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Protective effect of valproic acid on cultured motor neurons under glutamate excitotoxic conditions. Ultrastructural study

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that involves the upper and lower motor neurons and leads to the patient's death within 5 years after diagnosis. Approximately 2 per 100,000 people worldwide are affected every year. The only FDA-approved drug available for medical treatment is riluzole. It slows the disease progression and improves limb function and muscle strength for 3-4 months. Thus, looking for new therapeutic agents is a pressing challenge.

Valproic acid (VPA) is a short-chain fatty acid, widely used for the treatment of seizures and bipolar mood disorder. The beneficial effect of VPA has been documented in different neurodegenerative experimental models, including amyotrophic lateral sclerosis (ALS). The real mechanisms underlying numerous beneficial effects of VPA are complex, but recently it has been postulated that the neuroprotective properties might be related to direct inhibition of histone deacetylase (HDAC).

The aim of this ultrastructural study was to evaluate the beneficial effect of VPA on the spinal cord motor neurons (MNs) in a glutamate (GLU)-induced excitotoxic ALS model in vitro. It had been previously documented that chronic GLU excitotoxicity resulted in various MN injuries, including necrotic, apoptotic and autophagic modes of cell death. The present results demonstrated the neuroprotective properties of VPA associated with inhibition of apoptotic and autophagic changes of spinal MNs in a model of neurodegeneration in vitro.

Key words: *amyotrophic lateral sclerosis, valproic acid, neuroprotection, ultrastructural study.*

Introduction

Amyotrophic lateral sclerosis (ALS), known as Charcot's or Lou Gehrig's disease, is a fatal neurodegenerative disease, one of the heterogeneous group referred to as motor neuron diseases (MNDs). It primarily affects upper and lower motoneurons (MNs) of the cerebral cortex, brainstem and spinal cord, but surrounding glial cells, muscle cells, interneurons, and Renshaw inhibitory neurons might also be involved [5]. The generalized weakness and progressive muscle atrophy usually lead to death by respiratory muscle failure within five years after the disease onset [42]. Most ALS cases occur sporadically, while about 5-10% of them are inherited and classified as familial form of the disease [1]. So far more than 16 causative

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genes responsible for ALS/MNDs have been identified. Among them, 10-20% are probably caused by mutations in the gene encoding Cu/Zn superoxide dismutase-1 (SOD1), one of the main free-radical scavenging enzymes that protect cells against oxidative stress [2,37].

Until now, there is no effective therapeutic intervention for ALS, but different potential therapeutic targets are widely discussed [48]. It has been recently suggested that acetylation and/or deacetylation play an important role in the pathogenesis of different neurological diseases, including ALS [26]. Thus, histone deacetylase (HDAC) inhibitors have proved to be promising treatment in the broadly defined neurodegeneration.

Valproic acid (VPA; 2-n-propylpentanoic acid) is widely used as an anticonvulsant and mood stabilizing drug. Although different results suggest that this agent may exhibit a neuroprotective effect, the mechanism of such protection is not yet clear. VPA may exert its beneficial effect on neuronal survival and synaptic plasticity by acting directly on neurons and indirectly through glial cells [27]. Being a simple fatty acid, VPA is a substrate for the fatty acid β-oxidation (FAO) pathway, which takes place primarily in mitochondria. Recently, it has been also demonstrated that VPA effectively inhibits HDAC [15], making it valuable for investigations into the therapeutic role of chromatin remodeling in various pathological states of the central nervous system.

The purpose of this study was to determine whether valproate exhibits efficient neuroprotection against neuronal damage caused by glutamate toxicity in the organotypic culture of rat's lumbar spinal cord.

Material and methods

The study was performed on organotypic cultures of lumbar spinal cord obtained from 8-day-old rat pups. The cultures were kept at 36.6°C in a medium consisting of 25% inactivated fetal bovine serum and 75% DMEM (Dulbecco Modified Eagle's Medium) supplemented with glucose to a final concentration of 600 mg% and with antibiotics. The medium was changed twice a week. On the 10-14th day *in vitro* (DIV), the well-differentiated cultures were subjected to 1) glutamate (GLU) alone in a concentration of 100 μM; 2) 100 μM GLU with 10 μM VPA; 3) 10 μM VPA alone. The control cultures were grown in standard conditions.

After 3, 6 and 11 days, the cultures were processed for electron microscopic study. They were rinsed in a cacodylate buffer (pH 7.2), fixed in a mixture containing 0.8% formaldehyde and 2.5% glutaraldehyde for 1 hour, postfixed in 1% osmium tetroxide, dehydrated in alcohols in graded concentrations and embedded in Epon 812. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a JEOL 1200EX electron microscope.

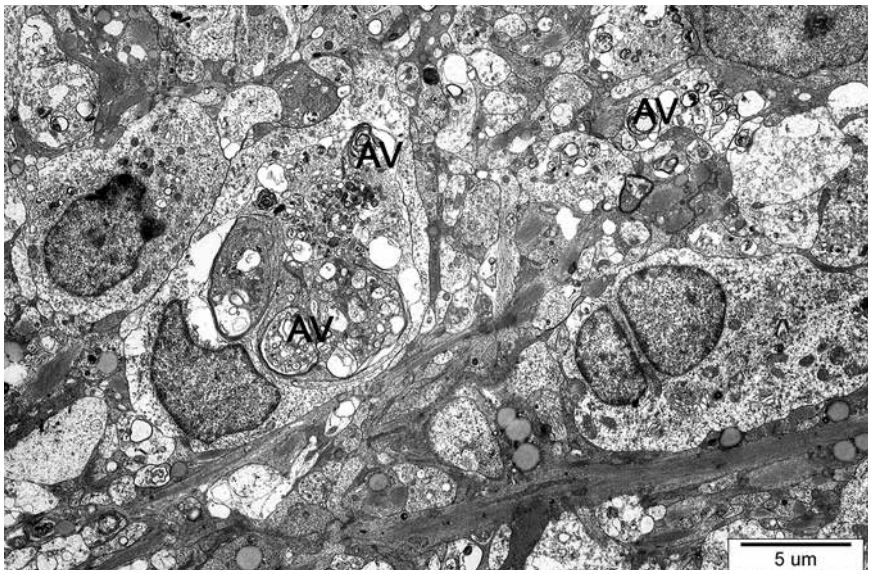

Fig. 1. Profiles of cellular processes and some neuronal cells with features of various degenerative changes, including numerous autophagic vacuoles (AV). Six days after 100 μM GLU.

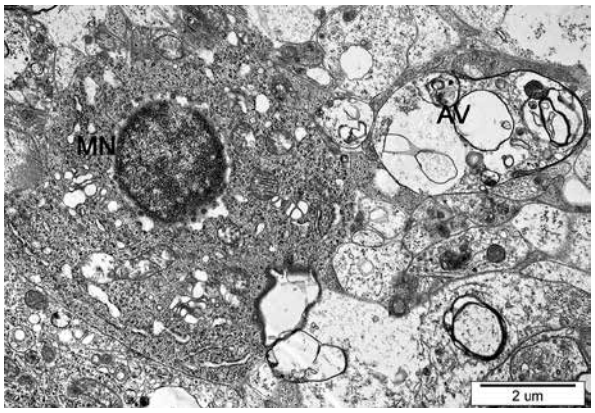

Fig. 2. Signs of apoptotic changes in motor neuron (MN), exhibiting condensed nuclear chromatin, increased electron density of cytoplasm and shrinkage of the whole cell body. Vesicular-autophagic changes (AV) seen in the vicinity of MN. Numerous damaged neuronal processes with signs of autophagy and destruction of organelles in surrounding neuropil. Eleven days after 100 µM GLU.

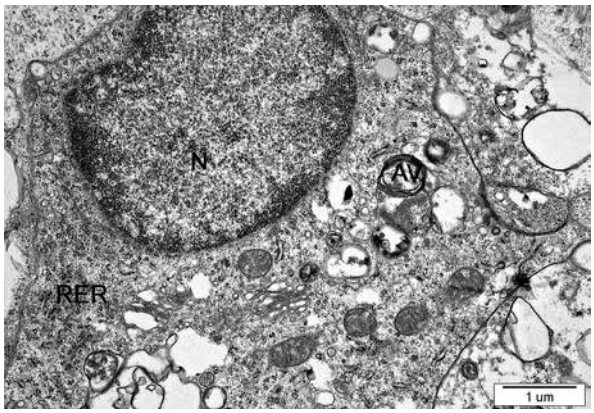

Fig. 3. Motor neuron with cytoplasm containing numerous autophagic vacuoles (AV) and dispersion of rough endoplasmic reticulum (RER). Nucleus (N) with condensed chromatin. Eleven days after 100 µM GLU.

Results

The spinal cord cultures exposed to GLU exhibited a range of advanced degenerative changes of MNs and destruction of neuronal processes in the surrounding neuropil. After 6 days of GLU exposure, the signs of various modes of cell death, including necrotic, apoptotic and autophagic changes, could be seen. Necrotic changes of MNs and their processes were characterized by extensive swelling and total destruction of organelles. The apoptotic bodies within large autophagic vacuoles were seen (Fig. 1). After 11 days of exposure to GLU, many MNs displayed signs of apoptotic changes in the form of a shrinkage of the cell body and condensation of nuclear chromatin (Fig. 2). Moreover, numerous autophagic vacuoles in the cytoplasm of MNs and neuronal processes were observed (Figs. 2 and 3). Less severe damage of MNs includes disaggregation and loss of rough endoplasmic reticulum and the Golgi complex, increase of cytoplasmic microvesicles and swelling of mitochondria (Fig. 4).

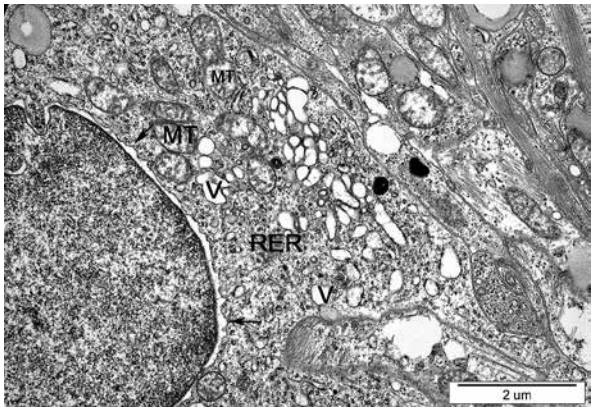

Fig. 4. Fragment of motor neuron exhibiting dispersed rough endoplasmic reticulum (RER), increased number of intracytoplasmic vesicles (V), distended perinuclear space (arrows) and slightly swollen mitochondria (MT). Eleven days after 100 µM GLU.

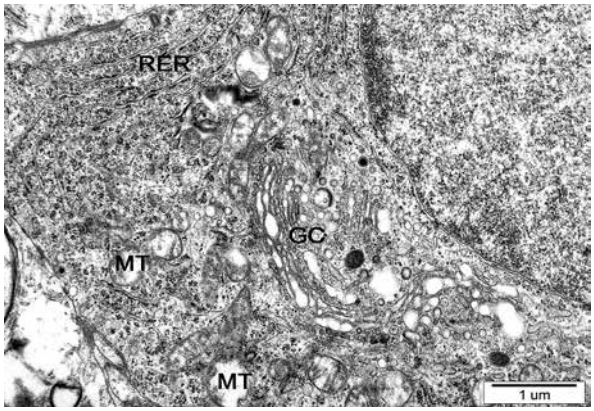

Fig. 5. Motor neuron with well-preserved nucleus and cytoplasm containing long, regularly arranged profiles of rough endoplasmic reticulum (RER), Golgi complex (GC), rich ribosomes and swollen mitochondria (MT). Three days after 100 µM GLU + 10 µM VPA.

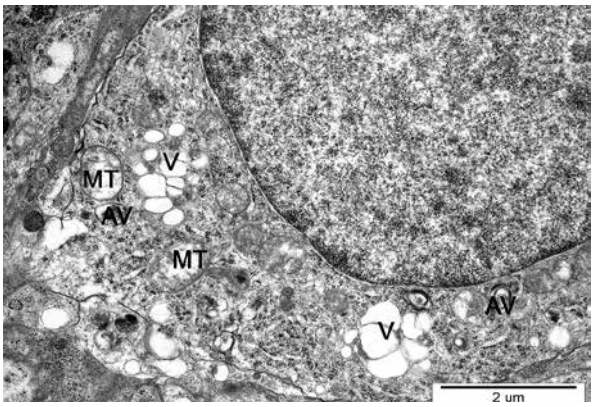

Fig. 6. Fragment of motor neuron with well-preserved nucleus and slight cytoplasmic abnormalities including presence of cytoplasmic vacuoles (V) with a few autophagic vacuoles (AV) and swollen mitochondria (MT). Three days after 100 µMGLU + 10 µM VPA.

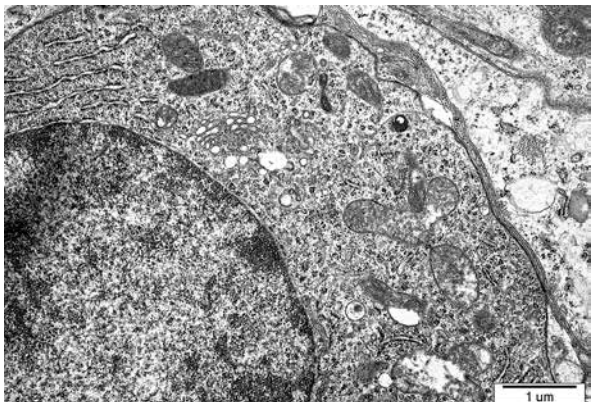

Fig. 7. Fragment of well-preserved motor neuron with normal organelles. Six days after 100 µM GLU + 10 µM VPA.

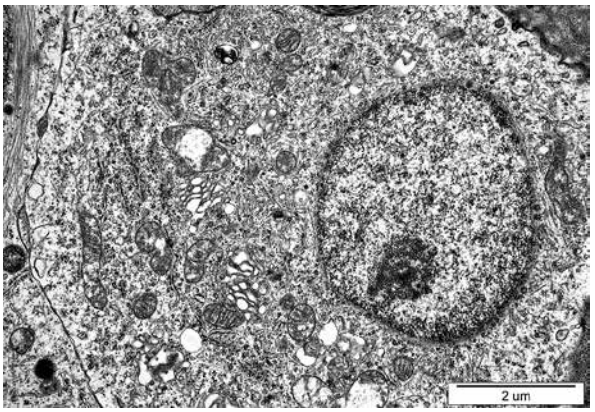

Fig. 8. Fragment of neuron, containing quite well-preserved organelles and nucleus with distinct nucleolus and dispersed heterochromatin. Six days after 100 µM GLU + 10 µM VPA.

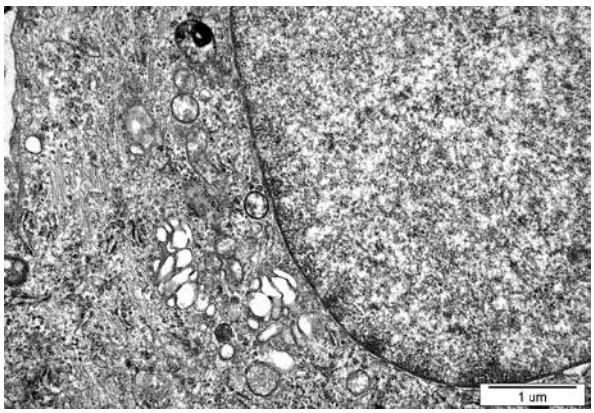

Fig. 9. Fragment of well-preserved motor neuron with large nucleus and cytoplasm containing normal organelles, microtubules and neurofilaments. Eleven days after 100 µM GLU + 10 µM VPA.

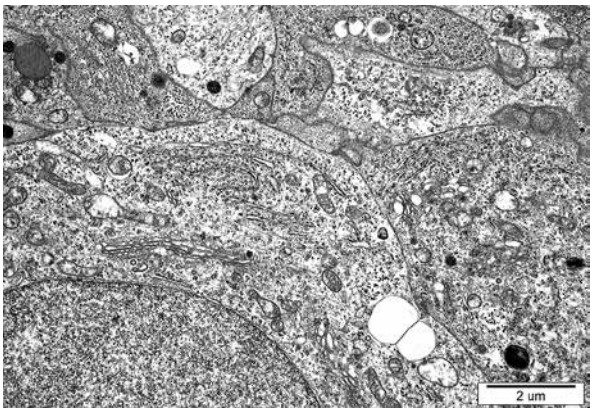

Fig. 10. Fragments of quite well-preserved neurons surrounded by neuropil with normal appearing neuronal and glial processes. Eleven days after 100 µM GLU + 10 µM VPA.

The treatment with VPA effectively prevents GLU-induced MN degenerative changes. Numerous well-preserved large motor neurons could be identified after 3, 6 and 11 days of co-treatment of VPA and GLU. After 3 days of VPA + GLU exposure the neurons exhibited perikaryal cytoplasm containing long profiles of rough endoplasmic reticulum, a well-developed Golgi complex (Fig. 5), and sometimes slightly swollen mitochondria and small autophagic vacuoles (Fig. 6). Quite well-preserved MNs, exhibiting a large nucleus with dispersed chromatin, distinct nucleolus and cytoplasm, containing well-organized rough endoplasmic reticulum and Golgi complex, were still recognizable after 6 days (Figs. 7 and 8) and 11 days (Figs. 9 and 10) of VPA + Glu exposure. Numerous microtubules and

neurofilaments in perikaryal cytoplasm were noticed (Fig. 9). The neuropil contained unchanged neuronal and glial processes (Fig. 10). Some large MNs exhibited only subtle ultrastructural abnormalities, such as slightly damaged mitochondria, focal cytoplasmic vacuolization and a few autophagic vacuoles. MNs with signs of apoptotic changes or autophagic degeneration could be seen only occasionally. Totally destroyed cells appeared sporadically.

Discussion

The mechanism underlying the pathogenesis of ALS remains unknown [28]. Genetic, molecular and environmental factors are believed to participate in the development of this progressive neurodegenerative process. Among various pathogenic factors, oxidative stress, mitochondrial dysfunction, apoptosis, glutamate excitotoxicity and proteasomal dysfunction are considered. However, the real cause of the disease, its natural history, classification, mechanism of progression and potential therapeutic targets are still under debate [13,29-31,35,36,41].

The cumulative evidence suggests that a mutation in Cu/Zn-superoxide dismutase (SOD1) protein contributes to the pathogenesis of familial ALS [38,44]. Dysfunction of neuronal mitochondria has been suggested to play an important role in MN degeneration [20]. The oxidative and endoplasmic reticulum (ER) stress [16,17] and deregulation of the ER mitochondrial calcium cycle [22] are described as the most likely causes of motor neuronal death, but it is believed that a complex mechanism of multiple toxic pathways is implicated in the ALS onset and progression [32]. Glutamate-induced excitotoxicity is also considered as one of the possible pathophysiological factors of motor neuron death [4,12].

Recently, a decreased histone acetylation level has been reported in the degenerating MNs in ALS experimental models [18]. Low histone deacetylase 6 (HDAC6) expression was reported at the onset and at the late stages of ALS in a mouse model [6]. Thus, it could be suggested that histone deacetylase inhibition may be a promising new therapeutic strategy for various neurodegenerative disorders, including motor neuron diseases (MNDs) [8,10]. As valproic acid (VPA), a short-chain fatty acid, effectively inhibits histone deacetylase [14,25] and delays apoptosis in degenerating neurons, it may be a good candidate for studies on its therapeutic role in different neurodegenerative conditions [3,19]. Its therapeutic potential has been documented in various cellular and animal models of neurologic, neurodegenerative, and neuropsychiatric disorders [7]. Combined treatment with lithium and VPA delays onset of clinical symptoms, reduces neurological deficits and prolongs survival in the SOD1 mouse model of amyotrophic lateral sclerosis [11]. Co-treatment of cerebellar granular cell cultures with lithium and VPA induced synergistic neuroprotective effects against glutamate excitotoxicity in a time-dependent manner [24].

Histone proteins organize DNA into nucleosomes, which are regular repeating structures of chromatin. This organization is required for the efficient packaging of large amounts of eukaryotic genomic DNA [45]. Acetylation and deacetylation of histone proteins play an important role in various cellular events, including epigenetic regulation of transcription. Histone acetyltransferases (HATs) catalyze acetylation, whereas histone deacetylases (HDACs) enhance deacetylation. Therefore VPA, via HDAC inhibition, might potentiate gene expression and promote a more transcriptionally active chromatin conformation. Thus, VPA protects neurons from excitotoxicity through inhibition of HDAC activity and suppression of apoptotic neuronal death associated with nuclear accumulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [21]. It was also suggested that VPA exerts neuroprotective effects through changes in a variety of intracellular signaling pathways, including upregulation of Bcl-2 protein with an antiapoptotic property [40]. Some results suggest that chronic treatment with valproate produce neuroprotection of cultured neuronal cells from damage caused by endoplasmic reticulum stress-mediated apoptosis [47] and oxidative stress [46]. It has been shown that combined treatment with histone deacetylase inhibitor and catalytic oxidant exerts additive neuroprotective effect in a transgenic mouse model of ALS [33]. Rouaux *et al*. [40] demonstrated that chronic VPA treatment *in vivo* prevented histone deacetylation in the spinal cord of symptomatic ALS mice. It delayed MN death and disease onset but did not significantly increase the mean lifespan of SOD1 (G86R) transgenic ALS mice and did not prevent distal pathology, i.e. neuromuscular denervation. However, despite the beneficial effect on MNs, VPA-treated animals died with

the neuropathological features typical for ALS. These results indicate that beneficial effects of VPA might be related to other factors than only a strict neuroprotection. On the other hand, neuroprotection of lower MNs is not sufficient for clinical therapy of ALS, because it does not prevent neuromuscular denervation.

In the mouse ALS model, CREB-binding protein, a transcriptional coactivator with histone acetyltransferase activity, was specifically reduced in motor neurons of the lumbar spinal cord. Consistently, decreased histone acetylation levels were observed in degenerating motor neurons [39]. Numerous studies have reported the beneficial effects of HDAC inhibitors on different aspects of neurodegeneration. Although treatment with VPA was found to protect motor neurons against glutamate toxicity in an organotypic culture of spinal cord and the ALS mouse model [43], another study using the same G93A mouse model found that long-term dietary VPA administration protected motor neurons but did not significantly affect the animal lifespan [9]. Overall, these data indicate poor efficiency of HDAC in treatment for ALS therapy despite the neuroprotective efficiency of HDAC. The randomized sequential clinical trial evidenced that VPA, at one dosage corresponding to the therapeutic dose used in epilepsy, did not show a beneficial effect on survival and disease progression in patients with ALS [34]. It has implications for future trials in neurodegenerative diseases with various histone deacetylase inhibitors.

Our ultrastructural study documented the neuroprotective efficacy of VPA in an experimental model of neurodegeneration related to GLU excitotoxicity in vitro. GLU-induced excitotoxicity has been implicated in the pathophysiology of various neurodegenerative diseases, including ALS. It was shown that valproic acid protected cultured cerebellar granule cells against GLU-induced excitotoxicity with concomitant transcriptional activation and induction of α-synuclein. This presynaptic protein was induced by valproic acid through histone deacetylase inhibition and participates in neuroprotection [23].

Our ultrastructural study evidenced that prevention of neuronal excitotoxic changes in vitro was related to inhibition of apoptosis and autophagy. The cultures subjected to GLU with VPA treatment exhibited well-retained parallel profiles of rough endoplasmic reticulum in the majority of motoneurons at 3 to 11 days after exposure. These results might support previous data indicating that neuroprotective effects of VPA might act via suppressing ER stress-mediated apoptosis. It could suggest that this mechanism may cause the VPA-induced therapeutic effects in neurodegenerative processes. Additional studies are necessary to clarify this issue.

Acknowledgments

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Disclosure

Authors report no conflict of interest.

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