of the spinal cord.

Straub-Napok, Szeged, 2008. December 3-5.

Paizs M, Engelhardt JI, Katarova Z, Siklós L: Parvalbumin upregulation reduces calcium increase and decreases the duration of inflammatory reaction in spinal motor neurons after acute lesion.

International Student Congress of Medical Sciences, Groningen, Hollandia, 2009. Jún. 2-5.

Paizs M, Engelhardt JI, Siklós L: Measurement of relative changes in immunohistochemical staining by light microscopy in neuronal tissue.

Magyar Idegtudományi Társaság XII. Konferenciája, Budapest, 2009. Január 22-24.

Paizs M, Engelhardt JI, Katarova Z, Gyenes A, Siklós L: Reduced calcium increase after axotomy in hypoglossal motor neurons of mice with increased parvalbumin level.

Magyar Idegtudományi Társaság XII. Konferenciája, Budapest, 2009. Január 22-24.

MATERIAL AND METHODS

Experimental animals and maintenance of the transgenic colony

To test the “calcium buffer” hypothesis, transgenic mice over expressing rat parvalbumin were developed, bred to homozygosity. Beside the demonstration of the presence of the transgene in the PV+/+ animals by PCR, hypoglossal- and spinal motor neurons, which are devoid of parvalbumin in the wild type mice were checked for the presence of the PV by light microscopic immunocytochemistry.

Axotomy/target deprivation of hypoglossal and oculomotor neurons

Twelve weeks old male B6/SJL- and PV+/+ mice were selected for the study. The animals were assigned for hypoglossal or oculomotor nerve cut experiments. Four animals were euthanized at 5 time points for both types of surgery and were processed for electron microscopic calcium histochemistry study. The hypoglossal nerve was transected lateral to the hyoid bone and a piece of 3-5 mm nerve segment was removed to prevent regeneration. For transection of left oculomotor nerve, animals were enucleated and the orbits were cleared of the remaining extraocular muscles and nerve segments.

Axotomy of the sciatic nerve

Twelve weeks old male B6/SJL-, Balb/C- and PV+/+ mice were selected for the study. Animals were assigned either for electron microscopic calcium detection study, or to light microscopic immunocytochemistry, or functional assay study. The sciatic nerve was transected at the mid-thigh, and a small piece (3-5 mm) was removed to prevent reinnervation. In these and in the above experiments the non-operated side served as an internal control.

Electron microscopic detection of calcium

The oxalate-pyroantimonate histochemical reaction was used to study the distribution of calcium at subcellular level. The specificity of the fixation procedure for calcium was checked by electron spectroscopic imaging (ESI). The relative cytoplasmic and mitochondrial volumes occupied by electron-dense deposits (EDDs) were determined on microscopic prints with a point counting technique.

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Inflammatory reaction can be quantified by systematic sampling and background corrected segmentation using immunohistochemistry

The acquisition of reliable conclusions from the pattern of the reaction products of immunohistochemical (IHC) procedures is hindered by the need for subjective judgments, in view of frequent inconsistencies in staining intensity from section to section or in repeated experiments. To diminish this problem, a procedure is presented, based on image analysis and the use of an internal reference area on the sections, which reduces the operator input and hence subjectivity, and makes the relative changes in IHC staining intensity in different experiments comparable. The reference area is situated at a position of the section which is not affected by the experimental treatment, thus, can be used to specify the baseline of the IHC staining. To compensate for another source of variability, the internal heterogeneity of the object, details are given of a systematic random sampling, which provides numerical data describing the intensity of IHC staining throughout the volume to be characterized. The procedure is illustrated by quantification of microglial activation after the lesion of the sciatic nerve.

Increased motoneuronal calcium buffer reduces local inflammation after acute lesion

The relationship between the increased calcium buffering capacity of motor neurons and the altered local inflammatory reaction has been examined in the spinal cord of PV^{+/+} and control (B6/SJL and Balb/c) mice after the lesion of the sciatic nerve. The temporal change of the inflammatory markers has been followed in 21 days after the operation with IHC supplemented with the characterization of the recovery of the hindlimb function. Calcium level of the motor neurons was determined at day 7, when the inflammatory reaction peaked.

The inflammatory reaction commenced as early as day 1 after operation in the spinal cord, either the MCP1 chemotactic signal, or the altered CD11b expression is considered. These reactions lasted at least 3 weeks long, however with a different decay time: the decrease of the release of the MCP1 by the injured motor neurons preceded the attenuation of the microglial reaction at their vicinity. Nevertheless, the decrease of both of these inflammatory markers was significantly faster in the PV^{+/+} mice compared to the control strains.

An important result of the present experiments is – similar to the hypoglossal motor neurons of the PV^{+/+} mice – that the PV upregulation attenuated the increase of the intracellular calcium after axotomy in the spinal motor neurons, as well. The calcium measurements have been performed at postoperative day 7, at the time when the calcium increase in the wild type hypoglossal neurons was significantly higher than in those of the PV^{+/+} mice.

The reduction of calcium increase in motor neurons is indeed capable of reducing local inflammation, furthermore, according to the observed rate of improvement of the hindlimb function in these animals in the same period, leads to a faster functional recovery.

DISCUSSION

The role of calcium and CaBPs in neuronal degeneration

It is well documented, that abnormal and sustained increases in intracellular calcium induce a series of calcium-dependent enzymatic processes, which participate in cellular destruction. CaBPs within the cells are commonly located at critical positions: in the vicinity of plasmalemmal calcium pumps and channels, around the mitochondria and endoplasmic reticulum, organelles known to play key roles in Ca²⁺ signaling and buffering. Thus, these proteins may slow down the diffusion of calcium ions away from the influx regions, or facilitate their recycling into internal stores, and thereby reduce the amplitude of calcium concentration changes in the cytosol. In the present study, we decided to assay the changes in calcium level after injury in motor neurons with an artificially elevated PV content, and to compare the results obtained from the same population of neurons with a naturally low PV content in wild-type animals.

Calcium level changes induced by axotomy

In hypoglossal motor neurons of B6/SJL mice, as large as 1.7-fold calcium increase after axotomy was demonstrated, compared to the non-operated conditions, which was significantly different from the calcium increase in the oculomotor neurons of the same animals. In the PV^{+/+} mice there was no calcium increase either in the hypoglossal- or oculomotor neurons, providing evidence for that PV upregulation lends an “oculomotor-like” resistance .

In the spinal motor neurons of PV^{+/+} mice a similarly reduced calcium level was demonstrated after injury as in the hypoglossal motor neurons, suggesting that independent of the local neuronal environment, PV upregulation indeed contributes to the resistance against calcium increase.

The role of inflammatory factors in injury of motor neurons

In the spinal cord of the PV^{+/+} mice a reduced and a faster resolving inflammatory reaction could be demonstrated after acute injury compared to the B6/SJL mice. These observations, together with the reduced calcium increase in PV^{+/+} spinal motor neurons suggest that the magnitude of the primary lesion in the motor neurons determines the surrounding microclial activation, and stabilization of the calcium homeostasis in motor neurons may be indeed protective.

Quantification of the inflammatory markers by light microscopic IHC

With regard to the inflammatory reactions, a computerized method has been developed, based on light microscopic IHC and image analysis, which is able to reproducibly quantify the expression of certain inflammatory markers with high precision. The method has a general applicability.

The results confirm that elevated intracellular calcium buffer could attenuate both the *intracellular calcium increase (thus calcium-mediated degenerative processes)* and the *local inflammatory reactions* after acute injury, but leaves open the possibility that such a buffer may exhaust if the stress endures. Under chronic stress conditions, such as in animals with pathogenic mutations, which start working as early as *in utero*, an alternative way of reducing calcium levels, based e.g. on special ion channel blockers, is suggested.