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Characterizations of heterotopic neurons in the spinal cord of amyotrophic lateral sclerosis patients

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Abstract This report concerns a comparative immunocytochemical, ultrastructural and morphometric investigation on heterotopic neurons in the white matter of the spinal cords of 19 patients with amyotrophic lateral sclerosis (ALS) and 18 age-matched neurologically normal individuals. The study revealed that the heterotopic neurons were scattered in the white matter, often adjacent to gray matter, that they immunoreacted with the antibody to synaptophysin, and that there were synaptic apparatuses on the surface of their somata and their neuronal processes. Bunina bodies and ubiquitin-positive inclusions such as Lewy body-like inclusions and skein-like inclusions, characteristic of anterior horn neurons of ALS, were present in the cytoplasm of the patients' heterotopic neurons in the anterior or lateral column of the white matter. These findings suggest that heterotopic neurons in the anterior or lateral column have the characteristics of alpha motor neurons. The average number of heterotopic neurons observed in ALS patients was generally less than in normal subjects. This reduction was correlated with the severity of neuronal loss. The heterotopic neurons in ALS were less susceptible to the degenerative process as compared with spinal cord anterior horn cells. We assume that in this disease the heterotopic neurons may be degenerated and their number diminished after or concomitantly with the depletion of anterior horn neurons.

Key words Amyotrophic lateral sclerosis · Heterotopic neuron · Alpha motor neuron · Immunocytochemistry · Ultrastructure

Introduction

The abnormal location of large numbers of neurons is characteristic of genetic mutations which impair the proper migratory processes of developing neurons. By contrast, the presence of small numbers of heterotopic neurons is fairly common even in normal adult individuals [7, 9]. Heterotopic neurons may be the result of aberrant neuronal migration during spinal cord development, and they possibly contribute to the development of motor neuron disease [7]. However, there is controversy regarding the presence of heterotopic neurons in spinal cord of patients with amyotrophic lateral sclerosis (ALS) [7, 9]. Moreover, the origin of heterotopic neurons in the white matter of spinal cords remains to be established. In this report we present the results of comparative immunocytochemical, ultrastructural and morphometric investigations on heterotopic neurons in the white matter of the spinal cord of normal individuals and ALS patients. This study was undertaken in an effort to elucidate the origin of heterotopic neurons, and to determine a possible relationship between the number of such neurons and the extent of neuronal loss of anterior horn cells in ALS.

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Materials and methods

This study was carried out on the spinal cords of 19 patients with ALS (ages at death: 41–83 years, average: 64.7 years), and 18 age-matched neurologically normal control individuals (ages at death: 35–81 years, average: 64.2 years). Additionally, 10 spinal cords of control subjects ranging in age from 44 to 75 years (average: 57.0 years) were used for ultrastructural studies. In all instances, tissue blocks were obtained at autopsy from each level of the lumbar spinal cords (L1–5), fixed in 10% formalin and embedded in paraffin. Paraffin sections (6 μm) were stained by conventional methods, including H&E, silver impregnation, cresyl violet, and Masson-trichrome. H&E-stained sections were used to count the number of heterotopic neurons. The white matter of the spinal cord was subdivided into anterior, lateral and posterior columns (Fig. 1). The ALS patients were classified in three groups based on the degree of neuronal loss of anterior horn cells in the lower lumbar spinal cord. The loss was judged as mild (6 cases) when around 20 large anterior horn neurons remained per anterior horn, moderate (7 cases) when around 10 neurons remained, and severe (6 cases) when there were less than 5. The number of heterotopic neurons present in the white matter of each section at each lumbar level from L1 to L5 was counted, excluding those in immediate contact with gray matter. The quantitative data obtained were analyzed by the analysis of variance (ANOVA: Sheffe's method) using a computerized statistical program.

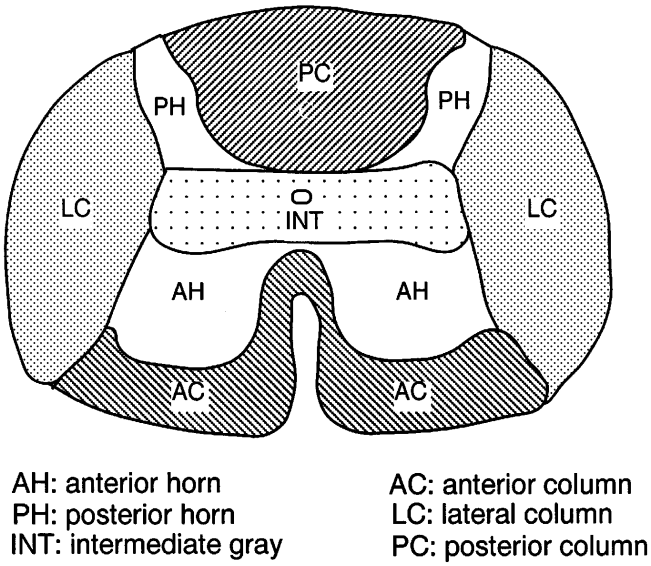


Fig. 1 Subdivision of the spinal cord

Immunocytochemistry

Immunocytochemical assays were performed with the following primary antibodies, all of which were diluted in TRIS-saline (50 mM TRIS, pH 7.6 and 150 mM NaCl): polyclonal antibody to glial fibrillary acidic protein (GFAP; Dako; diluted 1 : 1000); monoclonal anti-phosphorylated neurofilament (SMI-31; Sternberger Monoclonals; 1 : 10 000); monoclonal anti-synaptophysin antibody (Boehringer Mannheim; 1 µg/ml) and polyclonal anti-ubiquitin antibody (Sigma; 1 : 50). Sections of paraffin-embedded lumbar spinal cords were deparaffinized and incubated with the diluted primary antibodies overnight at 4°C. Sections from which antibodies were omitted served as reaction controls. Antibody binding was visualized by the labelled streptavidin biotin (LSAB) procedure (Dako).

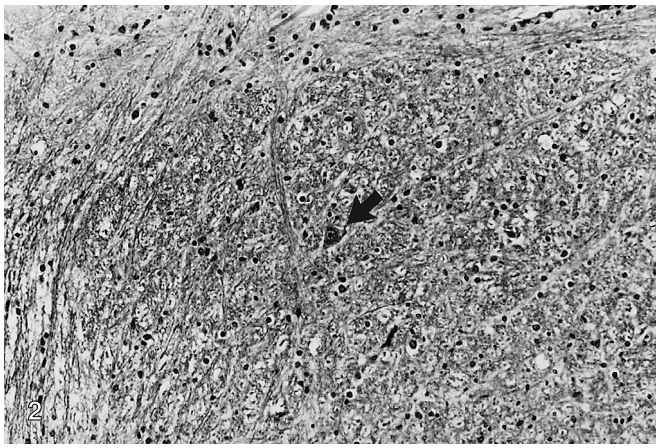
Ultrastructural study

The lumbar levels L1–5 of the 10 additional spinal cords of control subjects and of 13 spinal cords of ALS patients ranging in age from 41 to 83 years (average: 65.9 years) were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.4). Autopsies were performed on all patients within 6 h of death. After fixation, the anterior portion of the tissue at each level confined to lateral and anterior columns was cut into small pieces, postfixed in 1% osmium tetroxide for 2 h, dehydrated, and embedded in epoxy resin. The embedded specimens were cut into semithin (1 µm) sections that were almost large enough to contain the entire anterior portion of the spinal cord, including the white matter. The semithin sections were stained with toluidine blue, and after identifying heterotopic neurons by light microscopy, appropriate portions were properly trimmed and cut into ultrathin sections. These were stained with uranyl acetate and lead citrate for electron microscopic study.

Results

Control individuals

The heterotopic neurons were readily recognized in the white matter by H&E or Masson-trichrome staining because of the presence of characteristic structures such as nucleus, lipofuscin granules and Nissl substance (Fig. 2). They were also relatively easily differentiated from non-neuronal structures such as spheroids and corpora amylacea. The heterotopic neurons were immunostained by the anti-synaptophysin antibody (Fig. 3). Dot-like presynaptic terminals were distributed along the dendritic arborizations and around the neuronal somata, which closely resembles those of anterior horn and posterior horn neurons. The perikarya of heterotopic neurons were almost always negative for phosphorylated neurofilament protein.



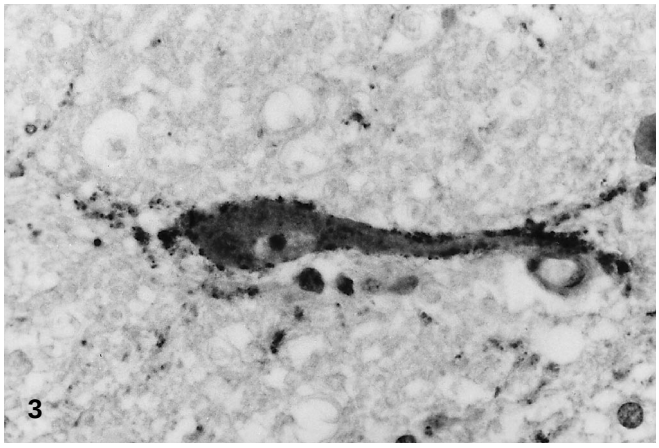


Fig. 2 Heterotopic neuron in the white matter adjacent to the anterior horn *(arrow)*. H&E, control individual, × 115

Fig. 3 The surface of the soma and the neuronal process of a heterotopic neuron is immunostained by the antibody to synaptophysin. Control subject, × 480

The heterotopic neurons were diffusely distributed in the white matter, being frequently adjacent to gray matter. As shown in the Table 1, the average number of heterotopic neurons per individual was 3.9 ± 3.4, and their density was significantly higher in the lateral column (7.4 ± 3.0) than in the anterior (1.6 ± 1.5; $P < 0.0001$) and posterior (2.6 ± 2.0; $P < 0.0001$) columns with no significant difference between the latter two columns ($P = 0.0788$). Regression analyses failed to reveal any significant correlation between the number of heterotopic neurons and the

Table 1 Number of heterotopic neurons in the spinal cord of control subjects and ALS patients (*ALS* amyotrophic lateral sclerosis, *NS* not significant)

	Controls Average (18 cases)	ALS patients							
		Average (19 cases)	*P* value*	Degree of neuronal loss					
				Mild (6 cases)	*P* value*	Moderate (7 cases)	*P* value*	Severe (6 cases)	*P* value*
Anterior column	1.6 ± 1.5	0.9 ± 1.0	NS	1.2 ± 1.1	NS	1.0 ± 1.1	NS	0.5 ± 0.8	0.0011
Lateral column	7.4 ± 3.0	4.4 ± 2.7	< 0.001	4.4 ± 3.3	< 0.0001	4.5 ± 2.5	< 0.0001	4.1 ± 2.4	< 0.0001
Posterior column	2.6 ± 2.0	1.3 ± 1.2	0.0046	1.4 ± 1.0	0.0149	1.1 ± 1.1	0.0003	1.4 ± 1.3	0.0149
Average	3.9 ± 3.4	2.2 ± 2.4	< 0.0001						

* ANOVA: analysis of variance, Scheffe's method

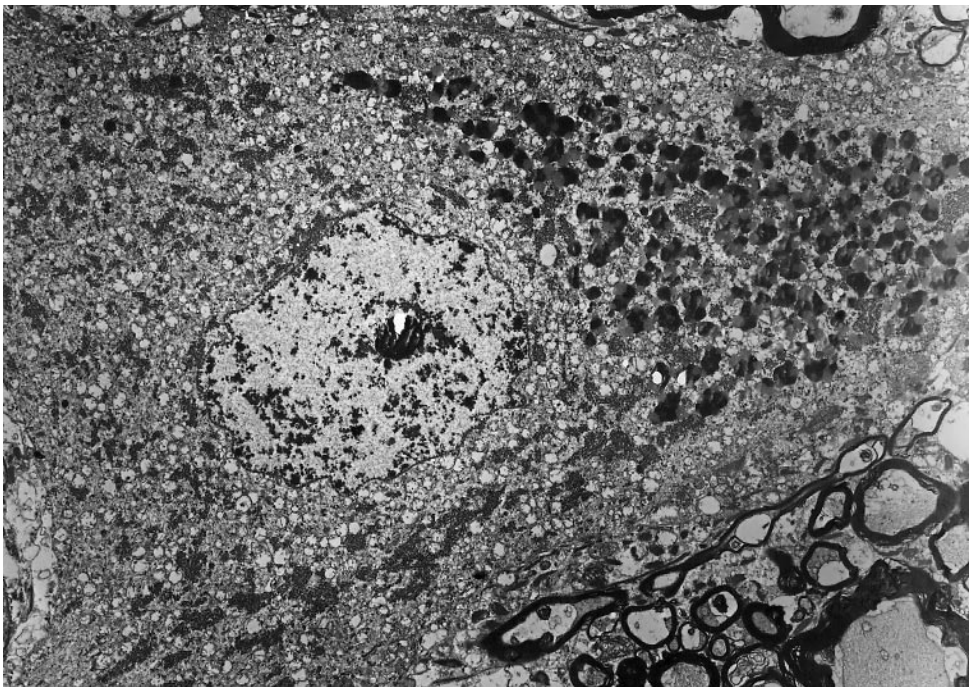


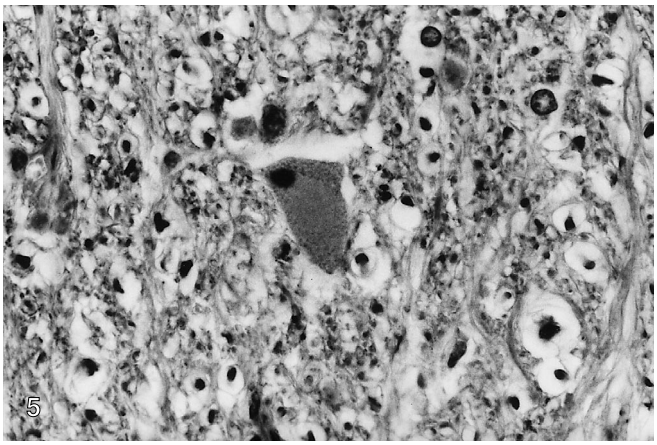
Fig. 4 Ultrastructure of a heterotopic neuron in the white matter of the spinal cord. Control individual, × 2075

age of control individuals. The ultrastructure of the heterotopic neurons consisted of prominent Nissl substance, abundant lipofuscin granules and cytoplasmic organelle such as mitochondria, lysosome and neurofilaments (Fig. 4). Synaptic apparatuses were observed on the surface of the somata and their processes.

ALS patients

The average number of heterotopic neurons per patient (2.2 ± 2.4) was significantly smaller than that of control subjects (3.9 ± 3.4; $P < 0.0001$). They were reduced in number in the lateral (4.4 ± 2.7; $P < 0.0001$) and posterior (1.3 ± 1.2; $P = 0.0046$) columns as compared with controls, but not signifcantly reduced in the anterior column (0.9 ± 1.0; $P = 0.3709$). The density of heterotopic neurons in the lateral column was higher than those in the anterior ($P < 0.0001$) and posterior ($P < 0.0001$) columns with no significant difference between the latter two ($P = 0.8577$). The number of heterotopic neurons in the anterior column of ALS patients with severe anterior horn cell depletion was significantly smaller than that of control individuals (0.5 ± 0.8; $P = 0.0011$), but this was not observed for patients with mild or moderate neuron depletion (1.2 ± 1.1; $P = 0.4597$ and 1.0 ± 1.1; $P = 0.1714$, respectively). In ALS patients with mild, moderate and severe depletion of anterior horn cells, the heterotopic neurons in the lateral and posterior columns were significantly reduced in number as compared with controls (Table 1). There was no significant difference in the number of heterotopic neurons in each column among the three groups of ALS patients classified by the degree of neuronal loss. Whether the number of heterotopic neurons in anterior and lateral columns was linked to the decrease of nerve cell densities in various motor nuclei of anterior gray horn was not clear.

The immunocytochemical assays revealed increased immunoreactivity of GFAP in the anterior horn and lateral column, and occasional ubiquitin-positive inclusions such



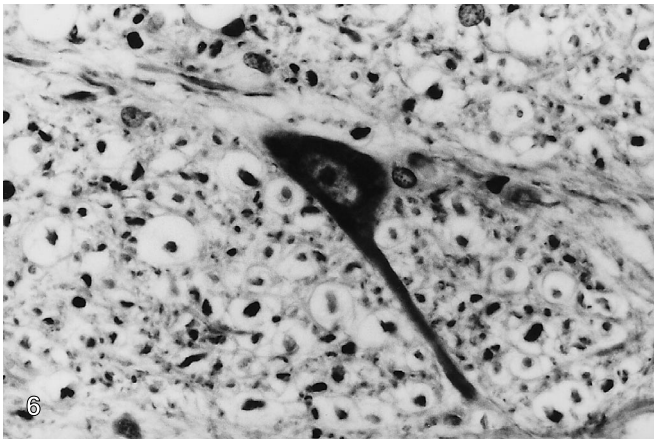


Fig. 5 Lewy body-like inclusion positively immunostained by the anti-ubiquitin antibody. Amyotrophic lateral sclerosis (ALS) patient, × 480

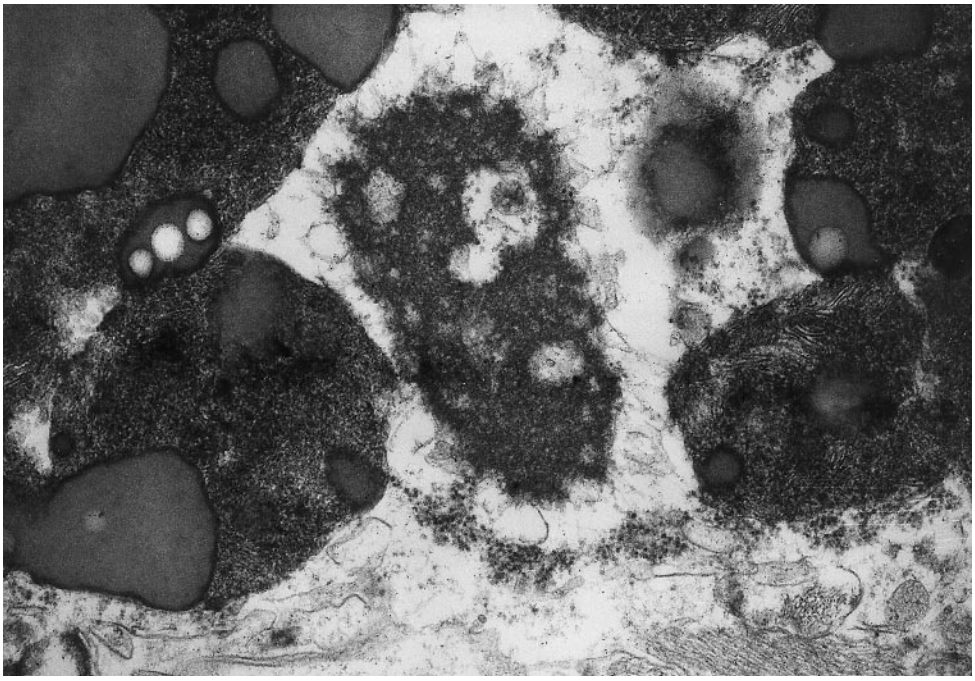
Fig. 6 Perikaryon and neuronal process of a heterotopic neuron immunostained by the antibody against phosphorylated neurofilament. ALS patient, × 480

as Lewy body-like inclusions (Fig. 5) and skein-like inclusions in the perikarya of the heterotopic neurons in the anterior or lateral column. Bunina bodies, skein-like inclusions and Lewy body-like inclusions were not observed in the somata of the heterotopic neurons in posterior columns. Immunoreactivity of the perikarya of heterotopic neurons of ALS patients with the antibody to phosphorylated neurofilament was usually negative as was the case in controls, but the somata of heterotopic neurons only occasionally showed strong immunoreactivity (Fig. 6). The surface of the somata and proximal processes of heterotopic neurons were immunostained by the anti-synaptophysin antibody. At the ultrastructural level, in addition to synaptic apparatuses on the surface of the somata and their processes, characteristic inclusions of the anterior horn neurons of ALS, such as Bunina bodies (Fig. 7) [17], skein-like inclusions (Fig. 8) [16], Lewy body-like inclusions [15], and other abnormal structures, including annulate lamellae (Fig. 9), lamellar structures, honeycomb like structures (Fig. 10) [14] and membraneous bodies were evident in the cytoplasm of the heterotopic neurons in the anterior or lateral column of the white matter. Bundles of neurofilaments and paracrystalline arrays similar to Hirano bodies were occasionally seen in the presynaptic spaces.

Discussion

Heterotopic neurons may occur anywhere in the central nervous system, including deep white matter of the cerebellum [12, 13], cerebrum [4, 10], and spinal cord [7, 9, 18] and they may be considered a normal variant [7]. During the development of the nervous system, neurons are usually generated at sites distant from their final location, where they arrive at the end of migratory processes. The

Fig. 7 A Bunina body consisting of electron-dense material with tubular structure or vesicles. ALS case, × 29 640



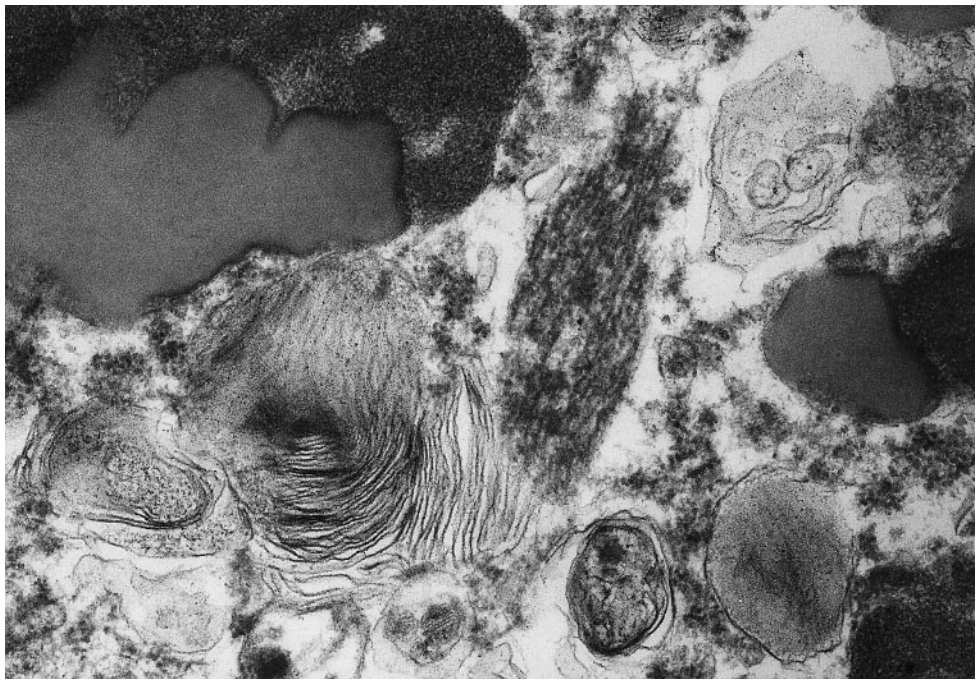
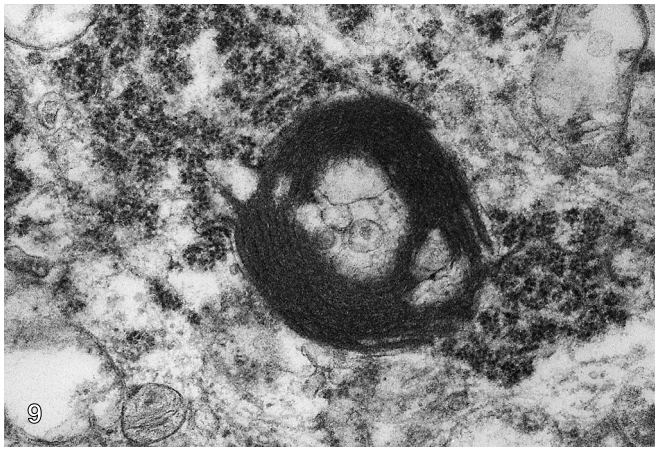


Fig. 8 Skein-like inclusion composed of bundles of tightly packed filaments running parallel to the longitudinal axis. ALS patient, × 40 000



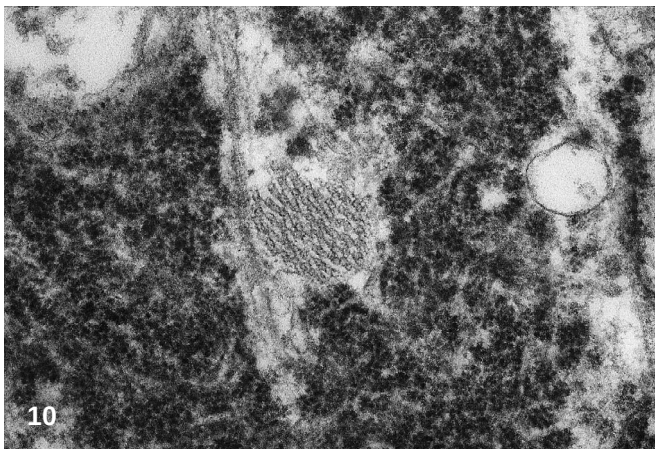


Fig. 9 Annulate lamellae. ALS patient, × 25 800

Fig. 10 Honey-comb like structure. ALS case, × 34 900

anomalous location of large numbers of neurons in the adult brain is a salient feature of several mutations or pathological conditions, in which the mechanisms controlling neuronal migration are disrupted [5]. However, small groups or single ectopic neurons are fairly common in various areas of the brain of normal adults of different species [1, 2, 11]. It is generally assumed that even in normal animals a number of migrating neurons are misrouted by obstacles such as blood vessels that are encountered along the migratory pathway [13]. However, it is thought that most of these neurons die before the end of development since they are unable to establish connections necessary for their survival [5].

Since heterotopic neurons are most numerous in the ventral outflow and lateral corticospinal tract regions at all spinal cord levels, they may be the result of aberrant neuronal migration during spinal cord development [7]. However, there is controversy regarding the origin of heterotopic neurons in the spinal cord of ALS patients [7, 9]. Thus, because of the lack of involvement of neurons located in the white matter of the spinal cord in ALS, spinal muscular atrophy (SMA), and poliomyelitis, Martin et al. [9] assumed that heterotopic neurons in the spinal cord may be sensory neurons, despite their frequent location in the ventral part of the white matter. On the other hand, Kozlowski et al. [7] considered that heterotopic neurons are alpha motor neurons, because the only ALS patient with severe loss of motor neurons had fewer such neurons than other cases, suggesting a possible concomitant loss with disease progression. However, contrasting the characteristic inclusions of anterior horn cells of ALS patients, none have been shown in the heterotopic neurons of such patients, either in H&E-stained sections or by immunocytochemical assays [7, 9]. On the other hand, as the present immunocytochemical and ultrastructural results document,

the perikarya of the heterotopic neurons of ALS patients contained Bunina bodies, Lewy body-like inclusions and skein-like inclusions, all of them characteristic of the anterior horn neurons of ALS. These findings suggest that heterotopic neurons present in the anterior or lateral column of the white matter have the characteristics of alpha motor neurons, although these groups of heterotopic neurons may be a very heterogeneous population.

The heterotopic neurons of ALS patients appear to be resistant to the degenerative process that affects motor neurons in this disease [7, 9]. The present data confirm that heterotopic neurons in ALS are less susceptible to damage as compared with spinal cord anterior horn cells. Nonetheless, Kozlowski et al. [7] reported that the heterotopic cells are more numerous and further displaced in individuals with ALS than in control subjects. More recently, Martin et al. [9] described no significant difference in the number of heterotopic neurons located deeply or superficially in the white matter of the spinal cord of ALS patients, individuals with other anterior horn cell disorders and controls. By contrast, the present results indicate that the average number of heterotopic neurons is reduced in the posterior and lateral columns of ALS patients, and that their reduction in the anterior column correlates with the severity of neuronal loss of anterior horn cells. The diminution in the number of heterotopic neurons in the posterior column of ALS patients is possibly due to the loss of neurons in the posterior horns [3, 6, 8]. Thus, it is conceivable that in ALS the heterotopic neurons may be degenerated and their number diminished after or concomitantly with the depletion of anterior horn neurons, since the heterotopic neurons are similar in some way to alpha motor neurons.

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