EFFECT OF ELEVATED CALCIUM LEVEL AND CALCIUM BINDING PROTEINS IN ACUTE AND CHRONIC DEGENERATION OF MOTOR NEURONS

Summary of the Ph.D. Thesis

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INTRODUCTION

Motor neuron degeneration could be used as a collective term for several neurological disorders that selectively affect motor neurons. These diseases include chronic pathological entities, for example, progressive muscular atrophy, hereditary spastic paraplegia or amyotrophic lateral sclerosis (ALS). However, this phenomenon is not limited to chronic manifestations, since acute forms like motor axonal neuropathy or injury related motor neuron degeneration are also well-known. Regardless of the initial cause and form of degeneration, these malicious events share common pathological processes, where calcium (Ca) elevation might be a prominent factor. These mechanisms can be observed simultaneously, furthermore, these processes may facilitate the deleterious effect of each other. Moreover, Ca may combine the individual pathological processes into a unified escalating mechanism of neuronal destruction. These findings suggest that Ca may play a central role in the pathobiology of the diseases.

However, while certain groups of motor neurons succumb to degeneration, oculomotor neurons and sacral motor neurons innervating sphincter muscles are spared along the disease progression. This resistance was shown to correlate with their elevated Ca binding protein (CaBP) content. Such protective effect might be based on moderating the Ca increase associated with injury either by reducing Ca influx or by buffering the excess Ca by CaBPs. These interventions might be able to reduce neighboring inflammatory reactions as well as it was shown by reduced microglial reaction around injured motor neurons with high CaBP content.

Several features may be responsible for the different susceptibility of certain types of motor neurons, however, the roles of intracellular Ca handling and Ca influx through the plasma membrane are generally emphasized. *T*ransgenic mice with upregulated motoneuronal CaBP, parvalbumin (PV) were challenged either in acute sciatic nerve crush injury setting, or chronically, by crossing to the mSOD1 transgenic mice, creating a double transgenic strain, which suggests PV upregulation could confer reasonable neuroprotection. One leading hypothesis

(excitotoxicity) for the selective death of motor neurons in ALS is based on the selective change of Ca permeability of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the plasma membrane of postsynaptic motor neurons. The AMPA receptors permeability to Ca is determined by the absence of the glutamate receptor subunit type 2 (GluR2). Although literature data are not fully consistent regarding the correlation of the susceptibility of motor neurons with the missing GluR2 subunit in their AMPA receptors, it was repeatedly reported that both mRNA for GluR2 subunit and GluR2 protein levels were decreased in vulnerable motor neurons.

AIMS OF THE STUDY

Based on experimental data on the ability of an AMPA receptor antagonist, talampanel (Tal), reducing Ca influx into motor neurons and ameliorating disease progression in the mutant superoxide dismutase 1 (mSOD1) animal model of ALS, clinical trial has been initiated to slow the evolution of the disease in ALS patients. However, the test was aborted, since the study did not meet its primary endpoint. In our previous experiment we gave evidence that the failure of the clinical study might be attributed to the late initiation of the treatment relative to the disease onset. To prove that the rationale of the treatment based on reducing Ca influx is still compelling, it needed to demonstrate that the pitfall of the treatment is not due to its inefficiency at the distant motor axon terminals where the disease probably starts. Thus, our first aim was:

1. **To demonstrate that the soma targeted treatment with Tal is equally effective to prevent Ca increase at the perikarya and at the axon terminals of motor neurons of mSOD1 mice if the treatment starts at an early phase of the disease.**

It is now widely accepted that neurons could be protected from Ca-mediated injury by reducing Ca influx. Previously we have shown that, alternatively, by elevating their CaBP content, an increased resistance could also be provided for motoneurons against acute injury or during chronic stress. Besides their Ca

buffering capacity, nearby inflammatory reaction may also have an effect on the fate of injured motor neurons. To demonstrate the possible connection between these two factors shaping the survival of motor neurons after injury, our second aim was:

2a. To visualize the magnitude and time course of neighboring microglial activation of injured motor neurons with differently elevated Ca content achieved by upregulated CaBP (in this case, PV) content.

2b. To standardize the measurement of the intracellular Ca content, a procedure was aimed to develop, which minimizes the variability of measurements by standardizing the thickness of sections used for analysis.

By increasing motoneuronal PV content, intracellular Ca elevation evoked by injury could be prevented and the local microglial reaction could be decreased. To investigate if injury-induced inflammation could be further reduced by mutually targeting microglia activation and stabilization of Ca level, our third aim was:

3a. To test the effect of diazoxide treatment on microglia activation in different motor nuclei after axotomy, since besides its anti-inflammatory effect, it was shown to protect neuronal mitochondria from Ca overload.

3b. To quantify microglia activation in motor nuclei, a double-immunostaining procedure was aimed to develop, which allows the determination of the volume of motor nuclei with fluorescently labeled motor neurons, and the simultaneous measurement of the relative volume of the non-fluorescently labeled microglial cells.

MATERIALS AND METHODS

Talampanel and diazoxide treatment

Transgenic mice expressing a G93A substituted form of the SOD1 gene, were treated with Tal or vehicle (Veh). Treatments were started at 10 or 17 weeks of age corresponding to the presymptomatic and the symptomatic stage of neuromuscular dysfunction. Tissue samples were harvested at 12 or 19 weeks of age. Balb/c non-

transgenic mice were assigned to three groups according to the type of surgery: unilateral eye enucleation, hypoglossal, or facial nerve axotomy. Five animals in each group were treated with diazoxide and five mice served as controls. Two lines of PV overexpressing (PV+/+) mice were used. The founder lines were bred to homozygosity and were characterized to prove that PV is transcriptionally and translationally expressed in the motor neurons of their spinal cords.

Immunohistochemical staining and quantification

Series of sections from the regions of interests were immunostained according to the avidin-biotin technique to display the relative location of the chemokine C-C motif ligand 2 (CCL2) and cluster of differentiation 11b (CD11b) stained profiles. Furthermore, PV or ionized Ca-binding adaptor molecule 1 (Iba1) and choline acetyltransferase (ChAT) in the lumbar motor neurons were immunostained with double fluorescent labeling. A combination of fluorescent and avidin-biotin technique was also developed. For quantitative assessment of the changes of immunostained cells were quantified by using an interactive macro developed in our laboratory. The significantly stained partial profile areas were determined, expressed as percentage of the area of interest, and the algebraic differences between the operated and the contralateral sides were summed up throughout the series of sections to arrive at a single number characterizing the net change in the area.

Transmission electron microscopic detection of Ca

The Ca content of the electron-dense deposits (EDDs) was tested by electron probe X-ray microanalysis. For this purpose, non-contrasted sections were prepared and examined. Then the relative volume of EDDs was determined by point counting methods. This protocol results in the partial volume of EDDs within a selected reference space assuming that the test object is a two-dimensional plane with zero thickness. Since this cannot be achieved physically a method for standardization was developed. The possible overprojection of the EDDs through

the thickness of the sections was visualized by electron tomography, which also indicated the necessity of the standardization of the thickness. A procedure for *in situ* measurement of actual section thickness in the electron microscope was adapted, based on the Bouguer-Beer-Lambert beam attenuation law. The precision of the method was tested by correlating the calculated thickness according to the attenuation law to the true thickness of the same section measured by atomic force microscopy (AFM).

Statistical analysis

All statistical analyses were performed with R statistical computing software with RStudio Integrated Development Environment for Windows. Data are presented as a mean value ± the standard error of the mean (s.e.m.).

RESULTS

Effect of talampanel on the Ca level of motor neurons of mSOD1 mice

Visual inspection showed an increased number of EDDs in mSOD1 mice, compared to controls, at both time points, which indicates an increased amount of Ca in the axon terminals of mSOD1 animals. Qualitatively, this Ca increase could be prevented by Tal, but only if the treatment was started presymptomatically. Quantitative assessment of the volume of the EDDs relative to the axoplasmic volume, expressed as percentage, revealed a 1.27-fold increase at the age of 12 weeks and a 1.75-fold increase at age of 19 weeks in the motor axon terminals of the interosseus muscle of mSOD1 animals compared to those of non-transgenic wild types. This Ca increase could be prevented by Tal application at 10 weeks of age but not at 17 weeks. Similarly, to the axon terminals, Ca increase in the spinal motor neurons of mSOD1 mice was significant at both time points, compared to non-transgenic controls. This could be prevented by Tal treatment only if the treatment was started presymptomatically. Motor neurons innervating the extraocular muscles, which represent groups of motor neurons resistant to Ca-mediated injury, were analyzed in the mSOD1 mice at ages of 12 and 19 weeks.

Qualitatively, relative to non-transgenic controls, neither structural alterations of the neuromuscular junctions and of the cell bodies could be observed, nor increased number of EDDs could be noticed. By expressing the relative amount of the EDDs neither an increased amount of EDDs in mSOD1 motor neurons compared to those from non-transgenic mice, nor their change after treatment could be documented at 12 weeks or at 19 weeks.

Controls of the electron microscopic calcium histochemistry

The analytical composition of randomly selected EDDs were irradiated by a focused electron beam, then the emitted secondary X-rays were collected. Their spectral composition was analyzed and the Ca peak was separated from the overlapping antimony peak. Thus, by demonstrating the presence of Ca specifically within the precipitates, the use of EDDs in determining the distribution of tissue Ca was justified. Electron tomographic reconstruction of the EDDs within the volume of the sections with thickness routinely used in electron microscopy revealed the possibility of their variable degree of overposition with variation in section thickness. Since this may considerably influence the size of the projected image of EDDs, and thus affect their calculated density, standardization of section thickness during the measurements was needed. AFM analysis of a series of microscopic sections revealed that the routinely used methods to select sections with a predetermined thickness are not suitable for standardization. However, the application of the Bouguer-Beer-Lambert law provides an accurate method to measure the thickness of each section *in situ* in the electron microscope. This method could be used to eliminate sections with non-standard thickness thus reduce the variance of the measurements of the density of the EDDs.

Sciatic axotomy-induced changes in the spinal cord of PV+/+ mice

Axotomy of the sciatic nerve induced a marked expression of CCL2 in the injured motor neurons which was paralleled by an increased microglial reaction at their vicinity. Microglial cells could often be seen at close positions to the CCL2-

expressing motor neurons. CCL2 immunostaining was detectable only in the motor neurons at the operated side. The relative change in the intensity of CCL2 staining in the operated side compared to the non-operated side in each animal was expressed quantitatively at postoperative days 1, 4, 7, 14 and 21. The net amount of staining ipsilateral to the injury increased gradually until day 7, and declined thereafter. The rate of decline, however, was different in the control and PV+/+ mouse strains: while the CCL2 expression in the PV+/+ mice returned close to the baseline already at day 14 after operation, the CCL2 level in the spinal cords of the control mice reached this level only at day 21 after axotomy. The difference between the CCL2 immunostaining in the control and the PV+/+ transgenic mice at day 14 was significant as well as the difference between the staining intensity in the PV+/+ animals at day 7 and 14 after the nerve cut. Similarly, the area fraction occupied by CD11b-positive profiles gradually increased in the spinal cords in all animals, regardless of their strain, peaked at day 7–14 after operation, and decreased afterward, which is evident at day 21. Similarly to the CCL2 staining, in PV+/+ animals the intensity of CD11b staining decreased faster than in the control strains, but reached significantly different level at day 21 only. It seems that the sequence of changes in CD11b staining intensity after the postoperative day 7, in the declining phase, follows that of CCL2 in the injured motor neurons with a time delay. Determination of Ca content of motor neurons of control and PV+/+ mice was performed at postoperative day 7, when the inflammatory reactions reached their maximum. Quantification of the relative volume of motoneuronal compartments occupied by the EDDs at the operated and control side revealed approximately a 2-fold Ca increase induced by the injury in each of the examined compartments of the control mice, while no Ca elevation could be measured in the PV+/+ animals.

Effect of diazoxide treatment on microgliosis in the axotomized motor nuclei

Motor neurons with different susceptibility against injury in the oculomotor-, hypoglossal- and facial nucleus were challenged with axotomy, then the anti-

inflammatory effect of diazoxide was tested. To express quantitative effect of diazoxide on axotomy-induced microgliosis, the exact boundaries of the affected nuclei should be known, which may require double staining. Since fluorescent double staining cannot be used for reliable quantification due to the unavoidable and uncontrollable changes in the imaging parameters, a combination of diaminobenzidine tetrahydrochloride (DAB)-based and fluorescent-based immunostaining protocol was developed. The fluorescent staining of the cell type to be quantified was substituted with DAB-based visualization, while the other cell type, used for anatomical orientation was still fluorescently stained. Using this technique, the exact boundaries of the motor nuclei involved in the analysis could be delineated, within which the microglial activation, relative to the ipsilateral, unoperated side, could be determined. These differences from section-to-section could be plotted along the rostrocaudal axis of each nucleus to characterize the injury-induced microglial activation. Then these numbers could be averaged in each experimental group which revealed a significant 3.75-fold decrease in the microglial area of the diazoxide treated group compared to the control group after target deprivation of the oculomotor nucleus. Similarly, statistically significant beneficial effect can be observed in the facial nucleus (165-fold decrease) and hypoglossal nucleus (72-fold decrease) as well. It is worth to note that the activation of microglial cells in the oculomotor nucleus without diazoxide treatment seemed to be more pronounced in the facial- and the hypoglossal nucleus.

DISCUSSION

Role of AMPA receptors in chronic motor neuron degeneration

In a previous experiment, we showed a protection of motor neurons from Ca-mediated degeneration by preventing Ca influx through AMPA receptors in mSOD1 transgenic mice with Tal. In these animals the treatment was successful only if it was started during the presymptomatic phase of the disease. The loss of

the treatment efficacy in early symptomatic mSOD1 mice could be attributed to the progressive nature of the degeneration, which might propagate from the upper to the lower motor neurons, imposing an excitotoxic burden on ventrolateral spinal motor neurons ("dying forward"), or retrogradely from the neuromuscular junction ("dying back"), or may occur independently. Our data indicate that in the cell bodies of spinal motor neurons and in motor axon terminals in the interosseus muscle Ca-elevation could be uniformly prevented by Tal treatment at a presymptomatic stage, furthermore, the efficacy of the treatment diminished equally in these positions at an early symptomatic stage. The only noted qualitative change was a mild mitochondrial degeneration, observed as swelling, in the axon terminals of mSOD1 mice at 19 weeks of age, which is consistent with the observation of neuronal death in chronic progressive excitotoxicity due to AMPA receptor overactivation. Thus, we may conclude that this soma-targeted protective treatment could also rescue semi-autonomously functioning motor nerve terminals, if applied early enough, in which the noted commencement of mitochondrial lesion at a later stage could be the consequence of a less efficient housekeeping support from the distant perikaryon. It is feasible that the similar stress conditions at the perikarya and the axon terminals could be handled less effectively in the terminals due to their limited homeostatic capacity compared to the somata, reflected in their higher increase of intracellular Ca content. As a consequence, when the Ca buffering of mitochondria in the axon terminals is overwhelmed, a Ca-dependent local death-cascade might be initiated leading to early synaptic degeneration. Since synaptic/axonal degeneration alone might be able to initiate the disease, the loss of efficacy of the treatment at an advanced stage might be the consequence of the retrograde spread of death machinery. This concept implies that the degeneration of motor neurons in mSOD1-induced stress is the consequence of compromised housekeeping support to the axon terminals from the perikarya under Ca-mediated stress. Such malfunction initiates a retrograde degeneration from the motor nerve terminals, the homeostatically weakest points of the motor neurons, i.e. both

compartments should be simultaneously protected. This suggestion is consistent with the observation that neuromuscular junction dysfunction rather than motor neuron loss is predictive of a more aggressive disease phenotype in SOD1 mice. In this scenario, if the Ca-mediated processes play a central role in degeneration, a lower or later commencing Ca increase is expected equally in the cell bodies and motor axon terminals of oculomotor neurons of mSOD1 mice, which are reported to be resistant in ALS. Indeed, in these experiments, at the time points when a 1.61-fold increase of perikaryal and a 1.27-fold increase of intraterminal Ca content of spinal motor neurons (12 weeks of age), as well as a 1.63-fold increase of perikaryal and a 1.75-fold increase of intraterminal Ca content of spinal motor neurons (19 weeks of age) were documented. No Ca increase could be measured in oculomotor neurons, which could be well explained by their higher CaBP content, such as PV.

Role of the neuron-microglia communication in neuronal degeneration

Since photobleaching and non-standardized excitation are the major controversy during quantification of fluorescent dyes, a development of a photostable visualization technique was necessary. This method suitable for proper quantification of the intensity and area of the region of interests with a secondary staining for anatomical mapping. This technique was tested in a neuroprotective paradigm using diazoxide, which acts on mitochondrial adenosin triphosphate-dependent potassium channels, therefore it is capable of modulating the mitochondrial homeostasis. This treatment successfully ameliorates the degeneration in different models. However, numerous components of the neuroprotective mechanism behind the anti-inflammatory effect remain unclear, attenuation of the microglial activation might be a viable protective strategy. Recent studies suggest that activation of the ATP-dependent potassium channels may cause hyperpolarization in the plasma membrane which prevents Ca entry into the cytoplasm via voltage gated Ca channels. We demonstrated the anti-inflammatory effect of diazoxide on acute neurodegenerative models challenging

motor nuclei with different susceptibility for degeneration. Our data indicate that microglial activation, induced with axotomy or target deprivation, could be alleviated with diazoxide treatment in the examined nuclei. However, anatomical mapping of the region of interest is a crucial parameter of the evaluation since anatomical boundaries of the nuclei or contralateral innervation capable of amending the outcome of the quantification protocol.

Protective effect of calcium binding proteins in motoneuronal injury

We demonstrated that an enhanced intracellular Ca buffer capacity of motor neurons in PV+/+ transgenic mice could attenuate the intracellular increase of motoneuronal Ca after acute injury, which could narrow the duration of the emission of distress signals by motor neurons, and could reduce the activation of microglial cells in their neighborhood. To gain additional experimental support for the view of the possible universal protective role of CaBPs, and to exclude species differences in the sensitivity of motor neurons against injury, the present experiments were extended to both control strains. At postoperative day 7, when the peak increase of intracellular Ca was expected, about 2-fold Ca increase could be observed in the spinal motor neurons of both control strains. Furthermore, no Ca increase could be observed in the spinal motor neurons of PV+/+ mice at this postoperative time, which suggests PV upregulation can transform the vulnerable cells to oculomotor-type resistant ones, at least as the intracellular Ca increase after acute injury is concerned. Since cytokine/chemokine signaling is known to precede the activation of further immune responses, in our study, CCL2 was selected as a marker of distress signals of motor neurons after acute lesion. Autopsy from ALS patients implies a possible graded microglial response after injury, since a correlation was found between increased microglial activation in the motor cortex and the severity of upper motor neuron signs. In the present study, differences in the axotomy induced microglial activation could be detected around motor neurons with different PV contents, potentially with different resistance against injury, which could be analogous to the graded response noted in chronic degeneration.

With regard to the temporal change of CCL2 expression and microglia activation following injury, our data reveal a transient increase of both parameters in the postoperative 3-week period. Our results demonstrate a reduced inflammatory reaction in the spinal cords of PV+/+ mice characterized by either of CCL2 or CD11b intensity. However, the measured CCL2 staining returns faster to the baseline, already at postoperative day 14, than CD11b at day 21. Assuming that motor neurons with different susceptibility against Ca stress start responding to the lesion in the same way, not the amplitude of the distress signal would be smaller in motor neurons with enhanced CaBP but its duration might be shorter. Thus, the cessation of the activation/migration of immune-competent cells, characterized by CD11b staining in our study, should follow the termination of the release of their driving signal, CCL2, with a time lag, as it could be seen. An attenuated Ca increase could be documented in spinal motor neurons of PV+/+ mice which is assumed to reflect an increased resistance against Ca-mediated degeneration after axotomy, and, thus, similarly to other models of acute injury where a graded microglial response was documented, a reduced activation of microglia could be evoked. Nevertheless, increasing the Ca buffer capacity of cells under chronic stress condition like in the mSOD1 model of ALS, may not provide sufficient protection, as it was shown in the double transgenic, PV+/+ × mSOD1 mice, in which the disease onset could be delayed but the progression of the disease could not be stopped. This inferior protection could be the consequence of the limited capacity of the buffer, which sooner or later saturates if the stress endures. Although the contribution of the added Ca buffers might not be sufficient for full protection, these data provide support for the idea that the strategy, addressing the stabilization of the Ca homeostasis, might be promising in attempts to rescue motoneurons.

SUMMARY

- In SOD1 transgenic mice, Tal treatment showed a significant protective effect in the axon terminals of susceptible muscles. The efficacy of the treatment is

related to the alleviation of extensive Ca influx via AMPA receptors with a Ca permeable, pathological feature. This neuroprotection lost its efficacy when applied in the symptomatic stage of ALS. However, both perikarya and distal axon terminals can be equally protected with a soma-targeted treatment.

- Oculomotor neurons might possess a special attribute which protects from Ca-mediated degeneration. This attribute might be related to the elevated CaBP content such as PV. Since these neurons show increased resistance against Ca-mediated degeneration, mSOD1 strain did not show elevated Ca level. Furthermore, Tal treatment was unable to decrease the Ca content further.
- Since nature provides us motor neurons with different CaBP contents, the effect of such proteins on neuroprotection could be tested in acute injury models using different motor nuclei. We showed that inflammatory processes represented by microglial activation are significantly lower in oculomotor neurons compared to the susceptible groups of motor neurons with low CaBP content.
- To attenuate the microglial reaction after acute injury, diazoxide treatment was applied. This neuroprotective trial shows a significant alleviation of inflammatory response in all examined motor nuclei. Efficacy of the diazoxide treatment might be related to the anti-inflammatory mechanism, however the pharmacological effect can be extended to the alleviation of Ca elevation as well.
- To test the interplay between Ca-mediated degeneration and microglial activation, PV+/+ mice were examined after unilateral axotomy. After the surgical procedure, intracellular Ca changes can be observed in the control strains only where the Ca shows significant elevation compared to the PV+/+ strain, therefore, we could confirm, that CaBPs are capable of reducing Ca-mediated degeneration.

- Furthermore, in the same PV-upregulated animals' chemokine signalization, represented by CCL2, was decaying faster, which resulted in a faster attenuation in the nearby microglia-mediated inflammation.

The results are in concordance with the hypothesis of the central role of Ca elevation in chronic degeneration and support its key role in acute motor neuron degeneration paradigms. Furthermore, we showed that both an AMPA receptor targeted treatment and an augmentation of the CaBP content are capable of reducing degeneration. Imbalanced Ca homeostasis might be establishing a local inflammatory reaction with the activation of microglial cells. However, this microglial recruitment could be attenuated by the augmentation of Ca homeostasis. According to our findings, measurement of the direct effect between inflammation and Ca elevation might give us new opportunities for neuroprotective trials and "eavesdropping" on the neuron-microglia crosstalk is strongly suggested to understand the pathomechanism behind motor neuron degeneration.

The Ph.D. thesis is based on the following *in extenso* scientific publications:

I. **Roland Patai, Bernát Nógrádi, József I. Engelhardt, László Siklós:** Calcium in the pathomechanism of amyotrophic lateral sclerosis – Taking center stage? *Biochemical and Biophysical Research Communications,* 2017; 483: 1031–1039. **Impact Factor: 2.466; Quartile Score: Q1 (Biophysics)**

II. **Roland Patai, Melinda Paizs, Massimo Tortarolo, Caterina Bendotti, Izabella Obál, József I. Engelhardt, László Siklós:** Presymptomatically applied AMPA receptor antagonist prevents calcium increase in vulnerable type of motor axon terminals of mice modeling amyotrophic lateral sclerosis. *Biochimica et Biophysica Acta – Molecular Basis of Disease*, 2017; 1863: 1739–1748. **Impact Factor: 5.476; Quartile Score: Q1 (Molecular Biology)**

III. **Melinda Paizs, Roland Patai, József I. Engelhardt, Zoya Katarova, Izabella Obál, László Siklós:** Axotomy Leads to Reduced Calcium Increase and Earlier Termination of CCL2 Release in Spinal Motoneurons with Upregulated Parvalbumin Followed by Decreased Neighboring Microglial Activation. *CNS and Neurological Disorders – Drug Targets, 2017;* 16(3): 356–367. **Impact Factor: 2.506; Quartile Score: Q1 (Medicine)**

Number of scientific publications providing the basis of the thesis:	**3**
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