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Synaptic loss in the proximal axon of anterior horn neurons in motor neuron disease

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Abstract This report deals with an ultrastructural investigation of the synapses of the proximal axons of normal-appearing anterior horn neurons of 7 patients with amyotrophic lateral sclerosis (ALS) and 4 patients with motor neuron disease who had no upper motor neuron and corticospinal tract involvement (lower motor neuron disease, LMND). Specimens from 12 age-matched individuals who died of non-neurological diseases served as controls. Proximal axons directly emanating from the normal-appearing neurons were examined: 42 axons were from ALS patients, 43 from LMND patients and 87 from controls. Our results show that the number of synapses on axon hillocks, as well as the lengths of the synaptic contact and of the active zone were reduced in both groups of patients ($P < 0.0001$), but no significant differences were seen between patients and controls with respect to the synaptic parameters of initial axon segments. There was no overall difference between ALS and LMND patients. These findings suggest that the electrophysiological functions pertaining to integration of electrical inputs into the axon and information transduction on the axon may be greatly impaired in the early stages of motor neuron diseases, and that the observed synaptic alterations may be pathological events, likely to be due to anterior horn neuron degeneration.

Key words Amyotrophic lateral sclerosis · Motor neuron disease · Axon hillock · Initial segment · Synapse

Introduction

The initial segment of the axon plays a crucial functional role as the site of initiation of the action potential [17, 30] and its undercoating may be related to that essential function. However, despite being a functionally important site, there have been no studies on possible alterations of the synapses located around the proximal axon, namely the axon hillock and the initial axonal segment of anterior horn neurons. In an effort to determine whether such synaptic changes occur in motor neuron disease (MND), we performed a comparative ultrastructural study of the axon hillocks and initial segments of axons emanating from the normal-appearing anterior horn neurons of patients with amyotrophic lateral sclerosis (ALS), individuals with MND who had no upper motor neuron and corticospinal tract involvement (lower motor neuron disease : LMND) and normal controls.

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Materials and methods

This study was carried out on the spinal cords of 7 patients (ages: 59, 59, 61, 61, 63, 78, and 81 years; average: 66.0 years) with clinically and neuropathologically proven ALS, 4 patients (ages: 49, 65, 72 and 75; average: 65.3 years) with similarly confirmed MND who had no upper motor neuron and corticospinal tract involvement (LMND), and 12 age-matched control individuals (ages: 44–80 years; average: 68.1 years) who had no neurological disease. All postmortem investigations were performed within 6 h after death. In all cases a tissue block was obtained at autopsy from the same level of the lower lumbar spinal cord (L4–5), and the anterior horns of each level were fixed immediately with 2% glutaraldehyde in phosphate buffer (pH 7.4). After fixation, the anterior horns were cut transversely into pieces approximately 1 mm thick, postfixed for 2 h with 1% osmium tetroxide, dehydrated, and then embedded flat in epoxy resin. Each embedded tissue block was subsequently cut into semithin (around 1 μm thick) sections that were almost large enough to contain an entire anterior horn. The sections thus prepared were stained with toluidine blue.

After light microscopic identification of the axon hillock and the initial segment emanating directly from the somata of morphologically normal-appearing anterior horn cells, appropriate portions of the semithin sections were cut into serial ultrathin sections. These were stained with uranyl acetate and lead citrate for electron microscopy. Photomicrographs of the anterior horn cells and the directly connected axon hillocks and initial segments were taken at a magnification of × 1,400 and then enlarged to a magnification of × 2,660 (Fig. 1). The total number of motoneurons analyzed was 172. Of these, 42 were from ALS patients, 43 from LMND patients and 87 from control individuals.

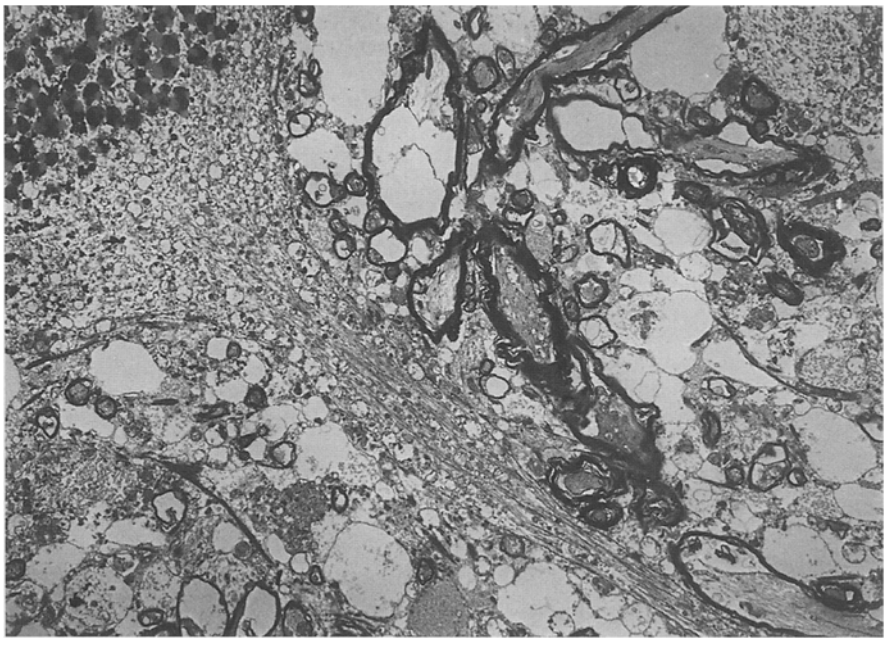

Fig. 1 A proximal axon directly emanating from the normal-appearing anterior horn neuron. An amyotrophic lateral sclerosis (ALS) patient. × 2,660

The axon hillock forms a cone-shaped projection from which the axon extends, and its distal portion merges gradually into the initial segment covered by a layer of electron-dense material (undercoating), which extends to the beginning of the myelin sheath, covering almost the entire circumference of the initial segment [24]. Each individual axo-axonic synapse seen on axon hillocks and initial segments was examined using photomicrographs taken at a initial magnification of × 20,000 and then enlarged to a magnification of × 38,000. A synaptic complex consists of a presynaptic bouton and a postsynaptic membrane separated by the extracellular space (Fig. 2). Synapses were identified by the presence of synaptic membrane thickenings that were associated with synaptic vesicles. We determined the cell body area of each motoneuron, as well as the number of synapses, the synapse length and the length of the active zone. The length of an individual synapse was defined as the entire length of the synaptic contact between the presynaptic and postsynaptic sites, and the length of the synaptic active zone as the length of the postsynaptic density (Fig. 2a). Discontinuous synapses were counted as a single synapse. The lengths of the synaptic contact and of the active zone were not calculated in those instances in which the distance between the axonal membrane and the presynaptic bouton was more than 0.1 μm.

The measurements were carried out using the Kontron computerized image analyzer (Munich, Germany) and the results expressed as mean ± standard deviation. The data obtained were analyzed by the unpaired *t*-test using a computerized statistical program.

Results

Control subjects

We examined a total of 87 proximal axons that emanated directly from the anterior horn neurons of this group of individuals. The mean cross-sectional area of the neurons which could be measured was 2,923.7 ± 992.7 μm² (n = 52); but each cell body area measured did not necessarily represent the maximum size of a given neuron. A total of 193 synapses were identified on axon hillocks and 53 on initial segments (Table 1). The mean number of synapses on axon hillocks (2.2 ± 2.1) was strikingly greater than on initial axonal segments (0.6 ± 0.9). Similarly, the mean length of the synaptic contact was larger in the former (4.73 ± 4.24 μm) than in the latter (0.94 ± 1.69 μm). In addition, the mean length of the active zone of the axon hillocks (1.22 ± 1.23 μm) was also larger than that of the initial segments (0.20 ± 0.38 μm). There was no significant relationship between the numerical value of the three parameters and cell body area, and regression analyses failed to reveal any significant correlation between the numerical data and the age of the control individuals. Neurofilaments were present in five presynaptic terminals. Accumulation of electron-dense mitochondria was found in three presynapses. A Hirano body and a spinule-like formation were observed in one presynapse each.

MND patients

Only proximal axons projecting directly from normal-appearing anterior horn neurons (i.e., those not showing degenerative changes such as central chromatolysis, pigmentary atrophy or simple neuronal atrophy) were included in the study. A total of 85 such proximal axons were investigated (Table 1). Of these, 42 were from ALS patients and 43 from LMND patients. The mean cell body area of the normal-appearing neurons of the MND patients (ALS and LMND patients) was 2,425.6 ± 854.4 μm² (n = 55). This value was smaller than that of the control individuals ($P < 0.01$). A total of 109 synapses were counted on axon hillocks and 44 on the initial segments. The mean number of synapses on the axon hillock was 1.3 ± 1.8. This figure is significantly smaller than that of the controls ($P < 0.01$). The mean length (2.20 ± 3.41 μm) of the synaptic contact of the axon hillocks was significantly shorter than that of the controls ($P < 0.0001$), as was the

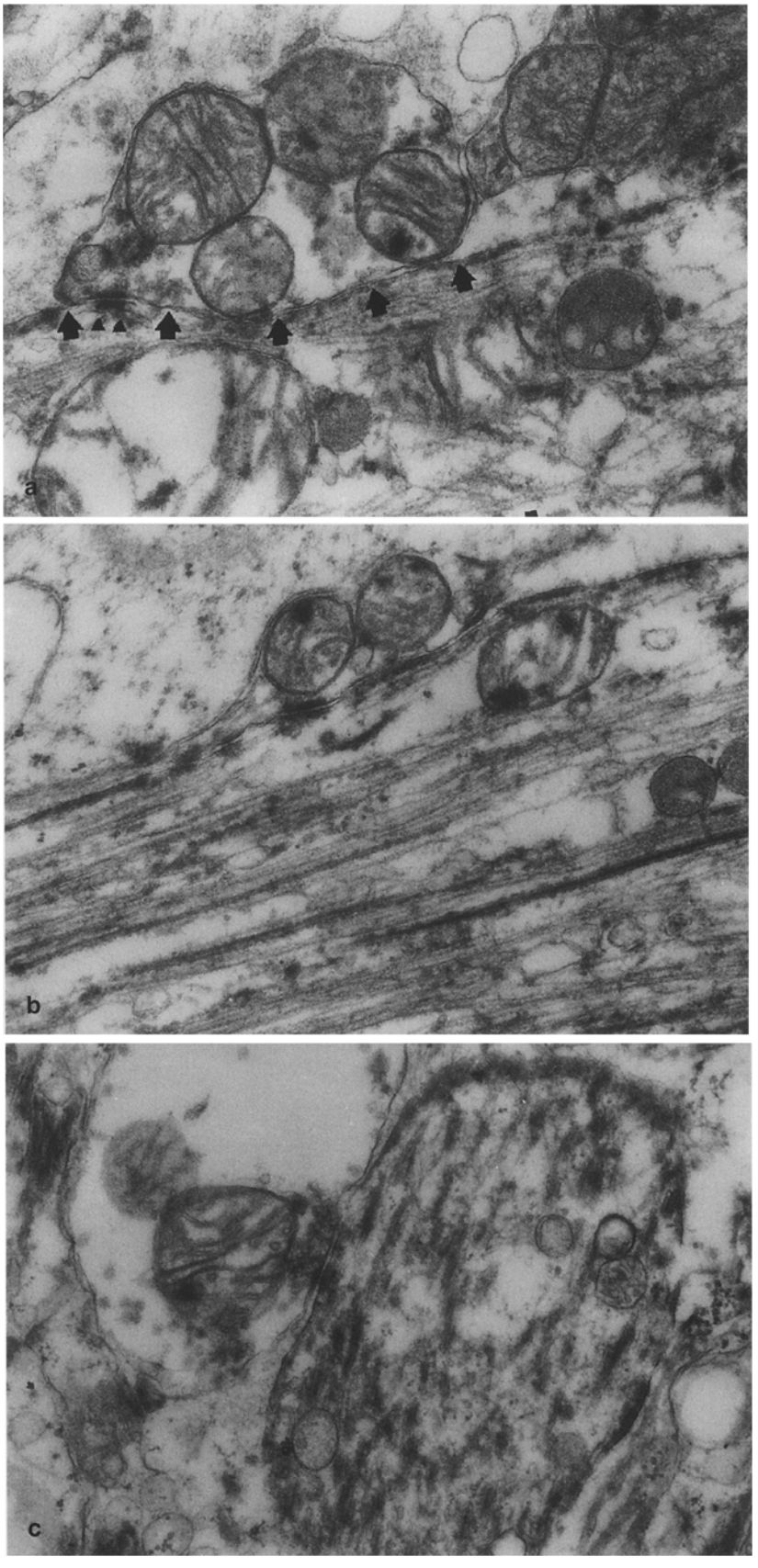

Fig. 2 **a** A synapse seen on the axon hillock consisted of a presynaptic bouton and a postsynaptic membrane separated by the extracellular space. *Arrows* indicate the entire length of the synaptic contact between the presynaptic and postsynaptic sites, and *arrowheads* the length of the active zone. **b** A synapse found in a longitudinal section of the initial segment. **c** A synapse observed in a transverse section of the initial segment. **a–c** ALS patient; × 38,000

Table 1 Comparison of neuronal size, number of synapses, synapse length and active zone length between control individuals and MND patients (*MND* motor neuron disease, *NS* not significant, *N* Number of neurons, *n* number of synapses)

	Cell body area (μm²)	No. of synapses/neuron		Synapse length (μm)		Active zone length (μm)	
		Axon hillock	Initial segment	Axon hillock	Initial segment	Axon hillock	Initial segment
Controls	2923.7 ± 992.7 (*N* = 52)	2.2 ± 2.1 (*n* = 193)	0.6 ± 0.9 (*n* = 53)	4.73 ± 4.24 (*n* = 193)	0.94 ± 1.69 (*n* = 53)	1.22 ± 1.23 (*n* = 193)	0.20 ± 0.38 (*n* = 53)
	*	*	NS	**	NS	**	NS
MND patients	2425.6 ± 854.4 (*N* = 55)	1.3 ± 1.8 (*n* = 109)	0.5 ± 0.9 (*n* = 44)	2.20 ± 3.41 (*n* = 109)	0.73 ± 1.28 (*n* = 44)	0.56 ± 0.86 (*n* = 109)	0.50 ± 2.83 (*n* = 44)

[a] Unpaired t-test: * $P < 0.01$, ** $P < 0.0001$

mean length (0.56 ± 0.86 μm) of the active zone ($P < 0.0001$). By contrast, the mean number of synapses on initial axonal segments (0.5 ± 0.9) did not differ from that of controls. Similarly, the mean length of their synaptic contact (0.73 ± 1.28 μm) and that of the active zone (0.50 ± 2.83 μm) were also not significantly different from those of the control subjects. Moreover, there were no significant differences with respect to the four parameters between ALS and LMND patients. Neurofilaments were seen in ten presynaptic terminals. Accumulation of electron-dense mitochondria was present in two presynapses. Hirano body and spinule-like formation were each found in three presynapses. The frequency of these presynaptic alterations was not statistically significant compared to controls (unpaired *t*-test).

Discussion

It has been suggested that proximal axonal swellings which consist of abnormal accumulations of neurofilaments, including spheroids of more than 20 μm in diameter, are associated with the pathogenesis of ALS [2, 3, 8, 25]. Probably the slow axonal transport of neurofilaments is impaired in this portion of the axon at an early stage of the disease process [21, 25]. As far as we are aware, there has been no systematic study on alterations of the synapses present on axon hillocks and initial axonal segments of the anterior horn neurons of humans. On the other hand, synapses have been identified on the initial segments of neurons in the prepiriform cortex [31], caudate nucleus [15], cortical pyramidal cells [26] and Purkinje cells [27] of animals. In addition, Conradi and Skoglund [4, 5], in their studies on the synaptology of spinal α-motoneurons during postnatal development of the cat, demonstrated by electron microscopy that the frequency and distribution of the different morphological types of synaptic boutons undergo changes after birth, and that boutons are no longer present in the initial motor axon segment of adult animals [5]. It should be noted in the context of the present investigation that Sasaki et al. [24] observed occasional synaptic boutons on the surface of axon hillock of proximal axons of anterior horn neurons of human spinal cords. However, no synapses were seen on initial axonal segments [24].

For carrying out computer-aided image analysis on electron microscope-generated photomicrographs, it is essential that the structure of the synaptic apposition zone is highly resistant to postmortem changes, as has been repeatedly documented [7, 9, 19, 20]. Thus, the use of spinal cords obtained at autopsies performed within 6 h after death allowed us to obtain the machine-generated data presented in this report. In contrast to earlier findings, the present results demonstrate that synapses are present not only on axon hillock but also, albeit at a lower frequency, on initial segments of normal-appearing anterior horn cells of neurologically normal individuals and of patients with MND. However, the mean number of synapses on axon hillocks, but not on initial segments, is significantly lower in the groups of patients with ALS or LMND compared with controls. By necessity our study had to be limited to normal-appearing cells only, since the neuronal processes of degenerated neurons are atrophic and thin [18, 23], which makes reliable ultrastructural analysis rather difficult. Nevertheless, as the results demonstrate, synaptic alterations can be detected on axon hillocks emanating from the morphologically normal-appearing neurons of MND patients.

The initial segment is an important site for the generation of action potential, and one of the major influences on motoneuron excitability is synaptic efficiency, which involves the interaction between presynaptic and postsynaptic elements [1]. The factors that control the synaptic efficiency of α-motoneurons include the number of synapses, the synaptic density, and the total postsynaptic membrane area on the motoneuron surface [1]. Immunocytochemical investigations using the anti-synaptophysin antibody, a constituent of the membrane of synaptic vesicles in presynaptic terminals, show a decrease of presynaptic terminals attached to distal dendrites, whereas those attached to the cell body and proximal processes of anterior horn cells are relatively intact in ALS and LMND [10, 14, 16, 22]. Electron microscopically, as our results indicate, the number of synapses on the axon hillocks emanating from the normal-appearing neurons, as well as their synaptic length and the length of the active zone are significantly reduced in patients with ALS or LMND. The observed reductions may either directly or indirectly influence the generator of the action potential. The electrophysiological functions as an integrator of electrical in-

puts into the axon and as an information transducer on the axon, together with abnormalities of interneuronal communication in the motor system, may already be greatly impaired in the early stage of anterior horn neuron degeneration, although a compensatory mechanism of synapses for reduced synaptic function could be considered to occur in the initial axonal segments.

The synaptic changes seen in the proximal axon raise the question of whether these abnormalities are primary or secondary disease process alterations. Pertinent to this question is our recent demonstration that the expression of synaptophysin, a synapse marker, is decreased in anterior horns of patients with MND, and that reduction is closely associated with the degree of anterior horn neuron loss [22]. Additionally, there is evidence from immunohistochemical and morphometric studies that the upper motor neuron system exerts little influence on anterior horn neurons [11, 16]. The novel finding that synaptic alterations in the proximal axons of normal-appearing neurons occur not only in ALS patients, but also in those with LMND is of particular significance. Moreover, there was no significant difference in the frequency of presynaptic degeneration in the proximal axons between controls and MND patients. This points to the possibility that in MND the synaptic loss of proximal axons, especially on the axon hillock, may be a pathological alteration, probably due to the degeneration of anterior horn neurons, although we cannot disregard the possible influence of afferent fibers such as interneurons [12, 28, 29] and primary sensory afferents (group Ia and group II) [6, 13] on the reduced presynaptic terminals.

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