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Ultrastructural changes in lumbar spinal cord in transgenic SOD1^{G93A} rats

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Abstract

The purpose of this study was to determine structural changes which trigger the onset and progression of amyotrophic lateral sclerosis in rats expressing a human SOD1 transgene with mutation G93A. Lumbar spinal cord of affected rats in early and late presymptomatic (PM, 60 and 93 days of age) and symptomatic (S, 120 days of age) stage of the disease were analyzed ultrastructurally. At 60 days the structure of lumbar spinal cord as well as alpha motoneurons type S and F appeared normal; however, careful examination revealed that approximately 15% of axons were filled with mitochondria that were abnormal in number, size and morphology. Grossly swollen mitochondria with disrupted cristae were a prominent feature in all large axons at 93 days of age. At this time swelling and dilated mitochondria were observed also in type S motoneurons, while type F had small, well preserved mitochondria. At symptomatic stage the alpha motoneurons showed moderate neuronal loss, mainly of the S type. The most interesting finding at this stage was the occurrence of motoneurons with morphological signs of apoptotic-like degeneration. Such apoptotic-like motoneurons were characterized by nuclear and cytoplasmic condensation, chromatin compaction and formation of uniformly dense, dark structures. Numerous axons with very dark, compact interior as well as apoptotic bodies were irregularly scattered throughout the neuropil. Our ultrastructural study indicates that dying motoneurons in transgenic mutant SOD1G93A rats exhibit reminiscent apoptotic morphology which is preceded by significant mitochondrial abnormalities mainly in proximal axons and S motoneurons. Different reaction of slow and fast motoneurons to degenerating factors requires further analysis.

Key words: *SLA, ultrastructural changes, slow motoneuron apoptosis.*

Introduction

ALS, the most common adult motor neuron disease, is a progressive neurodegenerative disorder leading to paralysis and death. The disease is characterized by the selective degeneration and death of lower motor neurons in the brainstem and spinal cord leading to muscle weakness and atrophy. In 5-15% of cases of human ALS, mutations of the SOD1 gene were identified. Transgenic mice and rats expressing some of these mutations, for example G93A mutation, develop progressive motor neuron disease similar to human familial ALS [9,10,19].

Although numerous reviews have appeared during the last decade on neuronal death in mice bearing human mutated SOD1 transgene, the molecular mechanism by which mutations in the SOD1 gene lead to selective death of motoneurons

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in familial ALS remain incompletely understood. The precise mode of motoneuron death in SOD1 mutant mice is still controversial. Immunohistochemical evidence has been mounting that neuronal death is a result of apoptotic machinery [7,18,21,23], but the actual ultrastructural phenotype of motoneuron death remains undetermined.

In contrast to several different types of neurons found in the brain, spinal alpha motoneurons have been classified as fast (F), slow (S) and intermediate [25,26].

F motoneurons innervate the glycolytic, white muscle fibres and are considerably larger than S neurons innervating the oxidative, red muscle fibres [25]. S motoneurons exhibit greater NADH activity and are more abundant in mitochondria, while phosphorylase activity is greater in F motoneurons [26].

The purpose of this study was to define ultrastructural changes in motoneurons at the onset and during progression of ALS in rats expressing human mutated $SOD1^{G93A}$ transgene, in relation to motoneuron type.

Materials and methods

Sprague-Dawley rats expressing human mutated $SOD1^{G93A}$ transgene and age-matched normal Sprague-Dawley controls were obtained from animal stocks bred in the Animal House of the Medical Research Institute of the Polish Academy of Sciences, as described in the preceding paper [10]. The animals were anaesthetized as described [10] and perfused with a solution of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.6. Tissues were kept in the same solution for further fixation. The L4 – L5 lumbar spinal cord was dissected out and postfixed with 2% osmium tetroxide in cacodylate buffer pH 7.6. After dehydration in graded alcohol, the tissue blocks were embedded in Spurr resin. Thick sections stained with methylene blue were examined with light microscopy. Thin sections stained uranyl acetate and lead citrate were examined with electron microscope JEM II.

Animals at different stages of the disease, i.e. presymptomatic (PS), age 60 and 93 days, and symptomatic displaying hind limb paralysis (S), age 120 days, were analyzed.

Results

In the control (normal) lumbar part of the spinal cord the main differences observed in the structure of the S motoneurons as compared with F motoneurons were the greater number of mitochondria, abundant rough endoplasmic reticulum and heterochromatic nuclei (Fig. 1 A, B). Motoneurons of F type had a lower number of mitochondria, more delicate and dispersed endoplasmic reticulum and larger, oval or round, euchromatic nuclei (Fig. 2 A, B).

In transgenic animals at the early PM stage (age 60 days) the ultrastructure of the lumbar spinal cord appeared normal; however, careful examination revealed that approximately 10-15% of the axons were filled with mitochondria that were abnormal in number, size and morphology (Fig. 3). Besides hypertrophic mitochondria we could distinguish giant mitochondria as well as mitochondria undergoing splitting (Fig. 4). At this stage in a few axons mitochondrial swelling was observed (Fig. 5). Such mitochondrial abnormalities were found in the proximal part of myelinated, as well as unmyelinated axons. It is worth noting that mitochondria were normal in both F and S motoneurons as well as in glial cells. At 93 days of age grossly swollen mitochondria with disrupted cristae were a prominent feature in all large axons (Fig. 6).

Dilated mitochondria appeared as vacuoles which were no longer carrying the remnants of cristae. Such vacuoles were so large that they occupied almost the entire axonal caliber (Fig. 7). Among vacuolated axons there were a few axons with dark appearance. They possessed a very dense, dark, compact, homogeneous interior (Fig. 8) with preserved dense mitochondria. At this late asymptomatic stage (age 93 days) swelling, disruption of the cristae and dilated mitochondria were observed in the S motoneurons (Fig. 9), while the F type cells had small, well preserved mitochondria.

At symptomatic stage there was still some degree of vacuolization of large axons, as well as an increase in number of axons with very dense, dark, homogeneous and compact material. The alpha motoneurons showed moderate neuronal loss, mainly of type S cells. The most interesting finding at this stage was the appearance of motoneurons with morphological signs resembling apoptotic death. Dying cells assumed fusiform shape, the nucleus showed uniformly dense, dark nucleoplasm, and cytoplasmic organelles became abnormally closely packed and condensed (Fig. 10, 11). Early (Fig. 12) and late apoptotic bodies, of various size, were observed in the neuropil as well as inside glial cells (Fig. 13).

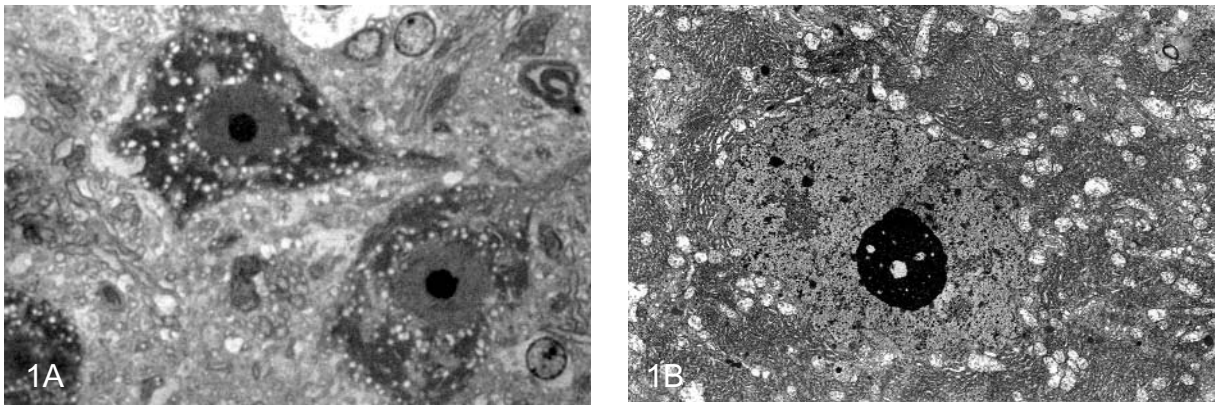

Fig.1. Alpha slow /S/ motoneuron: A) thick section in methylene blue staining x 1050; B) S motoneuron is characterized by an abundant rough endoplasmic reticulum and heterochromatic nucleus x 5000

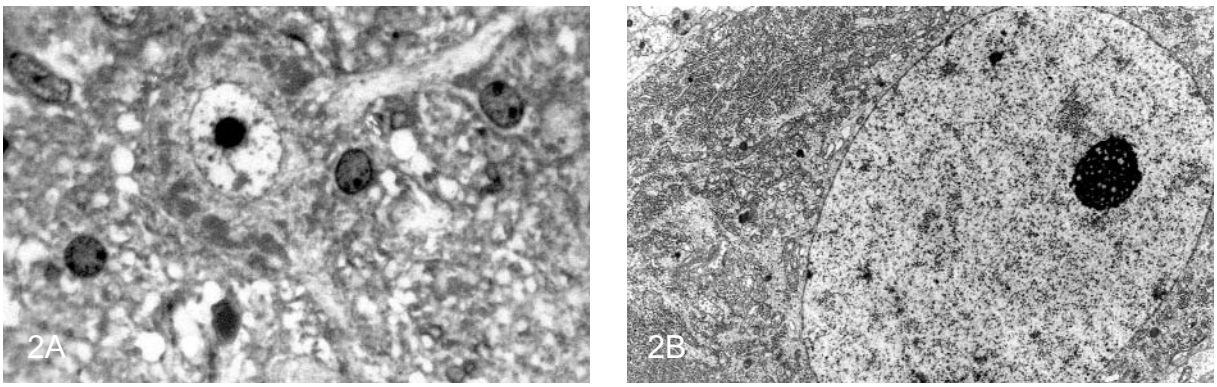

Fig. 2. Alpha fast /F/ motoneuron: A) thick section in methylene blue staining x 1050; B) F motoneuron has a large euchromatic nucleus and delicate dispersed endoplasmic reticulum x 6000

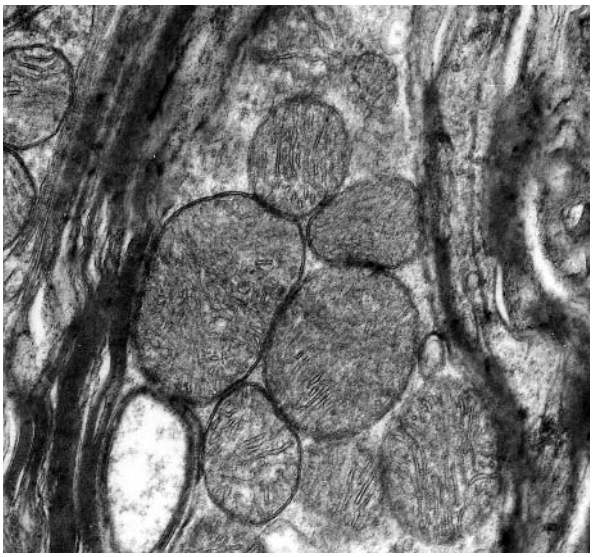

Fig. 3. PS stage. Axon filled with enlargement mitochondria x 24000

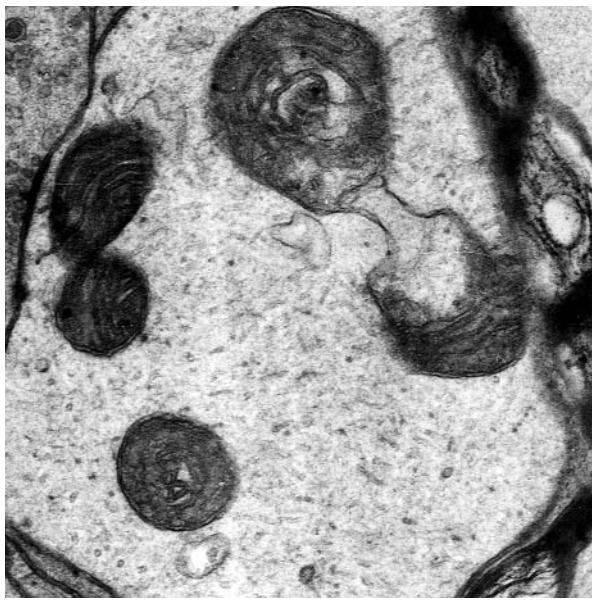

Fig. 4. PS stage. Mitochondrial splitting x 30000

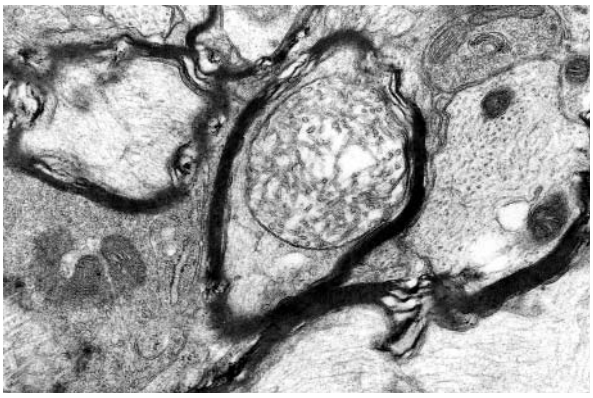

Fig. 5. PS stage. Swelling mitochondrion x 30000

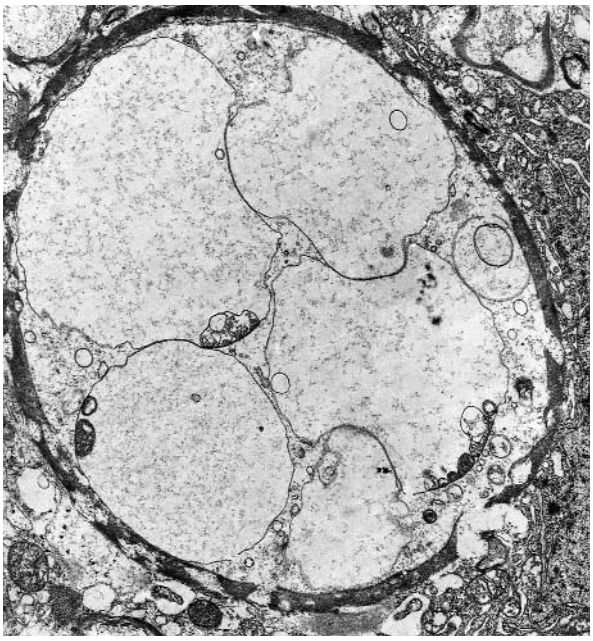

Fig. 7. PS stage. Large vacuoles occupy the entire axonal caliber x 6000

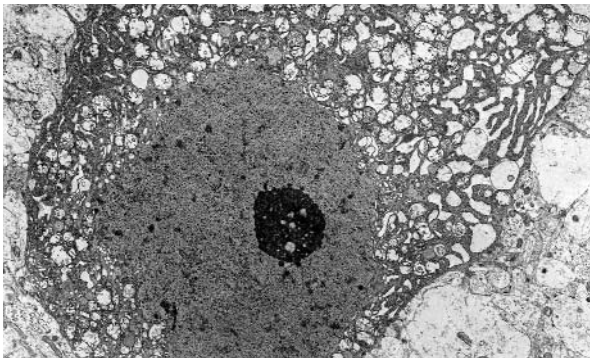

Fig. 9. PS stage. S motoneuron with swelling dilated mitochondria x 5000

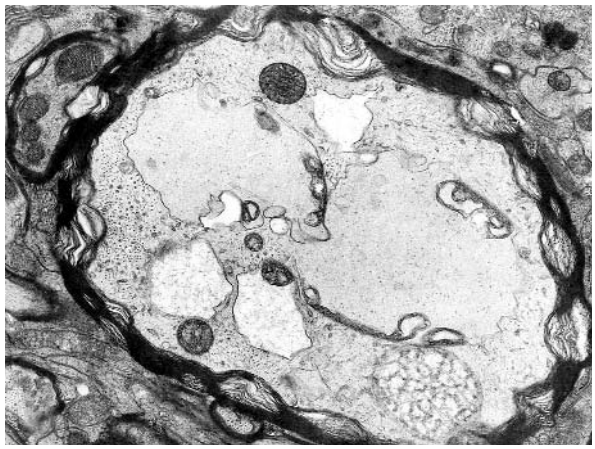

Fig. 6. PS stage. Mitochondria with disrupted cristae x 15000

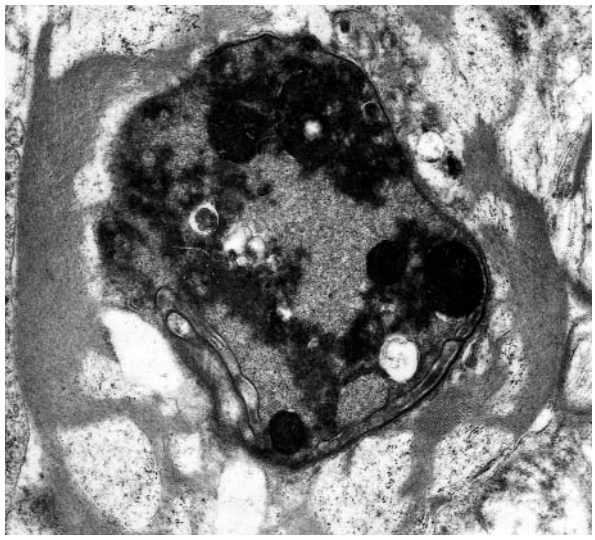

Fig. 8. PS stage. Axons with dark, dense interior x 12000

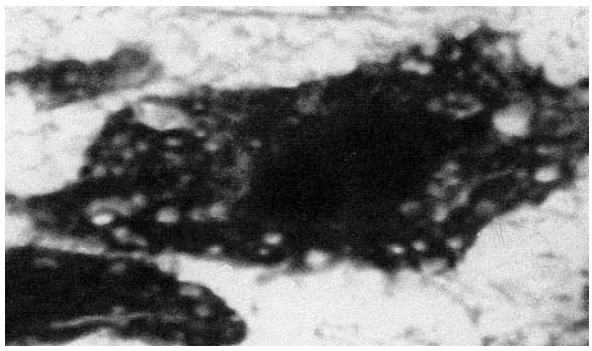

Fig. 10. S stage. Dying motoneuron with uniformly dense nucleoplasm and condensed cytoplasm. Thick section in methylene-blue staining x 1050

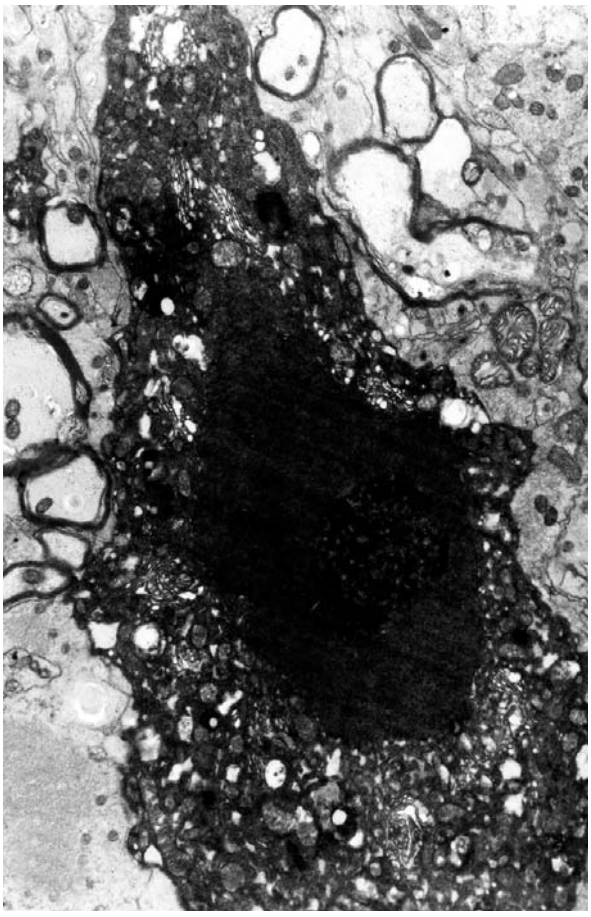

Fig. 11. S stage. Dying motoneuron with apoptotic-like morphology x 6000

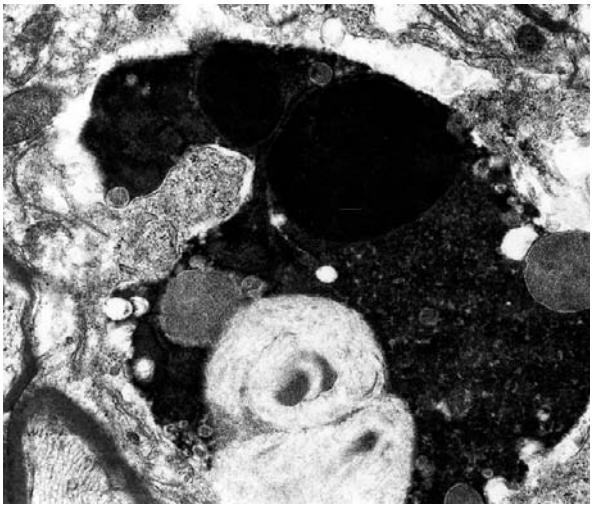

Fig. 12. Early apoptotic body x 24000

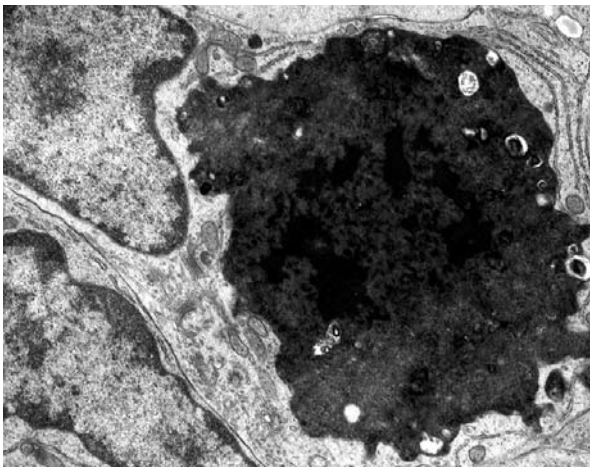

Fig. 13. Late apoptotic body inside glial cell x 12000

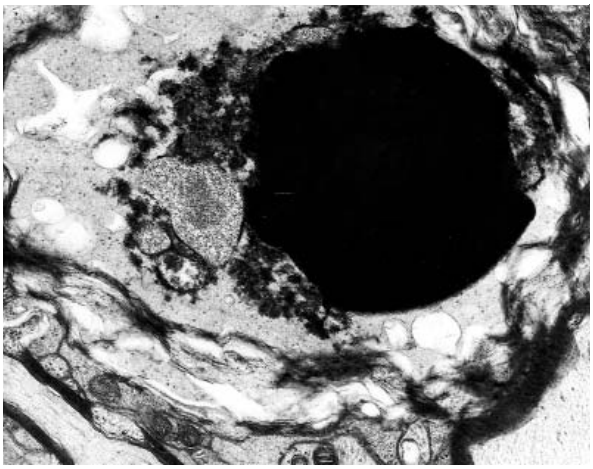

Fig. 14. Late apoptotic body inside myelin tube x 24000

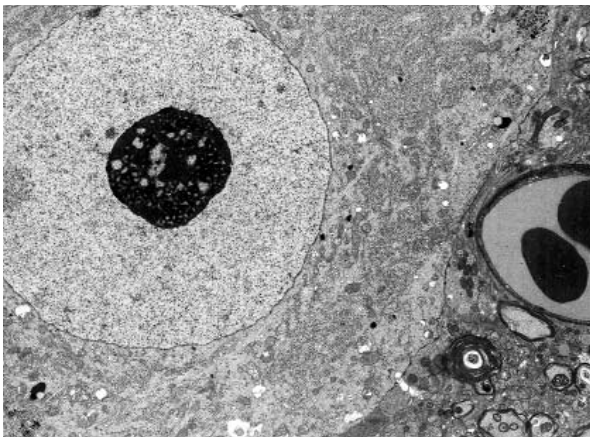

Fig. 15. F motoneuron in paralysis stage. Note the normal nuclear and mitochondrial structure x 5000

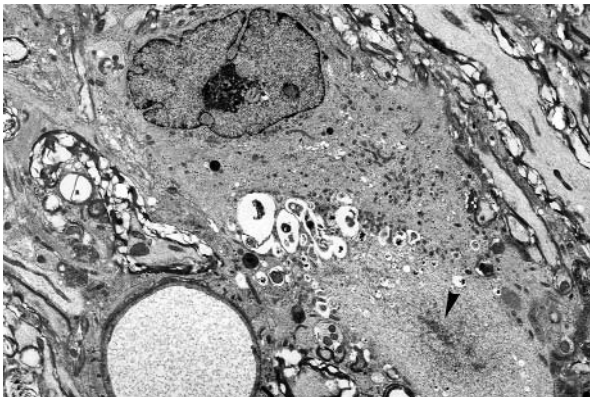

Fig. 16. Small motoneuron with autophagic vacuoles and filamentous inclusions (arrowhead) x 5000

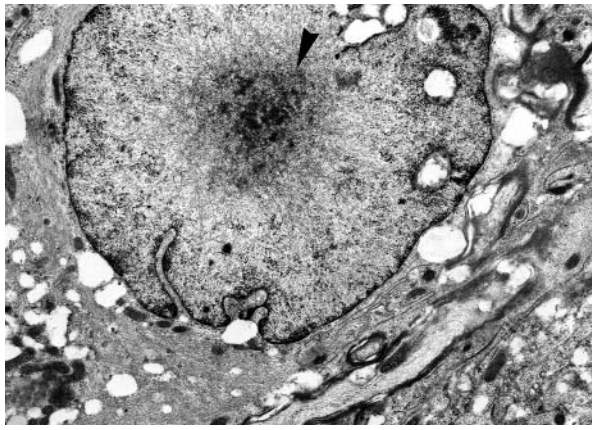

Fig. 17. Filamentous structures inside astrocytic nucleus (arrowhead) x 12000

Apoptotic bodies were also observed inside the myelin tube (Fig. 14). F motoneurons in the symptomatic stage appeared normal, but careful examination revealed fragmentation of the Golgi complex and disaggregation of membrane-bound polyribosomes into free ribosomes in the cytoplasm, making the cytoplasmic matrix more granular (Fig. 15). In the smaller motoneurons apoptotic degeneration was not observed, although they showed numerous structural abnormalities. Their eccentrically located nuclei exhibited deep invaginations. The cytoplasm contained fragmented Golgi complex, autophagic vacuoles and filamentous inclusions (Fig. 16). Filamentous inclusions were also observed in the nuclei of some astrocytes (Fig. 17).

Discussion

Our ultrastructural study indicates that mitochondria in the lumbar spinal cord of transgenic rats bearing human mutated $SOD1^{G93A}$ gene develop over the course of the disease major structural alterations, including variable degrees of swelling, vacuolization, cristae distortion and degeneration. Mitochondrial abnormalities appear as early as at the age of 60 days, preceding onset of the disease by two months. These findings are in keeping with previously presented data that in transgenic mice bearing human mutated SOD1 gene mitochondrial alterations in proximal axons are the first changes seen in the presymptomatic stage [11,13,14,24]. We also demonstrated for the first time that at the presymptomatic stage significant mitochondrial abnormalities appear in motoneurons of slow types while the fast motoneurons remain unaffected. These findings raise important questions: are the mitochondrial structural abnormalities a primary or secondary effect of SOD1 mutant protein, and why do these changes involve only a subset of axonal as well as slow motoneuronal mitochondria? The functional and morphological heterogeneity of mitochondria [3] may suggest that subpopulations of these organelles can carry out diverse processes within different motoneurons. The abundance of mitochondria as well as their larger size in slow motoneurons raise the possibility that they play a different role than those in fast motoneurons.

Mitochondrial dysfunction can lead to energy deficiency, ionic imbalance and oxidative damage and trigger the cell death program by releasing pro-apoptotic proteins residing in the mitochondrial interior [4,8,13,15,17]. Among the factors released from mitochondria are cytochrom C, the apoptotic inductor factor (AIF) and caspases [13,17]. The release of cytochrom C from the mitochondria to the cytosol plays a pivotal role in the apoptotic pathway that regulates the death of cells [15]. Accumulating evidence suggests that mitochondrial dysfunction causes motoneuron death in transgenic SOD1 mice by initiating the apoptotic pathway [8,20]. Consistent with massive mitochondrial degeneration seen in axons and slow motoneurons, the present study also shows that some axons at presymptomatic stage exhibit changes which mimic dark axonal degeneration. Such axons show very

dark, dense axoplasm and dark mitochondria with preserved architecture which are remarkably similar to those seen during apoptosis. Recently, Alvarez et al. [1] have suggested that dark axonal degeneration may be viewed as a form of cytoplasmic apoptosis. They proposed that neurons have at least two self-destruction programs. Like other cell types, they have an intracellular death program for undergoing apoptosis when they are not needed or injured. In addition they apparently have a second molecular distinct self-destruction program in their axons. This program is activated when the axon is severed and leads to rapid degeneration of the isolated part of the cut axon [22]. This idea correlates with our findings at symptomatic stage in which moderate loss of motoneurons occurs in parallel with the appearance of apoptotic motoneuron death and self-destructed axons.

Dying motoneurons exhibit features reminiscent of apoptosis manifested by cytoplasmic and nuclear condensation and compaction with tightly packed organelles within a dark cytoplasmic matrix. The appearance of early and late apoptotic bodies in affected lumbar spinal cord provides additional evidence of motoneuron apoptosis.

Recently, accumulating evidence suggests that neuronal apoptosis plays a role in ALS [7,18,21,23]. However, although high expression of apoptosis-related proteins has been demonstrated in human mutated SOD1 transgenic mice [3], a surprisingly low fraction of motoneurons exhibit apoptotic morphology. This phenomenon may be related to the swiftness of cell death in relation to the slowness of disease. Our study indicates that in the so-called apoptotic stage the dying motoneuron is approximately one quarter of its normal diameter, the cytoplasm and nucleus are extremely condensed and the cell body adopts a fusiform shape. In addition early and late apoptotic bodies are more frequently observed than cells in the first stage of apoptosis. The nuclear condensation in affected rats differs from classic apoptosis because chromatin is not organized into uniformly dense clumps as in developmental neuronal apoptosis [2,5,6]. However, recently Leist and Jaattela [16] have classified cell death into four types: apoptosis, apoptosis-like programmed cell death, necrosis-like programmed cell death and accidental necrosis (cell lysis). Apoptosis-like programmed cell death is less compact than classical apoptosis. These authors have concluded that structural differences reflect different cell death execution machinery of mitochondrial dysfunction. Three different possible independent signals emanate from mitochondria: cytochrom C, AIF and reactive oxygen species [13]. Activated caspases triggered by cytochrom C induce classical apoptosis. When caspases cannot work, caspase-independent AIF stimulate an apoptosis-like program of cell death. Taken together, our findings suggest that dying motoneurons in transgenic rats bearing human mutated $SOD1^{G93A}$ gene exhibit reminiscent apoptotic morphology which is preceded by significant mitochondrial abnormalities, mainly in proximal axons and slow motoneurons. The different pattern of degeneration of slow and fast motoneurons requires further analysis.

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