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Immunohistochemical analysis of spinal cord lesions in amyotrophic lateral sclerosis using microtubule-associated protein 2 (MAP2) antibodies

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Abstract We have studied microtubule-associated protein 2 (MAP2) expression in anterior horn neurons in the cervical and lumbar spinal cords of 19 cases of adult-onset sporadic amyotrophic lateral scerlosis (ALS) using immunohistochemistry. Specimens from 7 patients without neurological disease served as controls. MAP2 expression decreased in the anterior gray horn of all ALS cases and in the intermediate gray of several ALS cases. Such reduction correlated with the degree of degeneration or neuronal loss in anterior horn cells and with the clinical symptoms of limb weakness. Cytopathologically, the MAP2 immunoreactivity decreased corresponding to the occurrence of individual signs of neuronal degeneration, such as chromatolytic neurons, shrunken neurons and pigmented neurons. MAP2 expression was relatively well preserved in the specimens in which spheroids are conspicuous. The findings of this study demonstrate MAP2 to be an excellent marker for the detection and quantification of anterior horn degeneration in ALS.

Key words Amyotrophic lateral sclerosis · Microtubule-associated protein 2 · Semiquantitative analysis · Spheroid · Inclusion body

Introduction

Amyotrophic lateral sclerosis (ALS) is a human motor neuron disease that is characterized by a degeneration of motor neurons in the spinal cord, brain stem, and motor cortex. Typical symptoms consist of a degeneration of the motor neurons leading to denervation atrophy of the skeletal muscles and ultimately to paralysis and death. The etiology of the degeneration of the motor neurons in ALS is unknown. There are currently several theories regarding the pathogenesis of ALS [9], such as the excitotoxic hypothesis, the calcium channel autoantibody hypothesis, the oxidative stress hypothesis and the cytoskeletal hypothesis. Several immunohistochemical studies have been carried out on anterior horn cells in ALS [12], but until recently little immunohistochemical research has been done using microtubulue-associated protein 2 (MAP2) to estimate the somatic or dendritic changes in ALS.

MAP2 is one of the most abundant neuronal microtubule-associated proteins [36]. MAP2 promotes tubulin polymerization into the microtubules, and microtubule assembly or reassembly. MAP2 is a stringent marker for neurons. In the central nerve system, MAP2 is found specifically in the neuronal cell bodies and dendrites.

Here we report that MAP2 immunostaining markedly decreased in the anterior gray horn in ALS. Using immunohistochemical methods, we analyzed the neuronal change, axonal change and inclusion bodies, and compared the histological changes with clinical symptoms such as duration and muscle power in ALS.

Materials and methods

Tissue source

This investigation was carried out on the spinal cords obtained at autopsy from 19 cases of adult-onset sporadic ALS (average age 62.1 years) and 7 control cases (average age 67.1 years) (Table 1). The postmortem interval ranged from 2 to 21 h (average 5.4 h). Clinical involvement in the bulbar sign (BS), upper limb muscle strength (UL) and lower limb muscle strength (LL) of ALS cases are summarized in the Table 1. The limb muscle weakness was graded as follows: 0, muscle strength was almost completely preserved at 1 month before death; –1, muscle strength was mildly decreased but the patients could work throughout the clinical course; and –2, muscle weakness was so severe that the patients were bedridden. The bulbar sign was assessed at the advanced stage of the

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Table 1 Summary of clinical data and immunohistochemical results. Clinical grade (were scored as: *0* normal; *–1* moderately affected; *–2* severely affected; and the pathological grade (as: *0* no change; *–1* mild changes of neuronal loss, gliosis and volume loss; *–2* moderate changes; *–3* severe changes) MAP2 immunoreactivity is given in relative optical density; for C: *0* > 0.7; *–1* 0.5–0.7; *–2* 0.3–0.5; *–3* < 0.3; and for L: *0* > 0–6; *–1* 0.4–0.6; *–2* 0.2–0.4; *–3* < 0.2. Spheroids are graded as: *0* none; *+1* a few; *+3* many; *+2* between +1 and +3. [*UL* upper limb weakness, *LL* lower limb weakness, *BS* bulbar sign, *D* dementia (– or +), *LMN* lower motor neuron, *UMN* upper motor neuron, *C* cervical spinal cord, *L* lumbar spinal cord, *MAP2* microtubulue-associated protein 2, *IG* intermediate gray, *MAG* medial division of the anterior gray horn, *LAG* lateral division of the anterior gray horn, *CRF* chronic renal failure, *HCC* hepatocellular carcinoma, *LC* liver cirrhosis, *DM* diabetes mellitus]

Case no.	Age/ sex	Duration (months)	Clinical				Pathological				MAP 2						Spheroids			
			UL	LL	BS	D	LMN		UMN		C			L			C		L	
							C	L	C	L	IG	MAG	LAG	IG	MAG	LAG	MAG	LAG	MAG	LAG
ALS																				
1	60/F	11	–2	0	–2	0	–1	–1	–1	–1	0	–1	–1	0	–1	–1	+1	+1	+3	+3
2	59/M	13	–2	–2	–1	0	–2	–1	0	0	0	–1	–1	–1	0	0	0	0	+2	+3
3	67/M	18	–2	–1	–2	0	–3	–1	–3	–3	0	–1	–1	–1	–2	–2	+1	+1	+2	+2
4	55/F	24	–2	–2	–2	0	–3	–3	–3	–3	–2	–3	–3	–3	–2	–2	0	0	0	0
5	83/F	25	–2	–2	–2	0	–3	–3	–3	–3	–2	–2	–3	–1	–2	–2	0	0	0	0
6	71/F	26	–2	–2	–2	0	–3	–1	–3	–3	–2	–3	–2	–1	0	0	0	0	+1	+2
7	58/M	28	–2	0	–2	0	–3	–2	–3	–2	0	0	–2	0	0	0	+1	+1	+2	+3
8	51/M	32	–2	0	–1	+	–2	–1	–1	–1	0	–1	–1	0	–1	–1	+1	0	+3	0
9	61/F	36	–2	0	–2	0	–2	–1	–1	–1	0	–1	–1	0	0	–1	+1	+1	+3	+3
10	54/M	38	–1	–1	–2	0	–3	–2	–3	–3	–2	–2	–2	–2	–2	–1	0	0	0	+1
11	63/F	45	–2	–1	–2	0	–3	–2	–3	–3	–1	–1	–1	0	–1	0	0	0	+1	+1
12	77/M	51	–2	–2	–2	0	–2	–2	–1	–2	0	0	0	0	–1	–1	0	0	0	+1
13	61/F	53	–2	–2	–2	0	–3	–3	–3	–2	–1	–2	–2	0	–2	–2	0	0	+1	0
14	45/F	60	–2	–2	–2	0	–3	–2	–3	–3	–2	–2	–2	0	0	0	0	0	0	0
15	60/F	72	–2	–2	–2	0	–3	–3	–3	–3	–1	–2	–3	0	–1	–2	0	0	0	0
16	53/M	72	–2	–1	–1	0	–3	–3	–1	–1	–1	–2	–3	–1	–1	–1	0	0	+1	0
17	68/M	72	–2	–1	–1	0	–3	–1	0	0	–1	0	0	–1	–1	0	+1	+1	+3	+3
18	69/F	79	–2	–2	–1	0	–3	–3	0	0	–1	–1	–1	–1	–2	–3	0	0	0	0
19	65/M	108	–2	–1	–2	0	–3	–1	–2	–2	0	–1	–1	0	–1	–1	0	0	+2	+2
Control		Causes of death																		
C-1	48/M	CRF					0	0	0	0	0	0	0	0	–1	–1	+1	0	+1	0
C-2	61/M	HCC, LC					0	0	0	0	0	0	0	0	0	0	0	0	0	+1
C-3	62/M	HCC					0	0	0	0	0	0	0	0	0	0	0	+1	0	+1
C-4	69/F	Gastric cancer					0	0	0	0	0	–1	–1	0	–1	0	0	+1	+1	+1
C-5	71/F	CRF, DM					0	0	0	0	0	0	0	0	0	0	+1	0	+1	+2
C-6	73/M	Lung cancer					0	0	0	0	0	0	0	0	0	–1	0	0	0	+1
C-7	86/F	Lung cancer					0	0	0	0	0	–1	0	0	–1	0	+1	+1	+3	+3

disease, and was graded as follows: 0, normal; 1, moderately affected; and 2, severely affected. Dementia was recognized in one patient (case 8). Case 4 was previously reported by Kondo et al. [14].

The spinal cords were fixed for approximately 2 weeks in buffered 10% formalin, and embedded in paraffin. After embedding, 6-μm-thick sections were prepared. For routine neuropathological studies, the sections were stained with hematoxylin-eosin (H&E), Bodian's stain and Klüver-Barrera's (KB) stain. The pathological changes in the lower motor neurons were carefully assessed for the degree of gliosis, neuronal loss and volume loss of the anterior gray horn, and degeneration in the pyramidal tract was carefully assessed by the the presence of vacuolation and macrophages infiltration. The morphological changes were graded from 0 to –3 (Table 1).

Antibodies

The following antibodies were used in the immunohistochemical assays: mouse monoclonal antibodies (mAb) against MAP2 (clone AP-20, Boehringer Mannheim, 5 μg/ml; clone HM-2, Sigma, 1 : 500: HM-2 reacts with all known forms of MAP2, i.e., MAP2a, MAP2b and MAP2c, while AP20 recognizes an epitope on the high molecular weight MAP2 forms, i.e., MAP2a and MAP2b, but shows no reaction with MAP2c), a mouse mAb against phosphorylated neurofilament (NF; clone 2F11, DAKO, 1 : 500), a mouse mAb against ubiquitin (Ub; Chemicon, MAB 1510, 1 : 1000) and a rabbit antiserum against human cystatin C (Dakopatts; 1 : 1000).

Immunohistochemistry

Immunohistochemical staining was carried out using the indirect immunoperoxidase method. Anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (HRP) were purchased from Vector laboratories (USA). The sections were deparaffinized in xylene, hydrated in ethanol, and incubated with 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase. After rinsing the sections in tap water, they were completely immersed in distilled water and then were autoclaved for 10 min to enhance the immunoreactivities of

MAP2, cystatin C and Ub. After pretreatment, the sections were incubated with a primary antibody diluted in 5% nonfat milk in TBST (25 mM TRIS-HCl pH 7.6 containing 0.5 M NaCl, 0.05% NaN_3, 0.05% Tween 20) at 4°C overnight and then with an anti-mouse or anti-rabbit IgG antibody conjugated with HRP (1:200) in phosphate-buffered saline for 30–60 min at room temperature. The colored reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (0.02% DAB, 0.003% H_2O_2, 50 mM TRIS-HCl pH 7.6). The sections were counter-stained lightly with hematoxylin. Double immunostaining was carried out as follows. The sections were incubated with anti-MAP2 at 4°C overnight, and a brownish reaction product was developed with DAB. The sections were subsequently washed in 0.1 M glycine-HCl buffer, pH 2.2 for 2 h, water for 15 min and 50 mM TRIS-HCl for 30 min. The sections were then incubated with the second primary antibodies: anti-phosphorylated NF, anti-cystatin C or anti-Ub at 4°C overnight. After incubation with HRP-conjugated secondary antibodies, color development was performed with a mixture of 0.05% DAB, 0.01% cobalt chloride, 0.0003% H_2O_2 in 50 mM TRIS-CHl ph 7.6 to give a dark-bluish reaction product. Sequential sections were then stained with the same pair of antibodies but in the reverse order.

Semiquantitative analyses

To measure MAP2 immunoreactivity, the cross sections of the spinal cord at the cervical (C6) and lumbar (L3) were partitioned into four areas as shown in Fig. 1. The images of the sections were scanned by Scanjet IIc (Hewllet Packard, USA) and Adobe Photoshop software version 3.0J. A densitmetric analysis was performed using NIH image software version 1.60.

The quantity of MAP2-immunoreactive products was expressed as the optical density (OD). The relative amount of MAP2 in the intermediate gray (IG) was calculated as $(OD_{IG} - OD_P)$ divided by $(OD_{PG} - OD_P)$, MAP2 in the medial division of the anterior gray horn (MAG) was calculated as $(OD_{MAG} - OD_P)$ divided by $(OD_{PG} - OD_P)$ and MAP2 in the lateral division of the anterior gray horn (LAG) was calculated as $(OD_{LAG} - OD_P)$ divided by $(OD_{PG} - OD_P)$, where OD_{IG}, OD_{MAG}, OD_{LAG}, OD_P and OD_{PG} stand for the average OD per square in the IG area, in the MAG area, in the LAG area, in the posterior column (P) area and in the posterior gray horn (PG) area, respectively (Fig. 1).

The data obtained were assessed by Student's *t*-test and Welch's *t*-test. We analyzed the both halves of the corss sections, and since the values for each side were not significantly different, we show here just the data for the right half of the spinal cord.

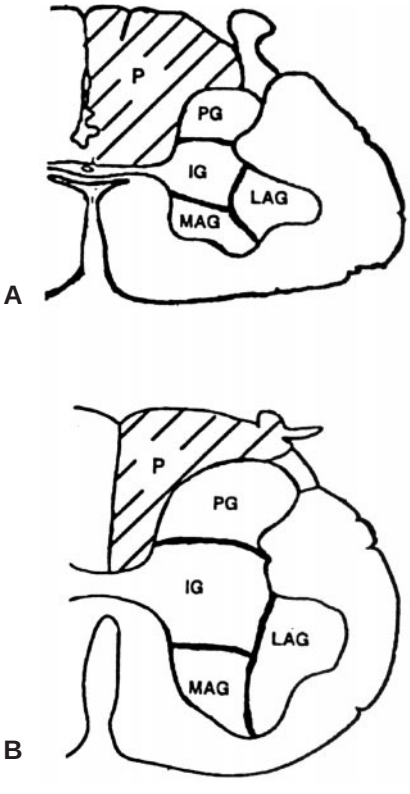


Fig. 1 Schematic drawing of the cervical spinal cord (**A**) and the lumbar spinal cord (**B**) [*IG* intermediate gray, *MAG* medial division of the anterior gray horn (whose neurons supply the truncal muscle), *LAG* lateral division of the anterior gray horn (whose neurons apply the limb muscle), *PG* posterior gray horn, *P* posterior column (whose density has been used for calibration of the background density)]

Results

The clinical data and immunohistochemical findings are summarized in Table 1. Immunohistochemistry using two distinct mAb against MAP2, consisting of clone HM-2 and clone AP20, gave identical results in the different cases examined. Thus, we show only the immunohistochemical findings for clone AP20 in the figures.

Controls

The gray matter of the spinal cords displayed diffuse, positive immunostaining for MAP2 (Fig. 2 A, D). The dendrites and somata of the anterior horn cells stained densely for MAP2. The white matter was not immunolabeled. The OD patterns of MAP2 are shown in Fig. 3. MAP2 expression in the LAG area was relatively increased in the controls.

The axon, spheroids and globules (the latter being < 20 μm in diameter) were labeled by the NF immunostaining. Only a few of the spheroids were seen in the controls, especially in the elderly persons. The perikaryon and dendrites were not immunostained by NF in most cases.

Cystatin C-positive small granules were seen in the normal neurons of the anterior horns, but no Bunina bodies were detected. No ubiquitin-positive inclusion bodies (Ubl), such as skein-like inclusions and spherical inclusions, were observed.

ALS cases

MAP-2 immunoreactivity in the anterior gray horn was closely correlated with the preservation of the anterior horn cells. In the patients with mild neuronal cell depletion, MAP2 immunoreactivity was preferentially diminished in the lateral or ventro-lateral portion of the anterior gray horn in comparison to that of the medial portion (Fig. 2 B, E). On the other hand, the MAP2 immunoreactivity was diffusely decreased in the anterior gray horn, mildly decreased in the intermediate gray and only slightly de-

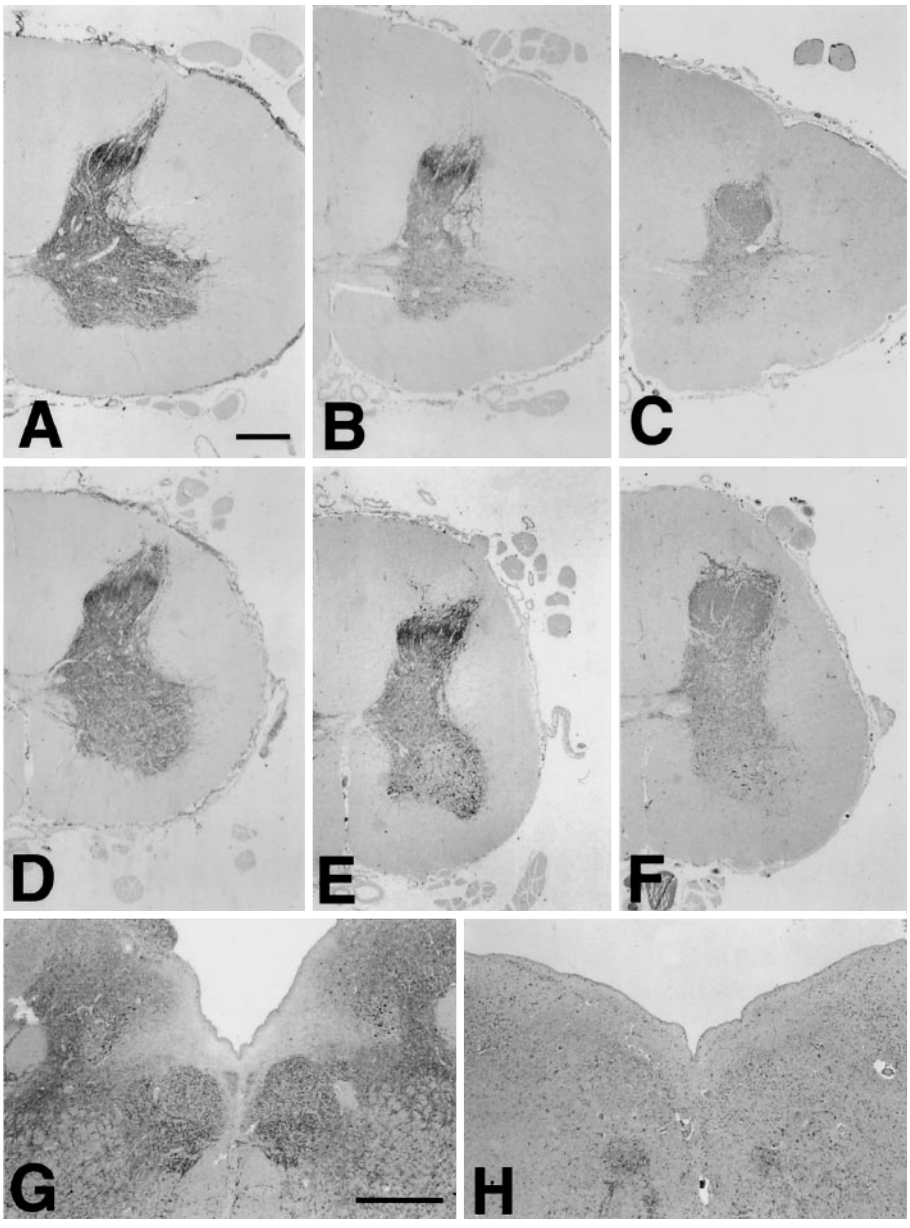


Fig. 2 Comparative immunohistochemistry of the 6th cervical spinal cord (**A–C**), the 3rd lumbar spinal cord (**D–F**) and medulla oblongata (**G, H**) of either control case (**A, D, G**) or ALS cases (**B, C, E, F, H**) probed with monoclonal antibody against MAP2. Positive immunoreactivity for MAP2 is localized in the gray matter from a control case (**A** and **D**, case C-4). MAP2 immunoreactivity of ALS with mild neuronal depletion (**B** and **E**, case 10) is selectively decreased in the lateral or ventrolateral portion of the anterior gray horn, compared with that of the medial portion. MAP2 immunoreactivity of ALS with severe loss of anterior horn neurons (**C** and **F**, case 15) is severely decreased in the anterior gray horn, mildly decreased in the intermediate gray and slightly decreased in the posterior gray horn. MAP2 immunoreactivity in the hypoglossal nuclei is markedly diminished in the ALS case with bulbar symptoms (**H**, case 17) in comparison to a normal case (**G**, case C-3) (*ALS* amyotrophic lateral sclerosis, *MAP2* microtubule-associated protein 2). *Bars* **A** (also for **B–F**), **G** also for **H**) 1 mm

creased in the posterior gray horn in the severe cases of ALS (Fig. 2C, F). In general, MAP2 immunoreactivity in the cervical spinal cords decreased more dramatically than in the lumbar spinal cords (Fig. 2B, C, E, F). When the semiquantitative data of MAP2 immunoreactivity in both the anterior gray horn and the intermediate gray were compared, every ALS cases showed a significant decrease, especially in the LAG area of both cervical (P = 0.0000019) and lumbar spinal cords (P = 0.0046) (Fig. 3). MAP2 immunoreactivity in the PG area appeared to decrease slightly in some severe ALS cases, whereas the semiquantitative data of the MAP2 in PG, when calculated as ($OD_{PG} - OD_{P}$), showed no significant difference between the ALS cases and the control cases (cervical spinal cord: P = 0.31, lumbar spinal cord: P = 0.56). MAP2 immunoreactivity was markedly reduced in the hypoglossal nuclei in the ALS cases with the bulbar sign (Fig. 2G, H).

Cytopathologically, the appearance of the remaining motor neurons in the ALS cases ranged from normal-appearing neurons (Fig. 4C) to chromatolytic neurons (Fig. 4A), shrunken neurons (Fig. 4C) and pigmented neurons (Fig. 4C) [11, 31]. MAP2 immunoreactivity was well preserved in the perikarya and dendrites of the normal-appearing neurons (Fig. 4B, D, E), but was slightly decreased in the perikarya and dendrites of the chromatolytic neurons (Fig. 4B) and moderately to severely decreased in both shrunken neurons (Fig. 4D) and pigmented neurons (Fig. 4E).

NF immunoreactivity probed with 2F11 antibody was enhanced in the axons, globules, spheroids and some anterior horn cells. Numerous spheroids were seen particu-

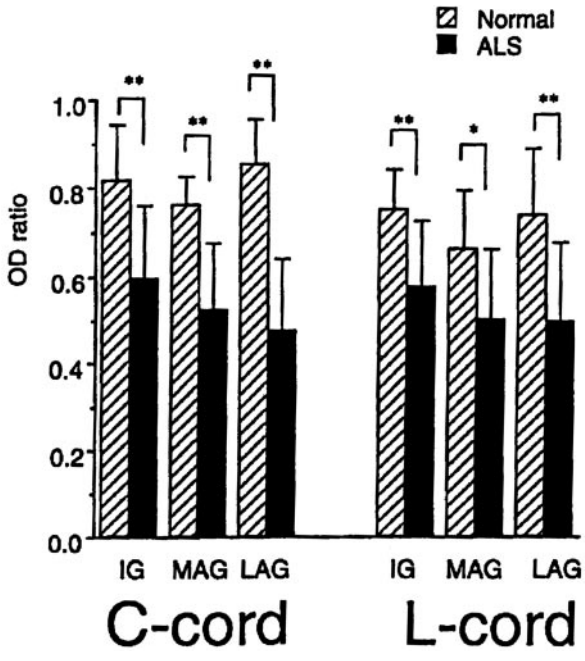


Fig. 3 The relative amount of MAP2 immunoreactivity in the anterior gray horn and the intermediate gray of the cervical and lumbar spinal cords (control $n = 7$, ALS $n = 19$), compared with controls by Student's *t*-test or Welch's test. * $P < 0.05$, ** $P < 0.01$. MAP2 immunoreactivity decreased significantly in both the anterior gray horn and the intermediate gray of the ALS cases, especially in the lateral division of the anterior gray horn in both the cervical ($P = 0.0000019$) and lumbar spinal cord ($P = 0.0046$) (*IG* the intermediate gray, *MAG* the medial division of the anterior gray horn, *LAG* the lateral division of the anterior gray horn, *C-cord* cervical spinal cord, *L-cord* lumbar spinal cord)

larly in the lumbar spinal cords of cases whose lower limb muscle strength was preserved (LLMP cases: cases 1, 3, 7, 8, 9, 17, 19). When the semiquantitative data on the MAP2 immunoreactivity were compared among the three groups: the control cases, ALS cases with numerous spheroids (ALS-NS: case 1, 2, 3, 7, 8, 9, 17, 19) and ALS cases with paucity of spheroids (ALS-PS), the MAP2 immunoreactivity of the anterior gray horn of the ALS-PS group was found to decrease significantly ($P < 0.05$), but no significant difference was observed between the control cases and the ALS-NS cases except for the data of the LAG area in the cervical spinal cords (Fig. 5). In case 9, a few spheroids were rimmed by dense MAP2 immunostaining, which seemed to represent a proximal axonal swelling (Fig. 6 A, B). In case 4, MAP2 immunoreactivity increased in the perikarya of the anterior horn cells containing a phosphorylated NF-positive inclusion (NFI), but no MAP2 immunostaining was seen in the inclusion itself (Fig. 6 C).

Immunoreactivity for cystatin C was detected in Bunina bodies, as reported by Okamoto et al. [25], and the MAP2 immunoreactivity was mildly to severely decreased in the perikarya of the anterior horn cells bearing Bunina bodies (Fig. 6 D). Ub immunoreactivity was localized in skein-like inclusions and spherical inclusions, while the MAP2 immunoreactivity slightly decreased in the perikarya of the anterior horn cells containing Ubl (Fig. 6 E). Bunina body and Ubl were not immunostained with MAP2 antibodies as previously reported by Murayama et al. [24].

Discussion

Our study points to MAP2 as being an excellent marker for the detection and quantification of the anterior horn degeneration in ALS, which is difficult to visually estimate by H&E and KB staining. By measuring the size of the anterior horn cells, Oyanagi et al. [26, 27] demonstrated that the degeneration of the anterior horn in ALS occurred from the lateral devision of the anterior horn and the intermediate gray and was also affected in the advanced stage, while the degeneration of the cervical spinal cord preceded that of the lumbar spinal cord. Some recent studies have shown that synaptophysin immunoreactivity decreased from the ventro-lateral portion of the anterior horn of ALS [19, 29]. Our MAP2 immunohistochemical results support these observations and allow us to quantify them more easily. In addition, MAP2 immunoreactivity decreased slightly in the posterior gray horn in some severe cases, which may imply that posterior horn was also affected in ALS.

It has been reported that chromatolytic neurons can be found in ALS with a rapid course, that shrunken neurons may well have been an artifact and pigmented neurons may result from the excessive accumulation of lipofuscin [11, 31]. These pathological findings were observed in a mosaic pattern in the same sections of the ALS. However, the pathological process shared in these cells and their lineages are not clear. The present study demonstrated that MAP2 immunoreactivity markedly decreased in the shrunken neurons and pigmented neurons. As a result, the MAP2 immunoreactivity closely correlated with the individual cytological alterations and also helped to characterize the distinct pattern of neuronal degeneration in the anterior horn cells in ALS.

Bunina bodies and Ubl are intracytoplasmic inclusions reported in the lower motor neurons of patients with ALS [17]. These inclusions have been carefully investigated because of the possibility that they may give us some clues to help resolve the pathogenic mechanisms involved in the selective degeneration of the upper and lower motor neuron in ALS. Our results indicate that many Bunina bodies exist in the pigmented neurons. MAP2 immunoreactivity decreased more severely in the perikaryon containing Burnina bodies than in the perikaryon containing Ubl, but MAP2 immunoreactivity was not directly affected by the appearance of these inclusion bodies themselves. Further cytopathological examinations are thus called for to the clarify the mechanism of formation of these inclusions.

In relation to the clinical symptoms, MAP2 immunoreactivity was better preserved in the LLMP cases than in the other classical ALS cases. However, MAP2 immunoreactivity was not necessarily preserved in the long-duration ALS cases. Hanyu et al. [10] demonstrated that

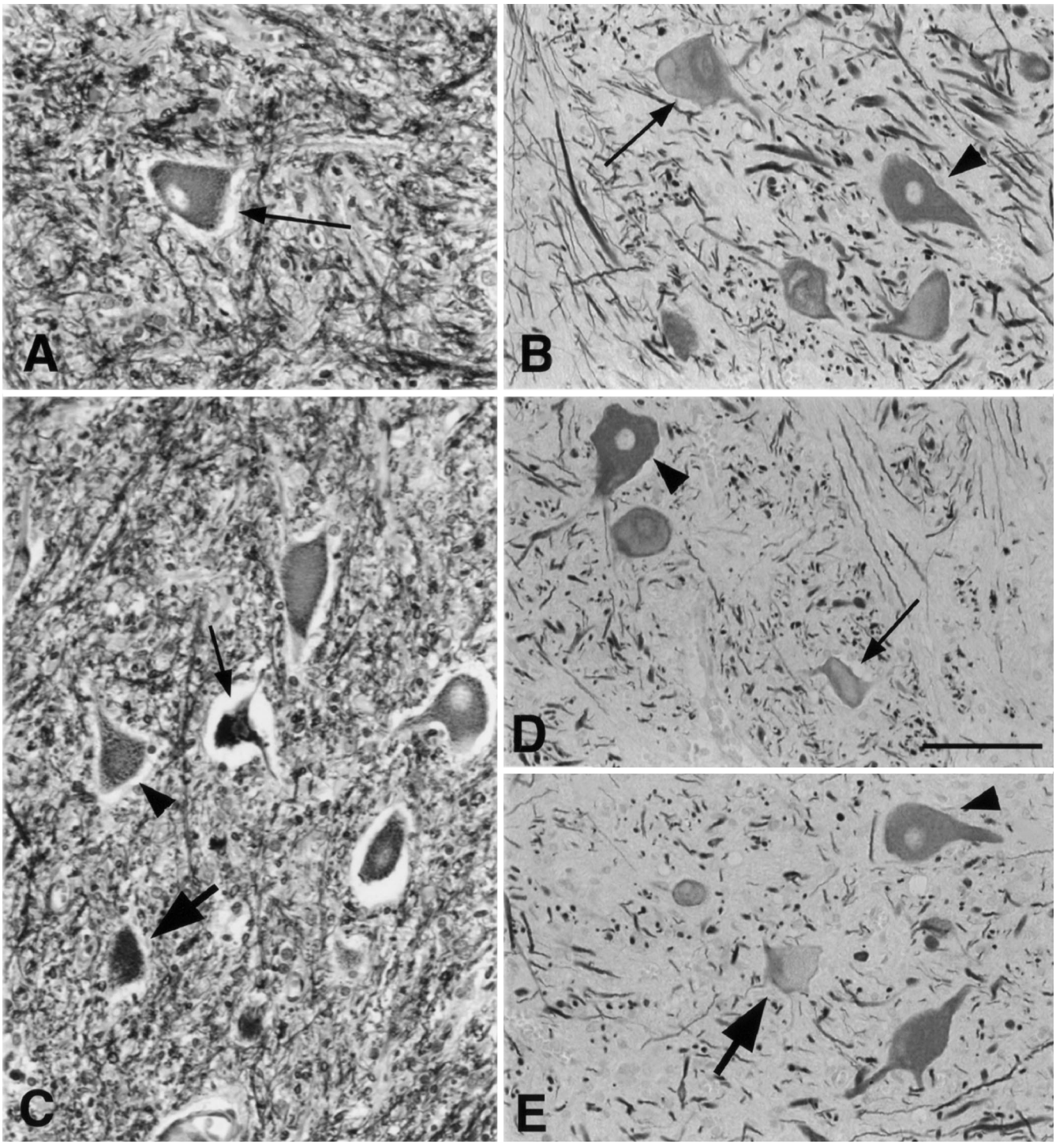


Fig. 4 A–E The cytopathological findings and immunohistochemistry of the anterior horn cells of cases with ALS. **A, C** The appearance of the remaining anterior horn neurons ranges from normal-appearing neurons (**C**, *arrowhead*) to chromatolytic neurons (**A**, *arrow*), shrunken neurons (**C**, *small arrow*) and pigmented neurons (**C**, *large arrow*). The pigmented neuron stained dark blue with Luxol-fast blue. MAP2 immunoreactivity is preserved in the perikarya and dendrites of a remaining normal-appearing neurons (**B, D, E**, *arrowhead*), but is slightly decreased in a chromatolytic neuron (**B**, *arrow*), moderately decreased in a shrunken neuron (**D**, *arrow*) and dramatically decreased in a pigmented neuron (**E**, *large arrow*) in ALS case (case 1). *Bar* **D** (also for **A–E**) 100 μm

the number of large myelinated fibers of C6 and C8 decreased significantly in ALS cases, which also correlated with the muscle strength of the ipsilateral upper limb as determined by manual muscle testing before death. Sobue et al. [32] proposed that the loss of anterior horn cells and the alteration of large myelinated fibers in ALS significantly correlated with the muscle strength and duration of symptoms. Furthermore, according to our results, anterior horn degeneration correlated more closely with clinical severity than with the clinical duration.

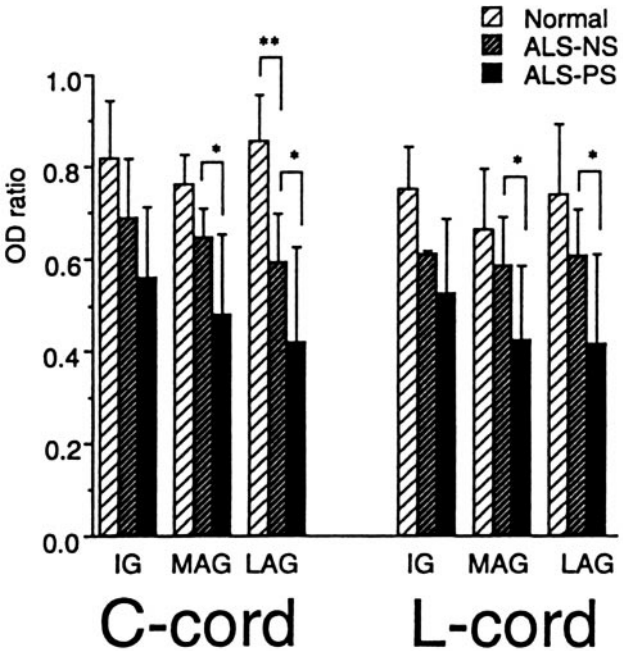
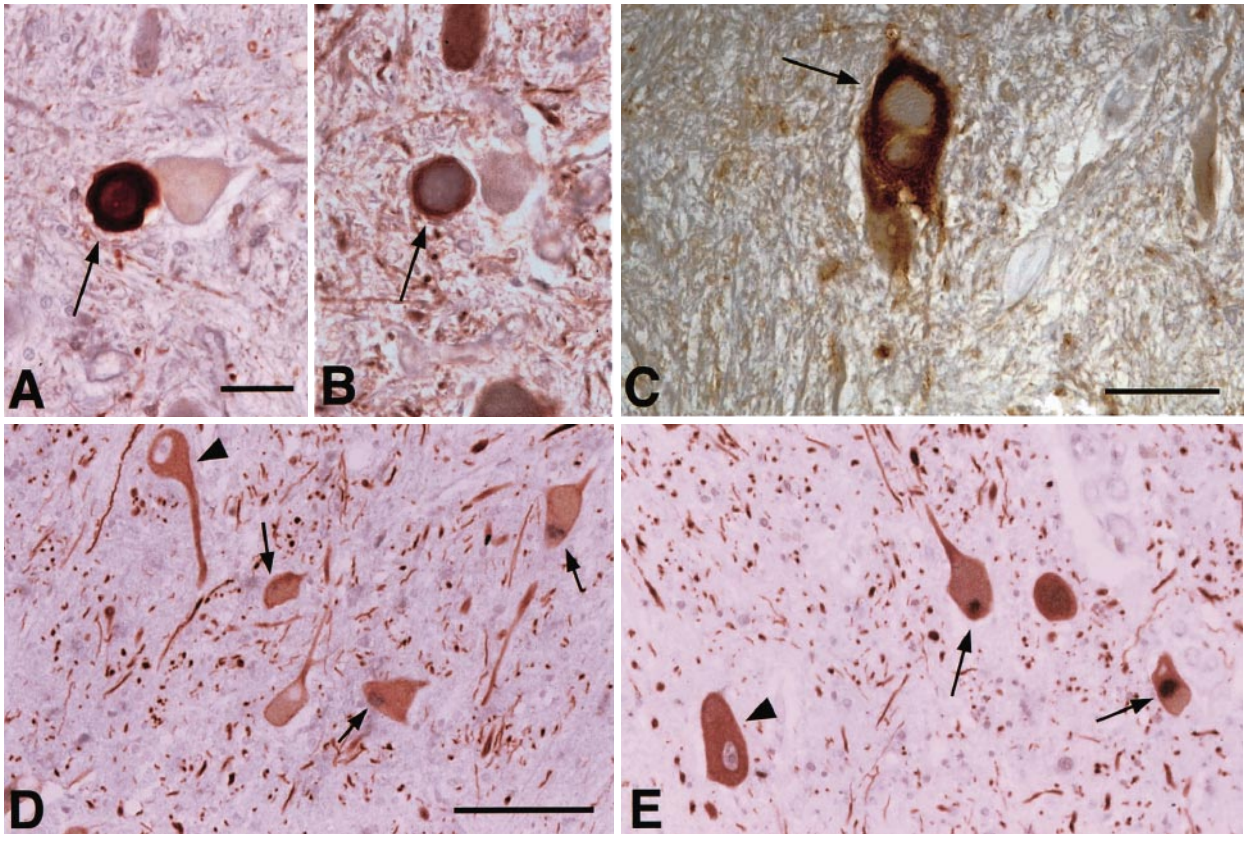


Fig. 5 The relative optical density for MAP2 in the anterior gray horn and intermediate gray of the cervical and lumbar spinal cord [control $n = 7$, ALS with numerous spheroids (ALS-NS) $n = 8$, ALS with paucity of spheroids (ALS-PS) $n = 11$], in comparison to the controls by Student's t-test or Welch's-test. $*P < 0.05$,. $**P < 0.01$. MAP2 immunoreactivity of the anterior gray horn of the ALS-PS decreased significantly ($P < 0.05$). but not significant difference is observed between controls and ALS-NS except for the LAG (abbrevation as in Fig. 3)

In recent pathological studies, the alteration of the Golgi apparatus [6, 21, 22, 34], synapse [19, 29], or dendrites [13] has been reported, but one of the most notable pathological findings in either familial or sporadic ALS is the abnormal accumulation or phosphorylation of NF in the perikarya and proximal axons of the spinal motor neurons. NF accumulation or phosphorylation has been considered to be a sign of degeneration [1, 16, 30], possibly preceding cell death in ALS [18, 20, 23, 33]. Although the accumulation or phosphorylation of NF is commonly observed in the early stage of ALS, this phenomenon has

Fig. 6 A–E Double immunostaining of the anterior horn cells in the cases with ALS. **A** Double immunostaining for MAP2 (*grayish blue*) and phosphorylated NF (*brown*), the spheroid is strongly immunostained for NF (*arrow*) (case 9). **B** In serial sections, the spheroid immunostained for NF (*grayish blue*) is rimmed with dense MAP2 immunostaining (*brown, arrow*). **C** MAP2 immunoreactivity (*brown*) increased in the perikaryon of the anterior horn cell containing a phosphorylated NF-positive inclusion (*grayish blue*) (case 4). **D** Double immunostaining visualizing MAP2 (*brown*) and cystatin C (*grayish blue*). Bunina bodies are immunostained by cystatic C (*arrow*), and MAP2 immunoreactivity is preserved in the mostly normal-appearing neuron (*arrowhead*), whereas this is mildly to severely decreased in the perikarya of the anterior horn cells containing Bunina bodies (case 8). **E** Double immunostaining visualizing MAP2 (*brown*) and ubiquitin (*grayish blue*). A skein-like inclusion and a spherical inclusion (*arrows*) are immunopositive to ubiquitin. MAP2 immunoreactivity is preserved in the mostly normal-appearing neuron (*arrowhead*), but is slightly decreased in the perikaryon containing ubiquitin-positive inclusions (case 8). *Bars* **A** (also for **B**), **C** 25 μm; **D** (also for **E**) 100 μm



been widely viewed as a secondary effect of neuronal degeneration, thus reflecting a defective axonal transport [5, 7]. However, some recent investigations have also suggested that the NF can play a causal role in ALS. Transgenic mice overexpressing NF proteins developed motor neuron disease [2, 3, 15, 37]; in addition, some of the human SOD1 mutant mice showed an accumulation of NF in motor neuorns [8, 28, 35]. Furthermore, variant alleles of the NF heavy subunit gene have also been found in some human ALS patients [4]. Our findings are consistent with the hypothesis that the alteration of NF is a crucial event for the pathogenesis since MAP2 immunoreactivity was relatively preserved in anterior horns which contained either many spheroids or NFI.

In conclusions, MAP2 was found to be useful for detecting and quantifying lower motor neuron degeneration in ALS. MAP2 immunoreactivity in the anterior horns of ALS was more closely related with clinical severity than clinical duration. Regarding the cytopathological aspects, MAP2 immunoreactivity was decreased especially in the shrunken neurons and pigmented neurons, some of which contained Bunina bodies.

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References

1. Carpenter S (1968) Proximal axonal enlargement in motor neuron disease. Neurology 18:841–851
2. Collard J-F, Côté F, Julien J-P (1995) Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. Nature 375:61–64
3. Côté F, Collard J-F, Julien J-P (1993) Progressive neuropathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. Cell 73: 35–46
4. Figlewicz DA, Krizus A, Martinoli MG, Meininger V, Dib M, Rouleau GA, Julien J-P (1994) Variants of the heavy neurofilament subunit are associated with the development of amyotrphic lateral sclerosis. Hum Mol Genet 3:1757–1761
5. Gajdusek DC (1985) Hypothesis: interference with axonal transport of neurofilament as a common pathogenetic mechanism in certain diseases of the central nervous system. N Engl J Med 312:714–719
6. Gonatas NK, Stieber A, Mourelatos, Chen Y, Gonatas JO, Appel SH, Hays AP, Hickey WF, Hauw J-J (1992) Fragmentation of the Golgi apparatus of motor neurons in amyotrophic lateral sclerosis. Am J Pathol 140:731–737
7. Griffin JW, Watson DF (1988) Axonal transport in neurological disease. Ann Neurol 23:3–13
8. Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng H-X, Chen W, Zhai P, Sufit RL, Siddique T (1994) Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. Science 264:1772–1775
9. Gutman L, Mitsumoto H 81996) Advances in ALS. Neurology [Suppl 2] 47:S17–S18
10. Hanyu N, Oguchi K, Yanagisawa N, Tsukagoshi H (1982) Degeneration and regeneration of ventral root motor fibers in amyotrophic lateral sclerosis. Morphometric studies of cervical ventral roots. J Neurol Sci 55:99–115
11. Hirano A (1982) Aspects of the ultrastructure of amyotrophic lateral sclerosis. Adv Neurol 36:75–88
12. Hirano A (1996) Neuropathology of ALS: an overview. Neurology [Suppl 2] 47:S63–S66
13. Kiernan JA, Hudson AJ (1993) Changes in shapes of surviving motor neurons in amyotrophic lateral sclerosis. Brain 116: 203–215
14. Kondo A, Iwaki T, Tateishi J, Kirimoto K, Morimoto T, Oomura I (1986) Accumulation of neurofilaments in a sporadic case of amyotrophic lateral sclerosis. Jpn J Psychiatry Neurol 40:677–684
15. Lee MK, Marszalek JR, Cleveland DW (1994) A mutant neurofilament subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. Neuron 13:975–988
16. Leigh PN, Dodson A, Swash A, Brion J-P, Anderton BH (1989) Cytoskeletal abnormalities in motor neuron disease. An immunocytochemical study. Brain 112:521–535
17. Lowe J (1994) New pathological findings in amyotrophic lateral sclerosis. J Neurol Sci [Suppl] 124:38–51
18. Manetto V, Sternberger NH, Perry G, Sternberger LA, Gambetti P (1988) Phosphorylation of neurofilaments is altered in amyotrophic lateral sclerosis. J Neuropathol Exp Neurol 47: 642–653
19. Matsumoto S, Goto S, Kusaka H, Ito H, Imai T (1994) Synaptic pathology of spinal anterior horn cells in amyotrophic lateral sclerosis: an immunohistochemical study. J Neurol Sci 125 :180–185
20. Mizusawa H, Matsumoto S, Yen S-H, Hirano A, Rojas-Corona RR, Donnefeld H (1989) Focal accumulation of phsphorylated neurofilaments within anterior horn cell in familial amyotrophic lateral sclerosis. Acta Neuropathol 79:37–43
21. Mourelatos Z, Yachnis A, Rorke L, Mikol J, Gonatas NK (1993) The Golgi apparatus of motor neurons in amyotrophic lateral sclerosis. Ann Neurol 33:608–615
22. Mourelatos Z, Hirano A, Rosenquist AC, Gonatas NK (1994) Fragmentation of the Golgi apparatus of motor neurons in amyotrophic lateral sclerosis (ALS). Clinical studies in ALS of Guam and experimental studies in deafferented neurons and in β,β′-iminopropionitrile axonopathy. Am J Pathol 144:1288–1300
23. Munoz DG, Greene C, Perl DP, Selkoe DJ (1988) Accumulation of phosphorylated neurofilaments in anterior horn motoneurons of amyotrophic lateral sclerosis patients. J Neuropathol Exp Neurol 47:9–18
24. Murayama S, Mori H, Ihara Y, Bouldin TW, Suzuki K, Tomonaga M (1990) Immunocytochemical and ultrastructural studies of lower motor neurons in amyotrophic lateral sclerosis. Ann Neurol 27:137–148
25. Okamoto K, Hirai S, Amari M, Watanabe M, Sakurai A (1993) Bunina bodies in amyotrophic lateral sclerosis immunostained with rabbit anti-cystatic C serum. Neurosci Lett 162:125–128
26. Oyanagi K, Makifuchi T, Ikuta F (1983) A topographic and quantitative study of neurons in human spinal gray matter, with special reference to their changes in amyotrophic lateral sclerosis. Biomed Res 4:211–224
27. Oyanagi K, Ikuta F, Horikawa Y (1989) Evidence for sequential degeneration of the neurons in the intermediate zone of the spinal cord in amyotrophic lateral sclerosis: a topographic and quantitative investigation. Acta Neuropathol 77:343–349
28. Rouleau GA, Clark AW, Rooke K, Pramatarova A, Krizus A, Suchowersky O, Julien J-P, Figlewicz D (1996) SOD1 mutation is associated with accumulation of neurofilaments in amyotrophic lateral sclerosis. Ann Neurol 39:128–131
29. Sasaki S, Maruyama S (1994) Decreased synaptophysin immunoreactivity of the anterior horns in motor neuron disease. Acta Neuropathol 87:125–128
30. Sasaki S, Maruyama S, Yamane K, Sakuma H, Takeishi M (1989) Swelling of proxial axons in a case of motor neuron disease. Ann Neurol 25:520–522

31. Schoene WC (1985) Degenerative disease of the central nervous system. In: Davis RL, Robertson DM (eds) Textbook of neuropathology. Williams & Wilkins, Baltimore, pp 788–823
32. Sobue G, Sahashi K, Takahashi A, Matsuoka Y, Muroga T, Sobue I (1983) Degenerating compartment and functioning compartment of motor neurons in ALS: possible process of motor neuron loss. Neurology 33 : 654–657
33. Sobue G, Hashizume Y, Yasuda T, Mukai E, Kumagai T, Mitsuma T, Trojanowski JQ (1990) Phosphorylated high molecular weight neurofilament protein in lower motor neurons in amyotrophic lateral sclerosis and other neurodegenerative disease involving ventral horn cells. Acta Neuropathol 79 : 402–408
34. Stieber A, Chen Y, Wei S, Mourelatos Z, Gonatas J, Okamoto K, Gonatas NK (1998) The fragmented neuronal Golgi apparatus in amyotrophic lateral sclerosis includes the trans-Golgi-network: functional implications. Acta Neuropathol 95 : 245–253
35. Tu P-H, Raju P, Robinson KA, Gurney ME, Trojanowski JQ, Lee VM-Y (1996) Transgenic mice carrying a human mutant superoxide disumutase transgene develop neuronal cytoskeletal pathology resembling human amyotrophic lateral sclerosis lesions. Proc Natl Acad Sci USA 93 : 3155–3160
36. Tucker RP (1990) The roles of microtubule-associated proteins in brain morphogenesis: a review. Brain Res Rev 15 : 101–120
37. Xu Z, Cork LC, Griffin JW, Cleveland DW (1993) Increased expression of neurofilament subunit NF-L produces morphological alterations that resemble the pathology of human motor neuron disease. Cell 73 : 23–33