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Astroglial alterations in amyotrophic lateral sclerosis (ALS) model of slow glutamate excitotoxicity *in vitro*

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Abstract

Chronic excitotoxicity mediated through defective glial and/or neuronal glutamate transport may contribute to several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS). This study was performed to determine the ultrastructural characteristics of astroglial changes concomitant with motor neuron (MN) degeneration in a model of slow excitotoxicity in vitro. The study was performed on organotypic cultures of rat lumbar spinal cord subjected to the glutamate uptake blockers threohydroxyaspartate (THA) and L-trans-pyrrolidine-2,4-dicarboxylate (PDC).

The chronic inhibition of glutamate transport by THA and PDC resulted in slow degeneration of the rat's MNs accompanied by distinct glial changes predominantly involving protoplasmic astrocytes. The presence of irregular vacuoles and vesicles in the astroglial cells was frequently observed. Occasionally the astrocytes exhibited proliferation and accumulation of abnormal profiles of smooth endoplasmic reticulum. In 3 weeks there were no signs of increased production of glial filaments in the protoplasmic astrocytes.

The results evidenced the coexistence of neuronal degeneration and astroglial abnormalities in an ALS model in vitro and suggested an active role of astrocytes contributing to the induction and propagation of MN degeneration.

Key words: *chronic excitotoxicity, glutamate uptake blockers, rat spinal cord in vitro, astroglial changes.*

Introduction

There is increasing evidence that astroglial cells participate in neurodegenerative processes in certain pathological conditions. Considering the mechanism of selective neuronal death in amyotrophic lateral sclerosis (ALS), the glutamate-mediated mechanism is thought to be responsible for the progressive loss of motor neurons (MNs) [34]. The widespread motor neuron degeneration in ALS is typically accompanied by a distinct reaction of the surrounding astrocytes [16, 18, 24, 40]. The origin of such pan-cellular pathology is not fully understood. However, increasing data have suggested an important role of astrocytes in excitotoxic damage of motor neurons in ALS [3]. It could be suggested that astrocytes contribute to excitotoxic neuronal injury by defect in glutamate transport [35, 36, 38].

The *in vitro* model of chronic glutamate excitotoxicity obtained by incubation of the organotypic spinal cord cultures with specific glutamate transporter inhibitors,

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originally developed by Rothstein et al. [32], is particularly useful for the study of ultrastructural characteristics of both neuronal and glial cells. Moreover, the organotypic cultures of rat lumbar spinal cord maintain neuron-astrocyte structural and metabolic interactions. The motor neuron cultures used to study ALS models may help to explain the mechanism of progressive nature of cell death in this neurodegenerative process [45].

Our previous ultrastructural studies performed on an *in vitro* model of slow glutamate excitotoxicity evidenced the different modes of MN death [22, 23]. The present ultrastructural study evaluated the contribution of glial changes to MN loss in organotypic cultures of rat lumbar spinal cord chronically exposed to specific glutamate uptake blockers: DL-threo-β-hydroxyaspartate (THA) and L-trans-pyrrolidine-2,4-dicarboxylate (PDC).

Materials and methods

Organotypic cultures were prepared from spinal cord obtained from 8-day-old rat pups. The lumbar spinal cords were dissected in sterile conditions and cut transversely into thin slices. The explants were placed on collagen-coated cover glasses with two drops of nutrient medium and sealed into Maximow double assemblies. The cultures were kept at 36.6°C in a medium consisting of 25% inactivated foetal bovine serum and 75% DMEM (Dulbeco 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 incubated with medium containing selective blockers of glutamate transport: DL-threo--β-hydroxyaspartate (THA, Sigma) and L-trans--pyrrolidine-2,4-dicarboxylate (PDC, Sigma) at concentration 100μM. After 2 and 24 hours, 3, 5, 7, 14 and 28 days post treatment the cultures were processed for study by 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.

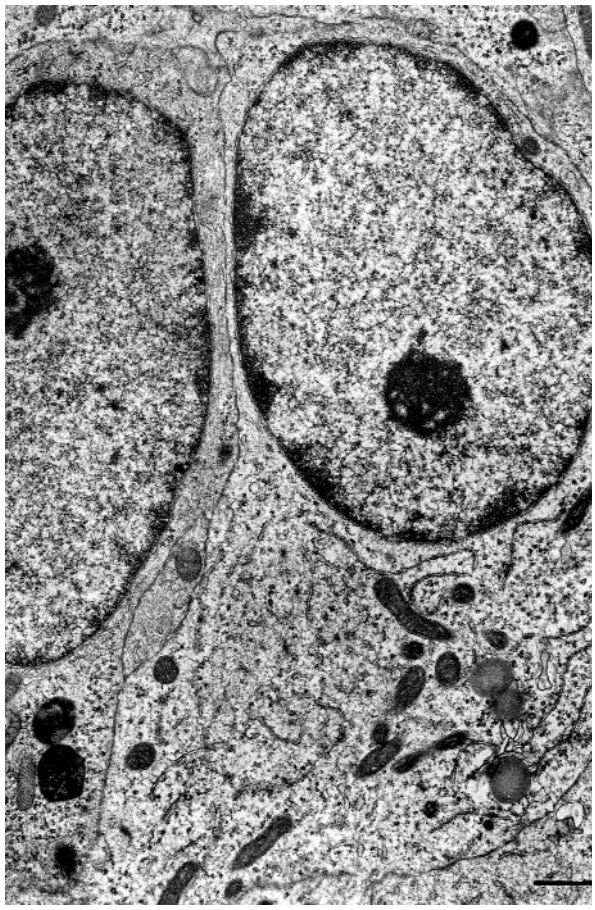

Fig. 1. Well-preserved cell bodies and processes of astroglial cells in control culture. Bar = 1 μm

Results

Up to the 28th DIV the control spinal cord cultures maintained well-preserved large MNs characterized by a large nucleus surrounded by abundant cytoplasm rich in organelles and numerous astroglial cells of protoplasmic type. For 28 days normal ultrastructural appearance of the astrocytic cells was observed in control cultures. These cells were characterized by moderately electron-lucent cytoplasm with dispersed ribosomes, narrow cisternae of endoplasmic reticulum, small mitochondria, occasional dense bodies or lipid droplets and eccentrically located round or oval nucleus with fine chromatin and small nucleolus (Fig. 1).

For 28 days the cultures treated with 100 μM THA or PDC displayed slowly progressing MN degeneration accompanied by distinct abnormalities of astroglial cells including predominantly protoplasmic type of

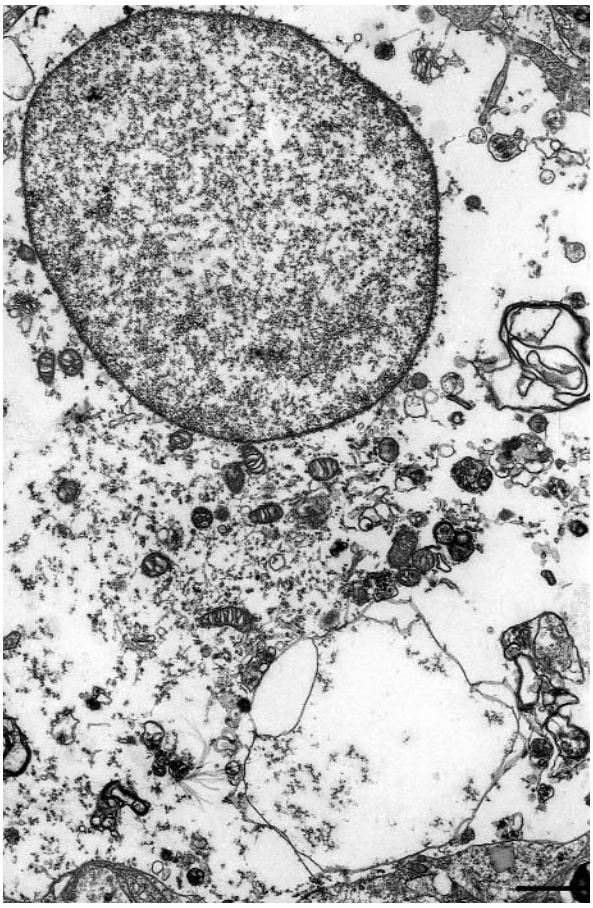

Fig. 2. Astrocyte displaying prominent swelling of the cytoplasm. 24 h of 100 μM THA incubation. Bar = 2 μm

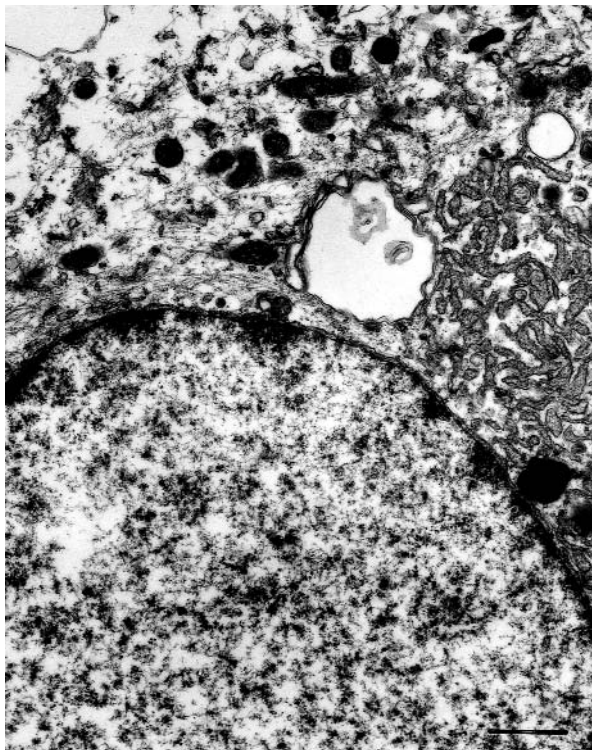

Fig. 3. Astrocyte with perinuclear aggregation of short abnormal channels of smooth endoplasmic reticulum and swelling and vacuolisation of peripheral part of cytoplasm. 5 days of 100 μM THA incubation. Bar = 1 μm

astrocytes. A large number of astrocytes exhibited distinct cytoplasmic abnormalities, whereas their nuclei were usually well preserved. Swelling of the cytoplasm accompanied by formation of irregular vesicles and vacuoles of different shape and size was seen as early as 24 h after exposure to THA (Fig. 2) and it was also prominent at day 5 after both THA and PDC treatment (Figs. 3, 4). The irregular vacuoles sometimes occupied the majority of the astroglial cytoplasm. Commonly, the peripheral part of the cytoplasm was most severely affected. Occasionally, degenerated organelles such as shrunken mitochondria, heterogeneous electron-dense bodies or multivesicular and autophagic vacuoles were observed in the astrocytes exhibiting swollen cytoplasm and nuclei with irregular dispersion of chromatin (Fig. 4). Some glial cells exhibited abnormal development of Golgi apparatus with increase of content of small and large Golgi vesicles. After 5 days and later some cells displayed proliferation of endoplasmic reticulum membranes with abnormal aggregation of their short channels (Fig. 3) or formation of long-branched profiles and multilamellar structures (Figs. 5, 6). After 14 and 28 days post THA and PDA exposure, swelling of the astrocytic cytoplasm diminished, whereas intracytoplasmic vacuoles of different size and shape increased in number (Figs. 7, 8). Membrane-limited large vacuoles occupied mostly peripheral parts of the cytoplasm or were distributed through the whole perikarya (Fig. 7) and the processes of the cells (Fig. 8). Some degree of cytoplasm condensation in the vacuolated astrocytes was noted; however, for 3 weeks there was no evidence

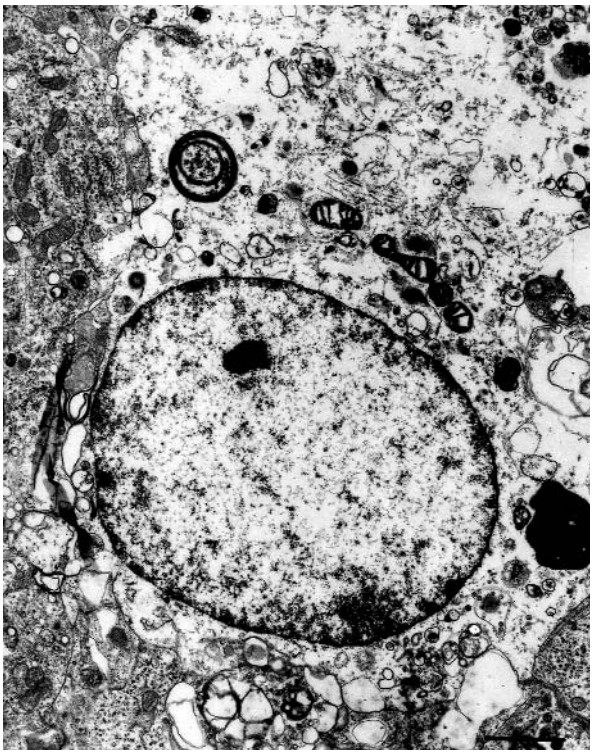

Fig. 4. Damaged astrocyte exhibiting degenerated organelles and autophagic vacuoles in cytoplasm and loss of nuclear chromatin. 5 days of 100 μM PDC incubation. Bar = 2 μm

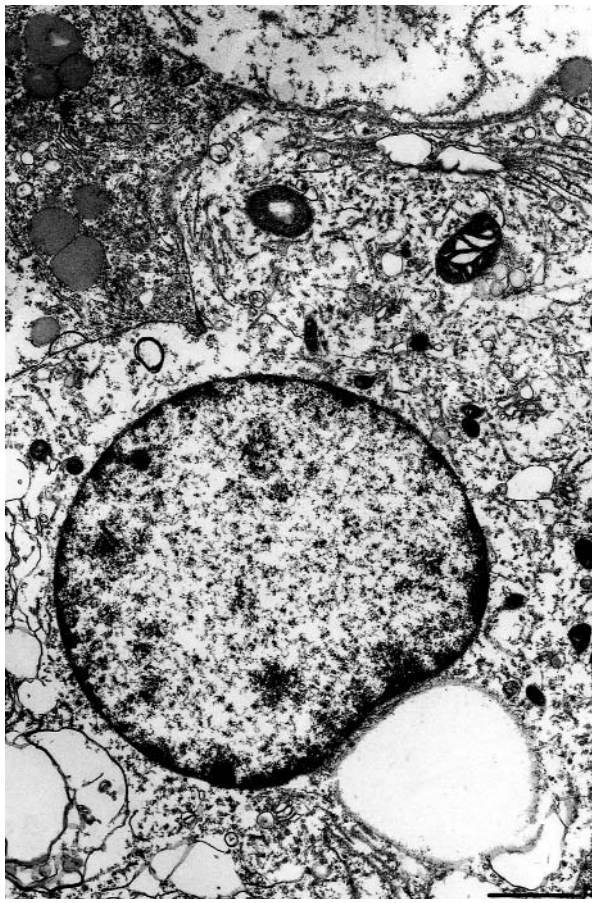

Fig. 5. Astrocyte exhibiting long channels of endoplasmic reticulum, irregular membranous profiles and vacuolar changes in cytoplasm and irregularly dispersed nuclear chromatin. 5 days of 100 μM PDC incubation. Bar = 2 μm

of production and increased accumulation of glial filaments in these cells, considered as protoplasmic astrocytes. Some hypertrophied fibrillar astrocytes filled with glial filaments and containing engulfed rest bodies of apoptotic or necrotic motoneurons (Fig. 9) were observed.

Discussion

Neuronal injury upon various pathological conditions is usually associated with a phenomenon known as "reactive astrogliosis", which has long been considered a non-specific response of glial cells to different noxious factors [26, 40]. The reactive astrocytes display characteristic morphological features in the form of enlarged nuclei surrounded by hypertrophic cell bodies with an increased amount of gliofilaments and marked immunoreactivity for glial fibrillary acidic protein (GFAP). These typical phenotypic changes are often accompanied by expression of cytoskeleton proteins, molecules of cell surface and matrix, proteases, growth factors and cytokines [8, 30].

The widespread astrogliosis is commonly observed in amyotrophic lateral sclerosis patients [16, 18, 24, 39]. A distinct astroglial reaction has also been demonstrated in a mouse amyotrophic lateral sclerosis (ALS) model [20] and in neonatal rat spinal cord after exposure to cerebrospinal fluid from patients with ALS [41]. Increasing data have supported the opinion of the important role of astrocytes in pathogenesis of neuronal death in various pathological states [49], including ALS [3]. Glial pathology is considered to be a potential pathogenic event in ALS as the glutamate-mediated

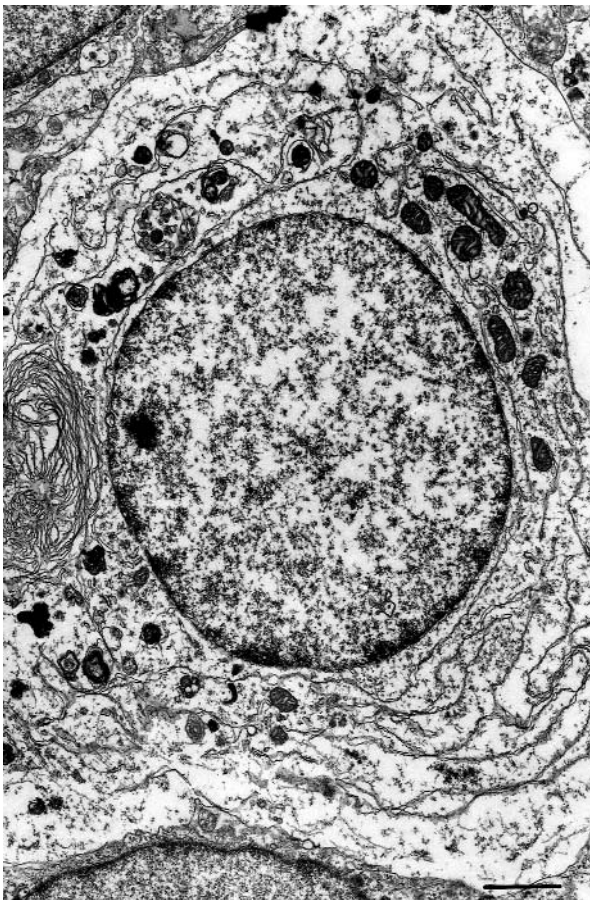

Fig. 6. Proliferation of long and narrow channels of endoplasmic reticulum and focal accumulation of smooth membranes accompanied by small dense bodies and autophagic vacuoles in astrocytic cytoplasm. 14 h of 100 μM THA incubation. Bar = 2 μm

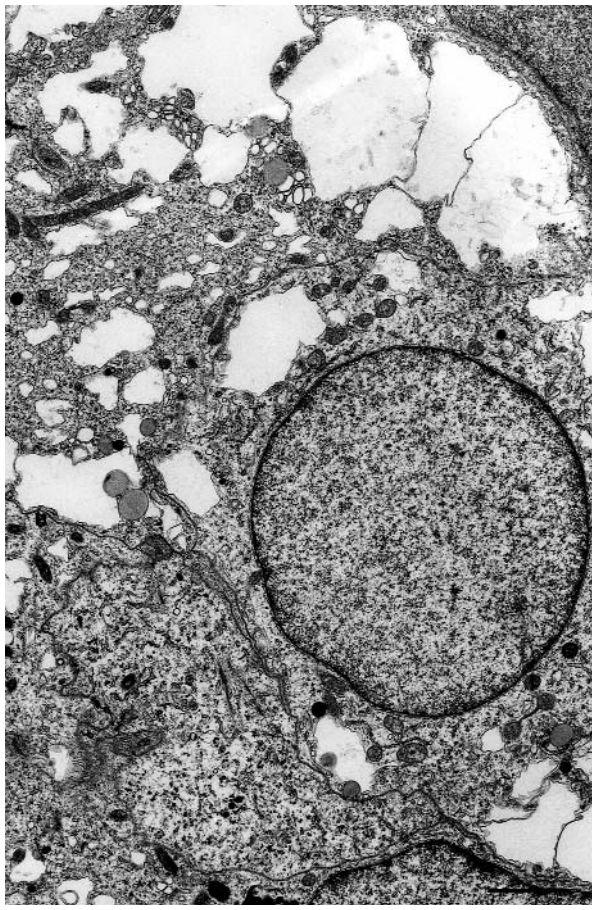

Fig. 7. Empty, irregular vacuoles of various sizes in condensed cytoplasm of astrocyte. 28 days of 100 μM THA incubation. Bar =2 μm

mechanism, including defective glial and/or neuronal glutamate transport, is widely accepted as responsible for progressive MN loss [34].

Glutamate (GLU) is the primary excitatory amino acid neurotransmitter in the central nervous system [6]. It has been documented that both astroglia and neurons are involved in glutamate synaptic transmission [2, 14]. Astrocytes participate in neuronal excitability by controlling the extracellular levels of GLU and release glutamine back to the neurons [4, 15, 36]. They also commonly express functional ionotropic (iGluRs) and metabotropic (mGluRs) glutamate receptors [13, 47].

The extracellular concentration of GLU depends on its efficient removal from the synaptic cleft by glutamate transporters of high affinity [50]. So far, a number of different glutamate transporters, located in both the plasma membranes of presynaptic terminals and astrocytes, have been identified in the central nervous system [25, 27, 33]. Two of them, GLT-1 and GLAST, are almost exclusively found in astrocytes [19, 43]. GLT1 (EAAT2) is responsible for up to 90% of all glutamate transport in adult tissue [7, 51], whereas GLAST (EAAT1) is mainly responsible for Glu transport in the developing nervous system [10, 42, 48].

It is suggested that chronic glutamate neurotoxicity due to non-effective glutamate uptake participates in various pathological states [6, 37] including selective loss of MNs in ALS [28, 35, 36]. Both elevated glutamate levels [44, 46] and

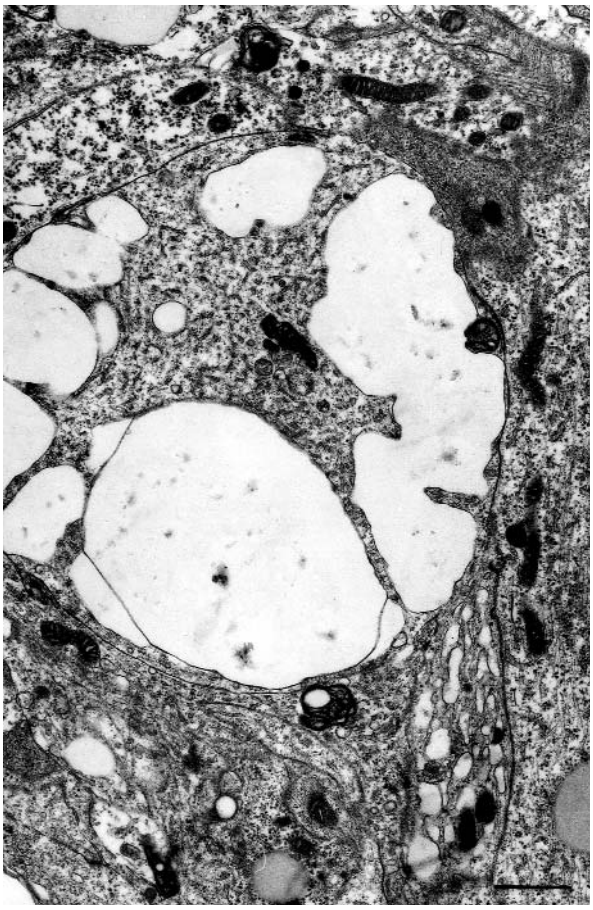

Fig. 8. Astrocytic process with accumulation of intracytoplasmic large vacuoles. 14 days of 100 μM THA incubation. Bar = 1 μm

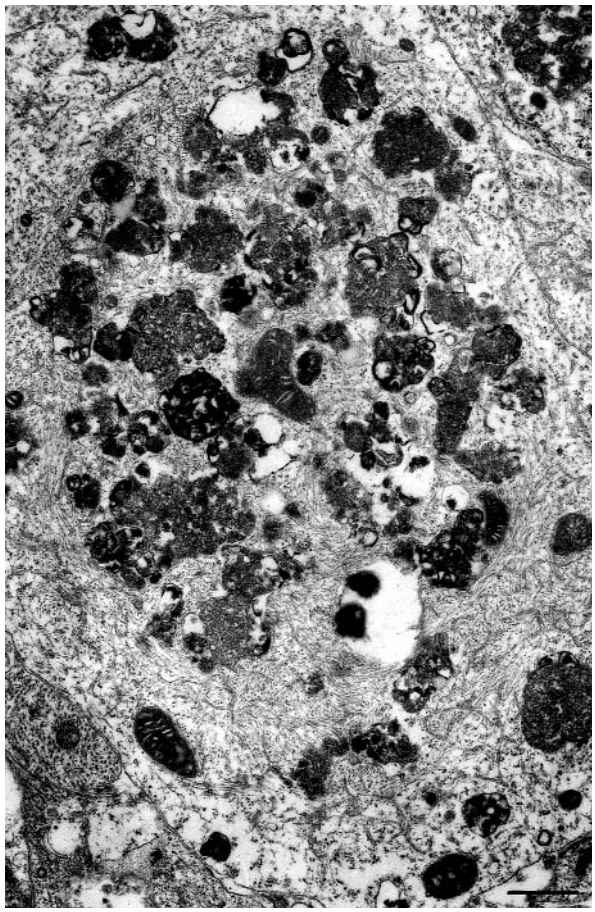

Fig. 9. Cytoplasm of phagocytic astrocyte filled with gliofilaments and multiple, electron-dense, polymorphic bodies. 14 days of 100 μM THA incubation. Bar = 1 μm

reduction of astrocytic glutamate transporter EAAT2 (GLT1) have been documented in patients with ALS [9, 31, 35, 36, 38]. A large decrease in glial glutamate transporter GLT-1 has also been observed in a cell model of familial amyotrophic lateral sclerosis [54] and in different animal models of ALS, including transgenic ones with the expression of high levels of mutated superoxide dismutase (SOD1) genes [5, 11, 12, 53]. The loss of EAAT2 transporters was detected in the spinal cord in SOD-1 G85R transgenic mice with ALS-linked SOD-1 mutation [5] and G93A transgenic rats [17]. Loss of glutamate transporters in ALS may be secondary to astrocytic activation. The damaged motor neurons produce mediators, i.e. reactive oxygen species that induce disruption of glutamate uptake by neighbouring astrocytes [29]. Astrocytes might potentiate excitotoxic motor neuron injury through the active release of glutamate as well. The reactive astrocytes in ALS show increased GFAP immunoreactivity and express inflammatory markers such as cyclooxygenase 1 and 2 (COX-1, COX-2) [21]. Some reports have indicated that glial cells in ALS can upregulate neuronal nitric oxide synthase (NOS) [1] and express inducible forms of NOS [38]. It has been postulated that oxidative and excitotoxic mechanisms might often operate in tight conjunction in neuronal injury in neurodegenerative disorders including ALS [52].

The present ultrastructural study evidenced the coexistence of MN degeneration and astroglial abnormalities in an ALS model *in vitro*. That suggested the participation of astroglial pathology in glutamate-mediated neurotoxicity in organotypic rat spinal cord cultures treated with 100 μM THA or PDC. The distinct glial changes predominantly involved the protoplasmic type of astrocytes and

consisted of the presence of irregular vacuoles and accumulation of abnormal profiles of smooth endoplasmic reticulum. During 3 weeks there was no increased production or accumulation of glial filaments typical for reactive astrogliosis. The evidence of distinct astroglial abnormalities different from typical reactive changes that accompany progressive MN damage supports the suggestion of a potential pathogenic role of glia in this progressive neurodegenerative process.

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