

# CDP-choline protects motor neurons against apoptotic changes in a model of chronic glutamate excitotoxicity *in vitro*

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## Abstract

Cytidine-5-diphosphocholine (CDP-choline, citicoline) is an endogenous nucleoside involved in generation of phospholipids, membrane formation and its repair. It demonstrates beneficial effects in certain central nervous system injury models, including cerebral ischaemia, neurodegenerative disorders and spinal cord injury.

Defective neuronal and/or glial glutamate transport is claimed to contribute to progressive loss of motor neurons (MNs) in amyotrophic lateral sclerosis (ALS). Our previous ultrastructural studies, performed on an organotypic tissue culture model of chronic glutamate excitotoxicity, documented a subset of various modes of MN death including necrotic, apoptotic and autophagocytic cell injury.

The aim of this ultrastructural study was to determine the potential neuroprotective effect of CDP-choline on neuronal changes in a glutamate excitotoxic ALS model *in vitro*. Organotypic cultures of the rat lumbar spinal cord subjected to 100  $\mu$ M DL-threo- $\beta$ -hydroxyaspartate (THA) were pretreated with 100  $\mu$ M of CDP-choline. The exposure of spinal cord cultures to CDP-choline and THA distinctly reduced the development of typical apoptotic changes, whereas both necrotic and autophagocytic THA-induced MN injury occurred.

These results indicate that CDP-choline treatment might exert a neuroprotective effect against neuronal apoptotic changes in a model of chronic excitotoxicity *in vitro*.

**Key words:** CDP-choline, neuroprotection, excitotoxicity *in vitro*, motor neuron degeneration.

## Introduction

A glutamate-mediated mechanism is accepted to be involved in progressive motor neuron (MN) loss in amyotrophic lateral sclerosis (ALS) [36,40,41]. Glutamate receptor overactivation evokes excitotoxic neuronal injury via various pathways, including necrotic and apoptotic mode of cell death [49]. The *in vitro* model of chronic glutamate excitotoxicity, originally introduced

by Rothstein et al. [37], has been widely used for the study of the mechanism responsible for progressive neuronal injury and neuroprotection.

Citicoline, also known as CDP-choline (cytidine-5-diphosphocholine), is an endogenous nucleoside that exhibits neuroprotective abilities in certain central nervous system (CNS) injury models [8,11,50]. The neuroprotective properties of CDP-choline seem to be

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related to its action on glutamate-mediated cell death, but the precise mechanism remains not fully understood [1,21]. Citicoline might decrease the extracellular level of glutamate by inhibition of neuronal glutamate efflux and increased astrocytic glutamate uptake. It has been suggested that the neuroprotective effect of this compound is related to inhibition of the glutamate-induced apoptotic pathway of cell injury [31].

The present study was performed to determine the neuroprotective efficacy of CDP-choline on development of MN neurodegeneration in organotypic cultures of rat lumbar spinal cord chronically exposed to DL-threo- $\beta$ -hydroxyaspartate (THA) – a specific glutamate uptake blocker.

## Material and Methods

The study was performed on organotypic cultures prepared from lumbar spinal cord obtained from 8-day-old rat pups. The explants were placed on collagen-coated cover glasses with two drops of nutrient medium (consisting of 25% inactivated fetal bovine serum and 75% Dulbecco Modified Eagle's Medium supplemented with glucose to a final concentration of 600 mg% and with antibiotics), sealed into Maximow double assemblies and kept at 36.6°C. The medium was changed twice a week.

On the 10-14<sup>th</sup> day *in vitro* (DIV), the well-differentiated cultures were subjected to the specific glutamate uptake

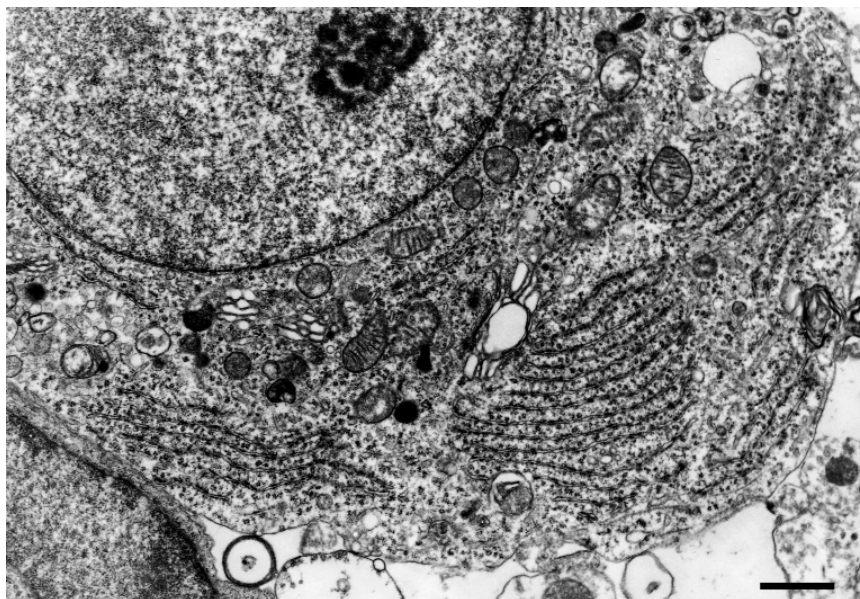
blocker DL-threo-beta-hydroxyaspartate (THA, Sigma) in a concentration of 100  $\mu$ M and to CDP-choline in three experimental groups: 1/ control group incubated with 100  $\mu$ M CDP-choline, 2/ cultures treated with 100  $\mu$ M THA, 3/ cultures incubated with medium containing THA but pretreated with CDP-choline in concentrations as before.

After 2, 3, 5, 7 and 14 days post treatment the cultures were processed for the electron microscope. They were rinsed in 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.

## Results

The rat spinal cord cultures exposed to CDP-choline alone exhibited quite well preserved motor neurons with a large nucleus with dispersed chromatin and abundant cytoplasm containing numerous organelles (Fig. 1). Normal astroglial cells and neuropil filled with neuronal and glial processes were observed (Figs. 2, 3).

Chronic THA exposure resulted in a distinct type of MN injury including necrosis with total destruction of cytoorganelles (Fig. 4), apoptotic changes with more

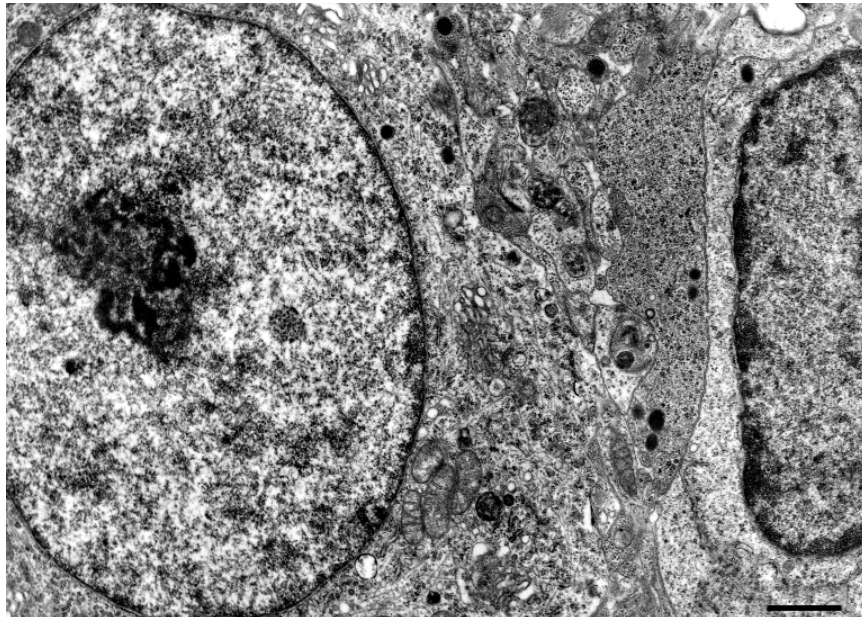


**Fig. 1.** Normal MN displaying large nucleus with dispersed chromatin, distinct nucleolus and cytoplasm containing numerous cytoorganelles. 5 days of 100  $\mu$ M CDP-choline incubation. Bar – 1  $\mu$ m

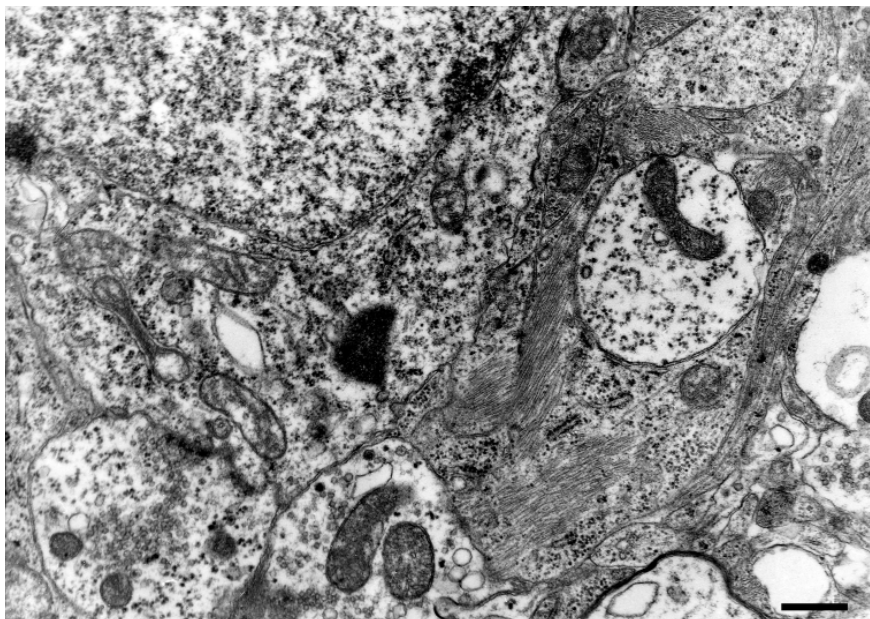
or less condensed chromatin and apoptotic bodies (Fig. 5) and autophagocytosis with the presence of numerous autophagic vacuoles containing destroyed organelles (Figs. 6, 7). Moreover, a few completely damaged neurons that shared apoptotic, autophagic and necrotic characteristics were observed.

In the spinal cord cultures treated with CDP-choline together with THA, evidence of inhibition of progressive MN neurodegeneration, especially limitation of apoptotic changes, was documented.

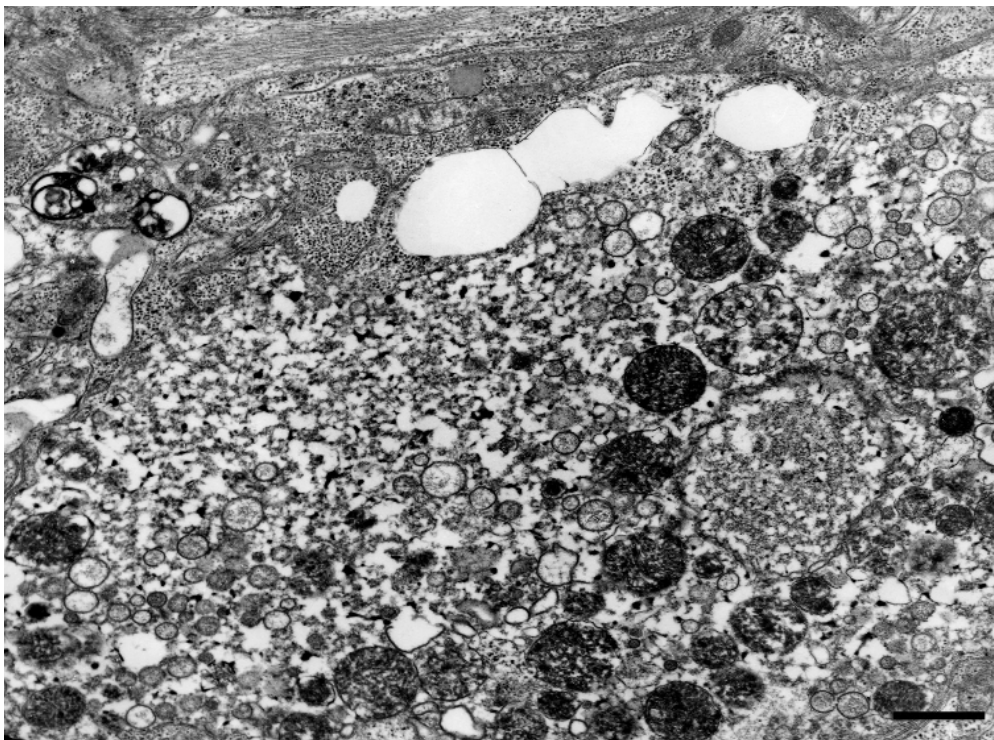
Typical apoptotic changes, characterized by peripheral condensation and margination of nuclear chromatin



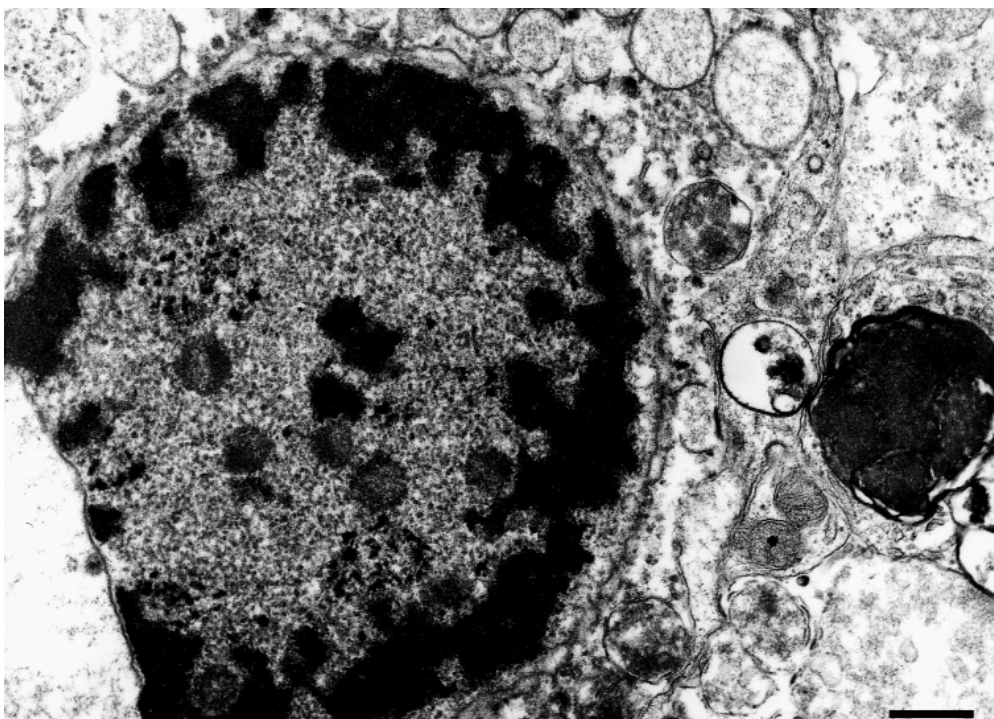
**Fig. 2.** Normal neuron and glial cells with well maintained organelles. 5 days of 100  $\mu$ M CDP-choline incubation. Bar – 1  $\mu$ m



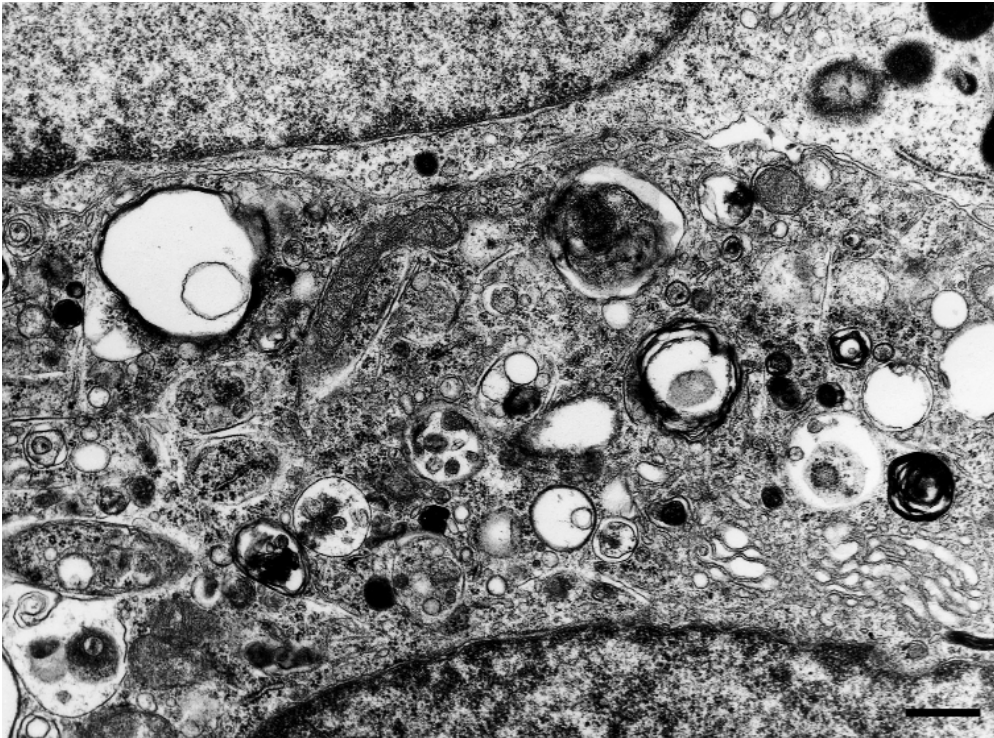
**Fig. 3.** Well-preserved neuron and neuropil with neuronal and glial processes and synapses. 5 days of 100  $\mu$ M CDP-choline incubation. Bar – 500 nm



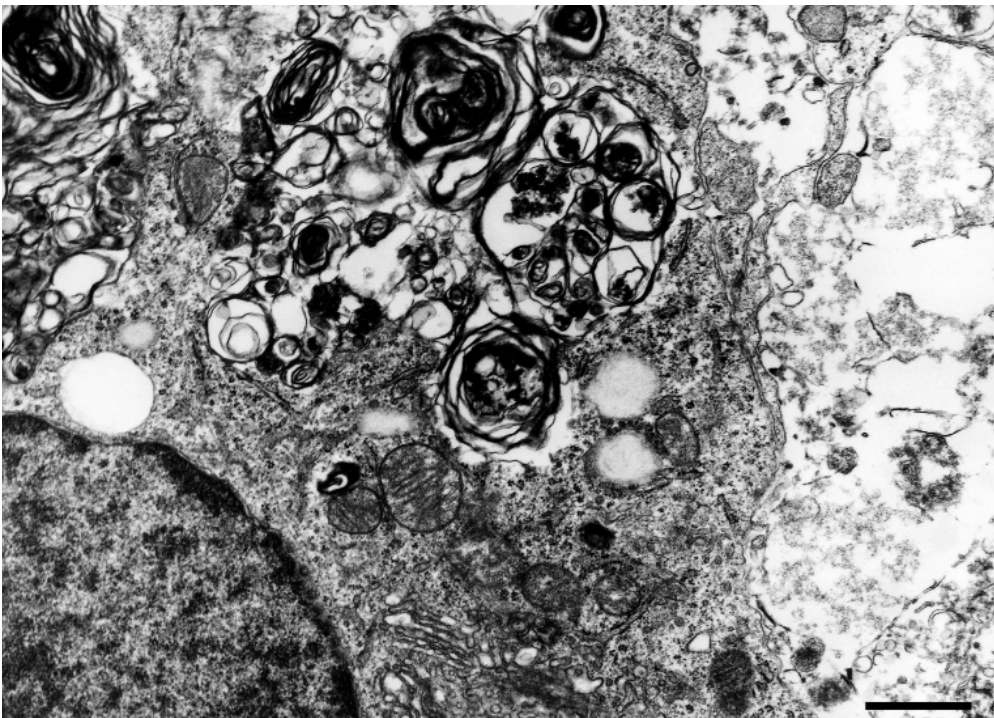
**Fig. 4.** Fragment of necrotic neuron containing destroyed organelles. 5 days of 100  $\mu$ M THA incubation. Bar – 1  $\mu$ m



**Fig. 5.** Degenerating neuron with clumps of condensed nuclear chromatin. Cytoplasm filled with autophagic vacuoles and swollen mitochondria with loss of cristae. Apoptotic body in vicinity. 24 hours of 100  $\mu$ M THA incubation. Bar – 500 nm

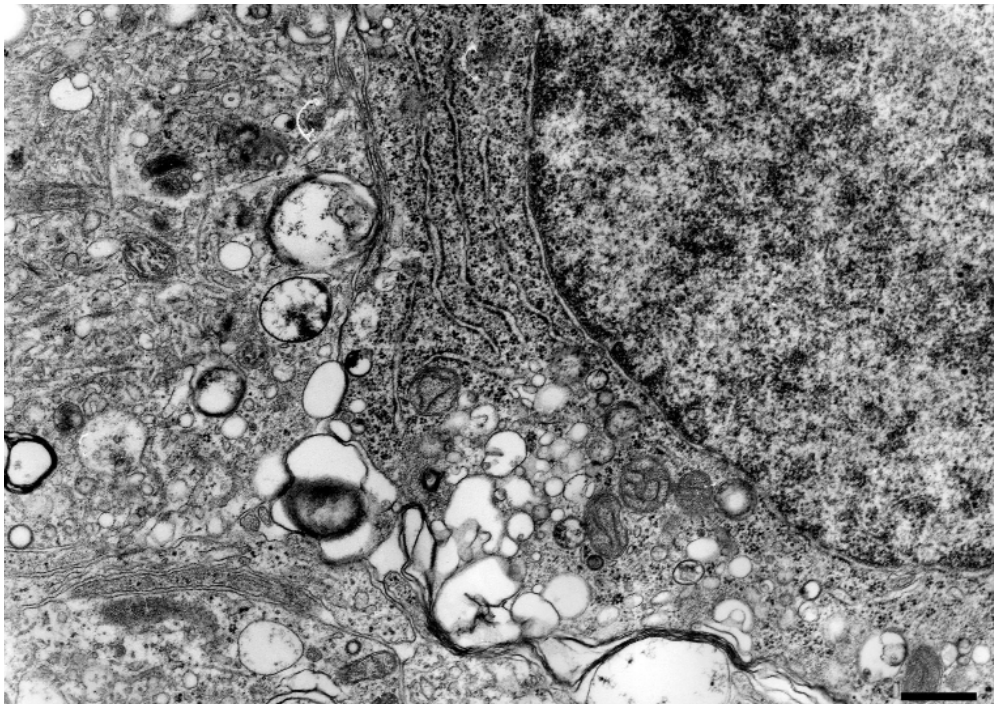


**Fig. 6.** Fragment of MN with cytoplasm containing numerous autophagic vacuoles. 24 hours of 100  $\mu\text{M}$  THA incubation. Bar – 500 nm

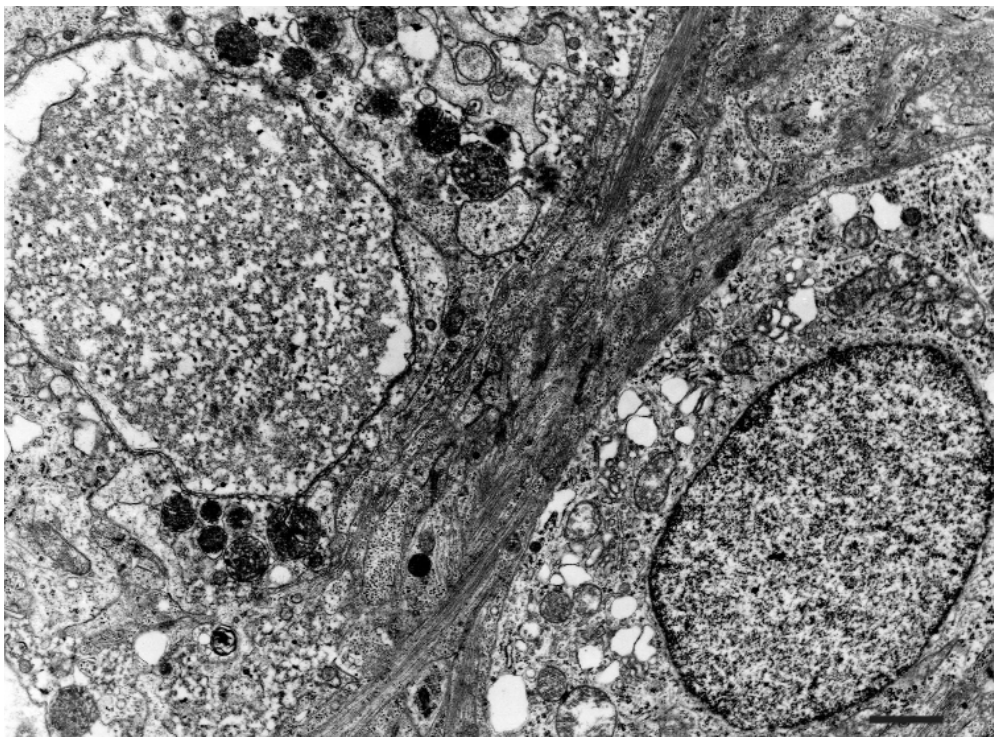


**Fig. 7.** Fragment of MN with cytoplasm containing numerous autophagic vacuoles, as well as other, sometimes quite well-preserved organelles and nucleus. 24 hours of 100  $\mu\text{M}$  THA incubation. Bar – 1  $\mu\text{m}$

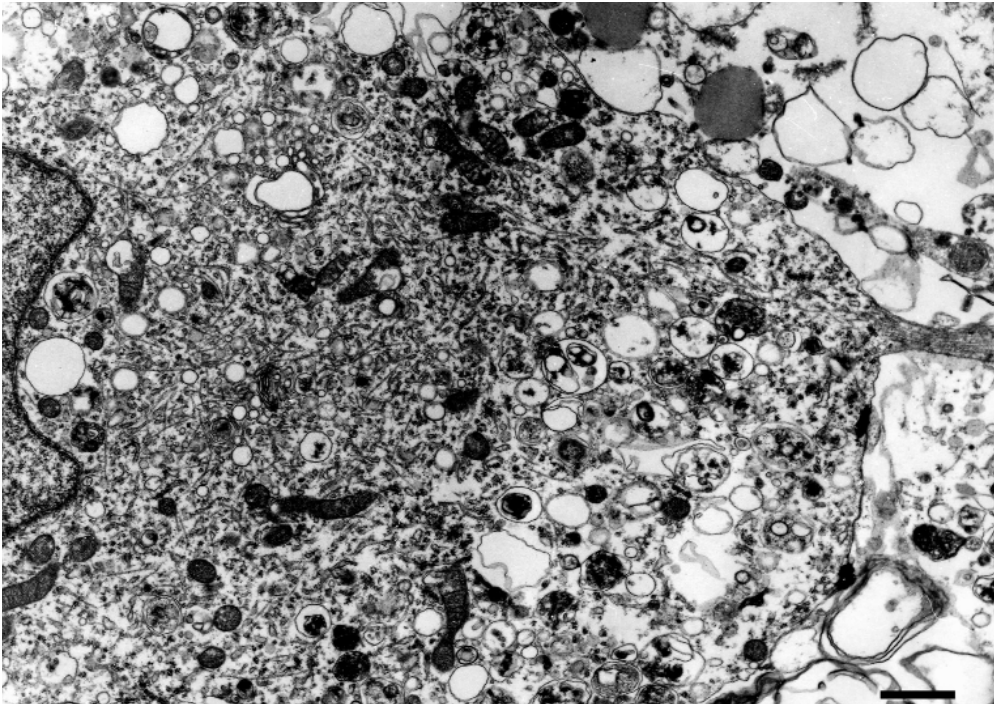




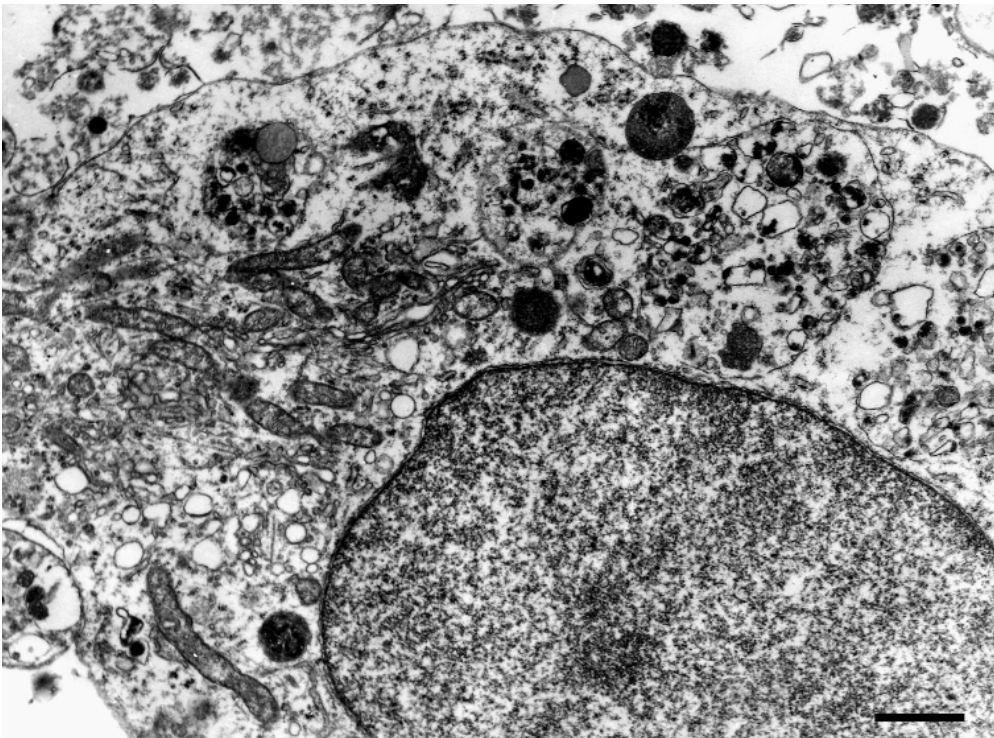
**Fig. 8.** Fragment of MN with normal nucleus and well-preserved channels of endoplasmic reticulum with focal accumulation of vacuoles. 5 days of 100  $\mu$ M THA and 100  $\mu$ M CDP-choline incubation. Bar – 500 nm



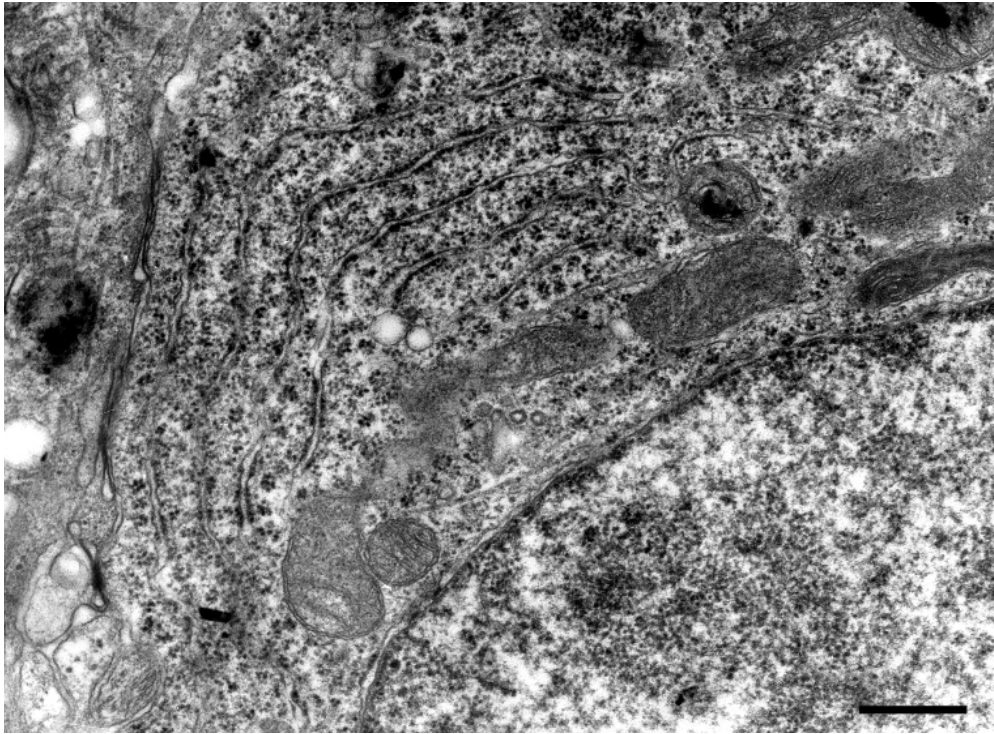
**Fig. 9.** Fragment of MN with large, well-preserved nucleus and slightly vacuolated cytoplasm. Necrotic changes in neighbouring cellular fragments. 5 days of 100  $\mu$ M THA and 100  $\mu$ M CDP-choline incubation. Bar – 1  $\mu$ m



**Fig. 10.** Fragment of neuron exhibiting necrotic and autophagic changes. 5 days of 100  $\mu\text{M}$  THA and 100  $\mu\text{M}$  CDP-choline incubation. Bar – 1  $\mu\text{m}$



**Fig. 11.** Fragment of neuron containing autophagic vacuoles sequestered within cytoplasm. Large, well-preserved nucleus with lucent dispersed chromatin. 5 days of 100  $\mu\text{M}$  THA and 100  $\mu\text{M}$  CDP-choline incubation. Bar – 1  $\mu\text{m}$



**Fig. 12.** Fragment of MN with normal appearance of cytoplasm and nucleus. 5 days of 100  $\mu\text{M}$  THA and 100  $\mu\text{M}$  CDP-choline incubation. Bar – 500 nm

or aggregation of nuclear chromatin in dense masses beneath the margin of the nucleus, could be seen only sporadically. Numerous large motor neurons exhibited quite well-preserved organelles (Fig. 8) or displayed only subtle mitochondrial swelling. Membrane-bound apoptotic profiles with fragments of compact chromatin and/or cytoplasmic structures were not seen in the cultures treated with CDP-choline.

Nevertheless, some neurons showed various degrees of cytoplasmic vacuolization and contained numerous vesicles and/or vacuoles of various size and damaged mitochondria (Fig. 9). Totally destroyed, necrotic cells appeared occasionally (Fig. 10).

More often MNs exhibited characteristics typical for autophagocytosis. These changes were observed especially after a longer time (7, 14 days) of the experiment. The cytoplasm of these neurons displayed many autophagic vacuoles of various size and shape, containing damaged organelles, i.e. ribosomes, mitochondria and/or unidentified vacuoles (Figs. 11, 12). In some large MNs, numerous secondary lysosomes and small dark bodies or autophagosomes were observed. A striking feature is that most MNs with advanced autophagocytic changes of the cytoplasm exhibited a well-preserved nucleus.

## Discussion

Glutamate-induced excitotoxicity is widely accepted to underlie the neuronal injury in certain acute or chronic neurodegenerative processes [9,12,30]. Both neurons and astroglial cells are involved in glutamate transmission [5,19,23,32,33]. It has been documented that astroglia express functional ionotropic (iGluRs) and metabotropic (mGluRs) Glu receptors and their dysfunction might result in ineffective Glu uptake and increase of its extracellular level [13,24,38].

Recently, the glutamate-mediated mechanism via defective glial and/or neuronal glutamate transport is widely accepted as responsible for progressive MN loss [36,38,40,41]. In ALS patients, elevated levels of glutamate in cerebrospinal fluid and selective reduction of EAAT2 glutamate astrocytic transporter have been documented [39,43]. The decrease or loss of GLT-1 glutamate transporter has also been observed in various animal models of ALS, including SOD-1 transgenic mice and a mutant SOD1 G93A rat model [7,45]. Morphological studies demonstrated the progression of morphological changes within CNS in a transgenic rat model of familial amyotrophic lateral sclerosis [17,20,35]. It seems that loss of glutamate



transporters in ALS might be secondary to astrocytic activation as alterations in glutamate transporters are accompanied by marked astrogliosis. Thus, cell death in amyotrophic lateral sclerosis results from the interplay between neuronal and glial cells [15].

It could be suggested that manipulation of glutamate transporters might be useful in therapeutic treatments in MN degeneration. Organotypic cultures of spinal cord, that maintain neuron-astrocyte structural and metabolic interactions, are especially useful to study the mechanism of progressive MN degeneration. Our previous ultrastructural studies performed on organotypic cultures of rat lumbar spinal cord chronically exposed to specific glutamate uptake blockers DL-threo- $\beta$ -hydroxyaspartate (THA) and L-trans-pyrrolidine-2,4-dicarboxylate (PDC) evidenced different modes of cell death, i.e. necrotic, apoptotic and/or autophagic degeneration [27,28], accompanied by distinct glial changes [29]. There is increasing evidence that both programmed cell death [48] and so-called "autophagic cell death" participate in cell degeneration in certain pathological conditions [25].

In the present study we tested the neuroprotective ability of citicoline – a naturally endogenous nucleoside that play an important role in formation and repair of cell membranes [52]. It has been documented that citicoline activates the biosynthesis of phosphatidylcholine (PtdCho) – a phospholipid essential for the maintenance of intra- and extracellular membrane [26,46]. Moreover, the hydrolytic products of citicoline (cytidine and choline) are involved in the formation of nucleic acids, proteins and acetylcholine [3,47]. Such properties allow citicoline to be used in clinical trials in certain central nervous system (CNS) disorders [50]. The neuroprotective efficacy of this agent was documented in patients with severe stroke, Alzheimer's disease, glaucoma and in various experimental models of CNS injury *in vivo* and *in vitro* [4,6,10,16,18,21,22,34].

It has been suggested that the neuroprotective action of citicoline might be related to a decrease of extracellular glutamate level. The protective effect of CDP-choline might also be associated with its actions on cell membrane stability [1,2,42]. The activation of phospholipases and degradation of membrane phospholipids have been reported in various neurodegenerative disorders [14,42,44,51].

The present study demonstrated that CDP-choline exerts neuroprotection in progressive MN injury in a model of chronic excitotoxicity *in vitro*. It inhibited mainly neuronal apoptotic changes, whereas necrotic and autophagocytic abnormalities were not reduced.

This confirms the suggestion that citicoline might protect neurons against the glutamate-induced apoptotic pathway [31], probably via a negative effect on activation of the caspase cell death pathway.

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