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Phosphorylated high molecular weight neurofilament protein in lower motor neurons in amyotrophic lateral sclerosis and other neurodegenerative diseases involving ventral horn cells*

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Summary. Lower motor neurons of the spinal cord of patients with amyotrophic lateral sclerosis (ALS), Werdnig-Hoffmann's disease (WH), X-linked recessive bulbospinal neuronopathy (X-BSNP) and multiple system atrophy (MSA), all of which were known to involve the lower motor neurons, were immunohistochemically examined by using a monoclonal antibody (Ta-51) specific to phosphorylated epitopes of high molecular weight subunits of neurofilaments. The incidence of Ta-51-positive neurons was significantly increased in ALS, WH and MSA, but not in X-BSNP. Ta-51-positive neurons showed a wide variety of morphological appearances, including neurons with normal appearance, central chromatolysis, simple atrophy and neurons containing massive neurofilamentous accumulation. In aged-control cases, similar Ta-51-positive neurons were observed, although to a much lesser extent. In ALS, spheroids and globules, which were strongly positive for Ta-51, were also significantly increased. Ta-51-positive motor neurons, spheroids and globules appeared in proportional to the number of remaining large motor neurons in ALS.

Key words: Phosphorylated high molecular weight neurofilament – Motor neuron – Amyotrophic lateral sclerosis (ALS) – Werdnig-Hoffmann's disease – X-linked recessive bulbospinal neuronopathy

Neurofilaments (NF), the intermediate filaments of mammalian neurons, are composed of three distinct polypeptides with approximate molecular mass of 200 kDa, 150 kDa and 68 kDa (NF-H200, MF-M150 and NF-L68, respectively). Monoclonal antibodies (mAb) specifically recognizing the phosphorylated epitopes of each subclass of NF are now available for investigation of the distribution of the phosphorylated NF in normal and diseased conditions [4, 5, 19–21, 36, 40, 41]. So far, the abnormal occurrence of phosphorylated NF in neuronal perikarya, or in proximal axonal segments and dendrites has been documented in Alzheimer's disease [9, 27, 37]. Pick's disease [27], Parkinson's disease [13], infantile neurodegenerative diseases [42], some neurodegenerative diseases [12], spontaneously occurring animal disorders [10], and several experimental conditions [2, 14, 39], including nerve transsection [25, 28].

Recently, several reports [22, 24, 26, 31] demonstrated that phosphorylated NF are abnormally increased in the perikarya of anterior horn motor neurons of ALS. In this report, we extended these findings by examining a large number of spinal cord samples from ALS patients and other neurodegenerative diseases that are known to affect spinal anterior horn motor neurons. Additionally, we characterized the abnormal distribution of phosphorylated NF in the ventral horn motor neurons.

Materials and methods

Lumbar spinal cord tissue was obtained from 40 cases with ALS (43–74 years old), 7 cases with Werdnig-Hoffmanns disease (WH) (3 months to 13 months old), [7, 18], 4 cases with X-linked recessive bulbospinal neuronopathy X-BSNP (51–72 years old) [35] and 11 cases with multiple system atrophy (MSA) associated with autonomic failure (48–75 years old). No cases with a familial history of disease were included in this study. Thirty-two cases who had died of non-neurological disorders between the age of 44 to 88 years, and 4 cases without neurological symptoms of age 2 to 9 months, served as age-matched controls. All the cases with neurodegenerative diseases had been diag-

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nosed clinically during life, and the diagnosis was confirmed by postmortem histological examination. Tissues of L4 spinal cord were fixed in 10% buffered formalin for 1 to 2 weeks and then embedded in paraffin.

In most cases, ten non-consecutive sections with 6-μm thickness for each case were obtained for the immunohistochemical study, and sections adjacent to those used for immunohistochemistry were stained with H&E to monitor the identify of immunoreactive cells.

The mAb Ta-51 was selected from a library of mAb specific to NF epitopes [19–21]. The specificity of Ta-51 for phosphorylated epitopes in human NF-H has been described previously [19–21]. Immunohistochemistry was performed by the avidin-biotin complex (ABC) method [34], using Vectastain Kits (Vector Laboratories Inc., Burlingam, Calif). The primary antibody (supernatant of hybridoma) was diluted to 1:20. To confirm the specificity of mAb Ta-51 against the phosphorylated epitope of NF-H in the tissue sections processed in the present procedure, deparaffinized sections were incubated with alkaline phosphatase from *E. coli* (Sigma Chemicals, Type III-N), or with buffer alone for 3 h as described previously [19–21], and then processed for immunohistochemistry. Preincubation with alkaline phosphatase abolished most Ta-51 immunoreactivity (Fig. 1), confirming that Ta-51 specifically recognized phosphorylated epitopes of NF in the present tissue preparation. We counted Ta-51-positive neurons in the anterior horns in gray matter anterior to a line perpendicular to the anterior sulcus. These counts were performed on at least five, but in most cases ten non-consecutive sections.

To estimate the number of spheroids and globules, photographs with a magnification of ×205 covering the entire ventral horns were prepared and counted with a particle size-analyzer (Zeiss, TGZ-3). According to conventional criteria [6, 11, 27, 28], spheroids were estimated as larger than 20 μm diameter, and globules smaller than 20 μm diameter.

To estimate the remaining neuronal population, neurons with nucleoli and with a diameter of ≥ 25 μm were counted on at least five, but mostly on ten non-consecutive sections. Thus-obtained data were analyzed by Wilcoxon's two-sample test.

Results

Neuronal perikarya

In ALS, mAb Ta-51, stained the neuronal perikarya of anterior horn motoneurons in the incidence of 0% to 37.5% of total neuronal cell counts (Fig. 3) which were significantly higher as compared with controls ($z = 4.30$, $P < 0.001$). Perikaryal Ta-51 immunoreactivity was observed in normal-appearing neurons, "chromatolytic-like" neurons, "simple atrophic" neurons, and neurons containing massive neurofilamentous accumulation (Fig. 2D, E). There were no specific morphological characteristics in the neurons which were Ta-51 positive. The staining pattern was also divergent among neurons; certain neurons were stained diffusely in the cytoplasm, some neurons were stained focally, i.e., only in the periphery of the neuronal soma (Fig. 2D, H). The population of Ta-51-positive cells was fairly well correlated with the population of the remaining motorneurons (Fig. 4).

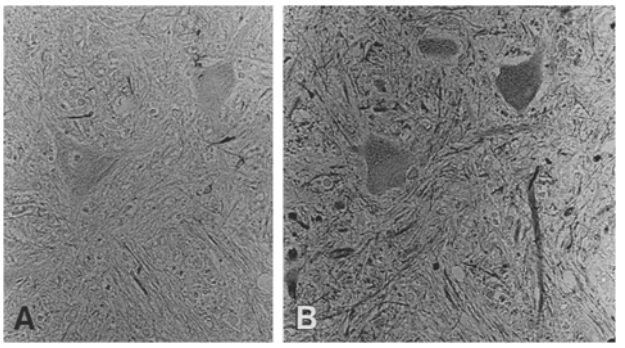

Fig. 1. Ta-51 immunoreactivity in the tissue section with (**A**) and without (**B**) alkaline phosphatase treatment. With the treatment, the immunoreactivity was almost completely suppressed. Counter staining with methyl green. ×256

In age-matched control cases, Ta-51-positive neurons were present but the incidence was much lower compared with those in ALS. Although the incidence was very low, "chromatolytic-like" neurons, neurons containing massive hyaline neurofilamentous accumulation and normal-appearing neurons with positive Ta-51 immunoreactivity were also seen in the age-matched controls (Fig. 2).

In WH, Ta-51-positive neurons were frequently seen, many of which were undergoing central chromatolytic changes (Fig. 2). However, normal-appearing neurons were also present. In contrast, age-matched infantile controls did not show Ta-51 immunoreactivity in the motorneuron perikarya (Fig. 3). MSA showed Ta-51-positive cells more frequently than in age-matched controls ($z = 3.48$, $P < 0.001$), but their incidence was much lower as compared with ALS. X-BSNP also showed occasional Ta-51-positive cells, but the incidence of those cells was not significantly increased as compared with age-matched controls ($z = 1.61$, N. S.).

Spheroids and globules

Spheroids and globules were strongly positive with Ta-51. In age-matched control cases, globules were seen mainly restricted to the medial-ventral area of the anterior horn, while the spheroids were very rarely seen (Fig. 5). In contrast to controls, spheroids and globules in ALS were located diffusely in the ventral horn, particularly in the lateral-anterior region (Fig. 5). The incidence of globules and spheroids in ALS was variable among the cases but was increased in some cases as compared to controls (Fig. 6). In cases with extensive neuron depletion, however, the spheroids and globules were rarely seen. The number of globules and speroids was directly proportional

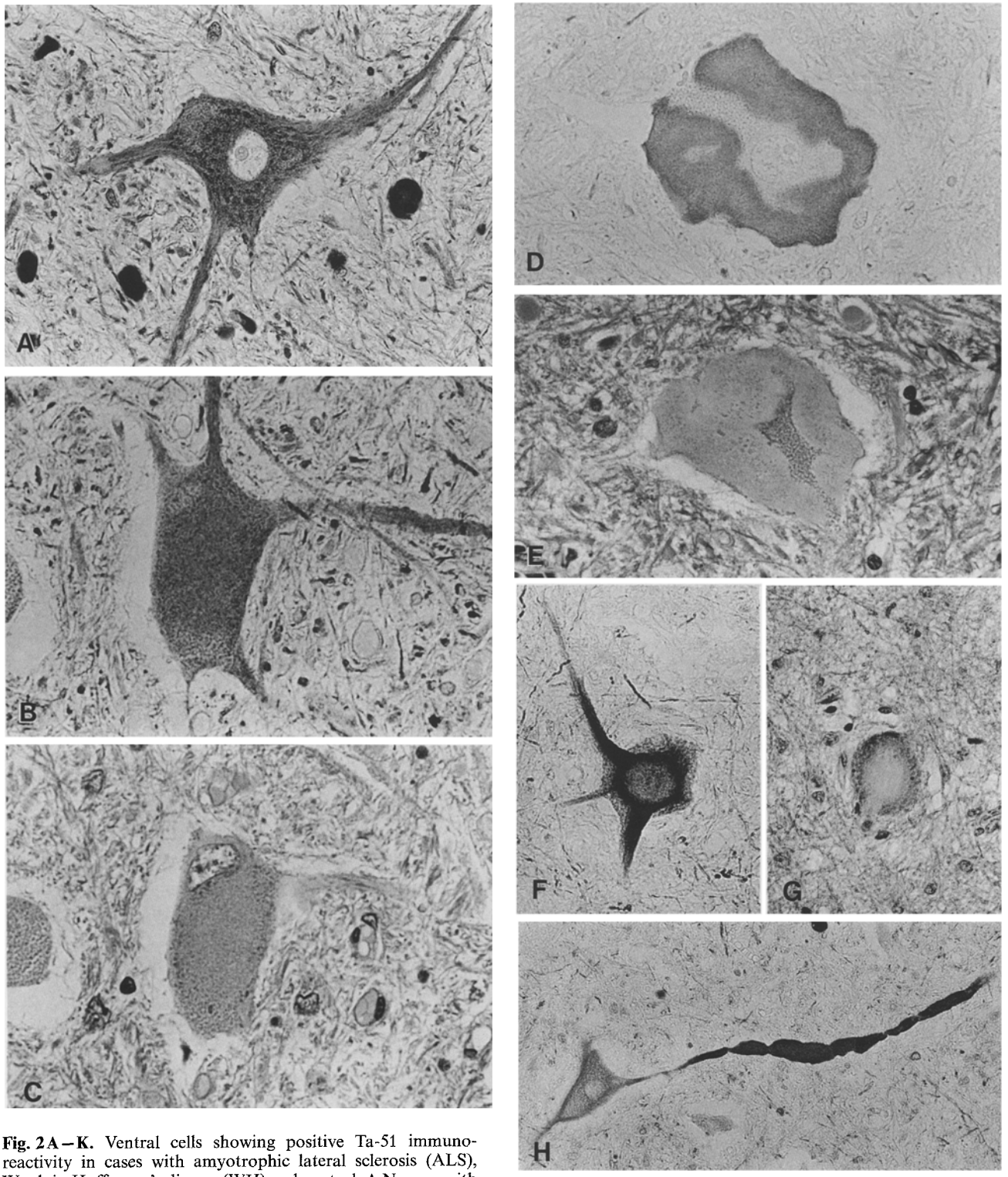

Fig. 2A–K. Ventral cells showing positive Ta-51 immunoreactivity in cases with amyotrophic lateral sclerosis (ALS), Werdnig-Hoffmann's disease (WH) and control. **A** Neuron with normal appearance in an ALS patient. **B** Neuron with central chromatolysis in an ALS patient. **C** H&E staining on the adjacent section of **B**. **D** Neurons containing neurofilamentous mass in ALS patients. **E** H&E staining on the adjacent sections of **D**. Only the neurofilamentous mass was Ta-51 positive. **F** Neuron with central chromatolysis in a patient with WH. **G** H&E staining on the adjacent section of **F**. **H** Neuron with axonal swelling in an ALS patient. Ta-51 immunostain is seen in the periphery of the perikarya. **I** Neurons with chromatolytic reaction and weakly positive Ta-51 immunoreactivity, observed in a control patient. **J** H&E staining on the adjacent section of **I**. **K** Neurons with possible dendrite swelling, and strongly positive Ta-51 immunoreactivity, observed in a control patient. **A, G** ×459; **I** ×434; **B–F** ×613; **J, K** ×579, **H** ×306

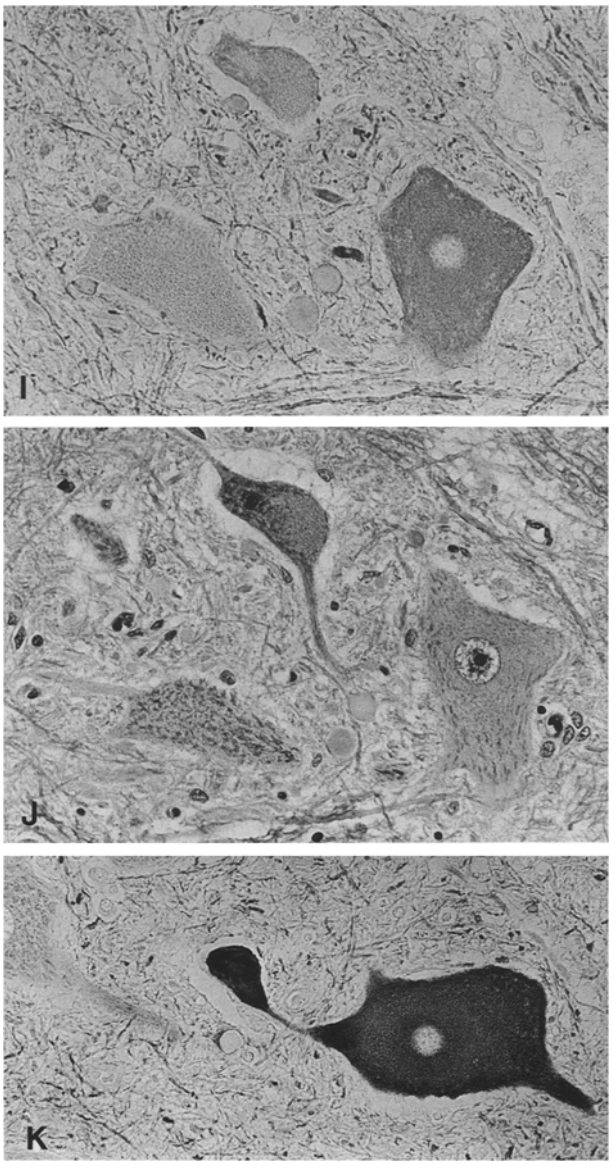

Fig. 2 I–K

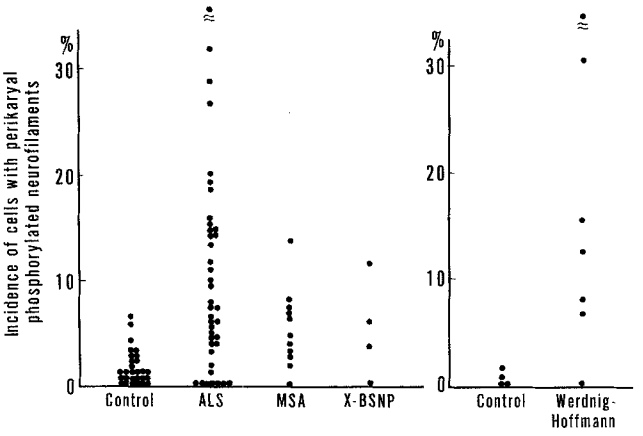

Fig. 3. Incidence of neuron with Ta-51-positive neuronal perikarya. Controls for ALS, X-linked recessive bulbospinal neuronopathy X-BSNP, multiple system atrophy (MSA) and for WH were age matched, respectively. ALS, MSA, and WH show the increased incidence of Ta-51-positive neurons as compared to controls ($z = 4.30$, $P < 0.001$; $z = 3.48$, $P < 0.001$; and $z = 2.00$, $P < 0.045$ respectively), but X-BSNP did not show a significant difference ($z = 1.61$, N. S.)

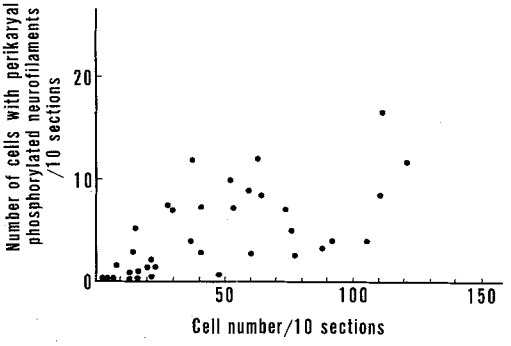

Fig. 4. Relationship between the population of Ta-51-positive neurons and number of the remaining large motor neurons in ALS. Relation coefficient was 0.666 ($P < 0.0001$)

to the number of remaining ventral horn neurons (Fig. 7).

In X-BSNP, anterior horn motor neurons were severely depopulated, and spheroids and globules were few in number and indistinguishable from those in control cases. Although the motor neuron population in MSA (particularly the large motor neurons) was relatively well preserved as compared to ALS or X-BSNP, globules and spheroids were not present in significant numbers.

In the infant without neurological symptoms, globules and spheroids did not occur. Spheroids in WH were rarely seen, but definitely increased in incidence in one case.

Discussion

Our results show that the L4 ventral horn of patients with ALS contain an excess number of motor neurons that express phosphorylated epitopes of NF-H in their perikarya. These findings confirm recent observations [22–24, 26, 31], but extend these findings to a larger number of ALS cases and cases of other neurodegenerative diseases involving ventral horn motor neurons.

In our series, Ta-51-positive perikarya were observed in neurons with a wide variety of morphological appearances; the staining pattern was also divergent among neurons. These divergent morphological ap-

Fig. 5. Distribution of spheroids and globules in the ventral horn in control (*upper panel*) and in a representative case with ALS (*lower panel*). Large spots represent spheroids, and small spots represent globules. As compared to a control case, spheroids and globules are diffusely distributed particularly in the lateral ventral horn

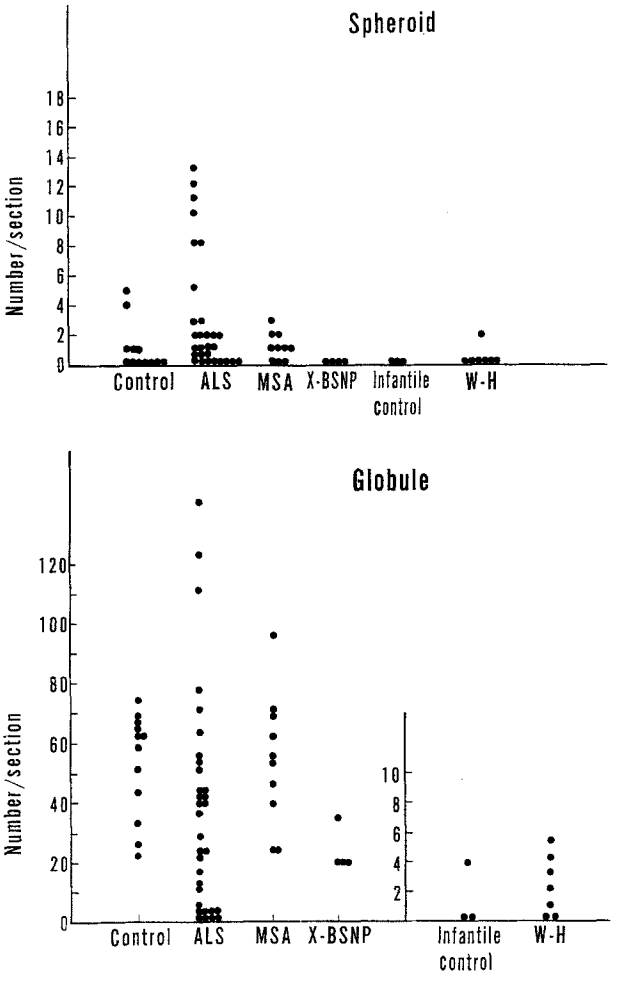

Fig. 6. Population of spheroids and globules in controls, ALS, X-BSNP, MSA and WH

pearances in Ta-51-positive neurons and divergent staining pattern suggests that the perikaryal occurrence of phosphorylated NF-H is not strictly linked to a certain morphological event in the motor neurons. The Ta-51-positive hyaline accumulations observed in ALS were also observed in MSA and aged-controls. Hence, they are not ALS specific as suggested by Leigh et al. [22].

Although the occurrence of Ta-51-positive globules and spheroids were prominently increased in some cases of ALS as previously reported [6, 11, 29, 31], the most significant difference between the ALS and control [8] was a topographical distribution of these swellings. In ALS, spheroids and globules were diffusely located in the ventral horn which coincided with the area of the lateral nuclei. This area consists mainly of large motor neurons and they are predominantly affected in ALS [32, 33]. In addition, the perikaryal occurrence of phosphorylated NF-H and the appearance of spheroids and globules, both correlated well with the number of remaining neurons, particularly with the number of large motor neurons of more than 25 μm in diameter; this supports the view that these two events reflect some aspects of the process of motor neuron loss in ALS.

Among non-ALS cases with an involvement of ventral horn cells, MSA and WH showed a significantly increased population of Ta-51-positive neurons and spheroids but X-BSNP did not show a similar occurrence. In X-BSNP, neuronal loss is extremely severe [35]; since large motor neurons are almost completely depleted, Ta-51-positive neurons also may be depleted even if a process similar to that in ALS is taking place. Alternatively, the sample number of X-BSNP may be too small to estimate the statistical significance of the difference from controls (Fig. 3). In MSA, the incidence of Ta-51-positive neurons was definitely increased, but remained at a lower level as compared with ALS or WH (Fig. 3). Neuron loss in MSA is known to occur in large motor neurons but

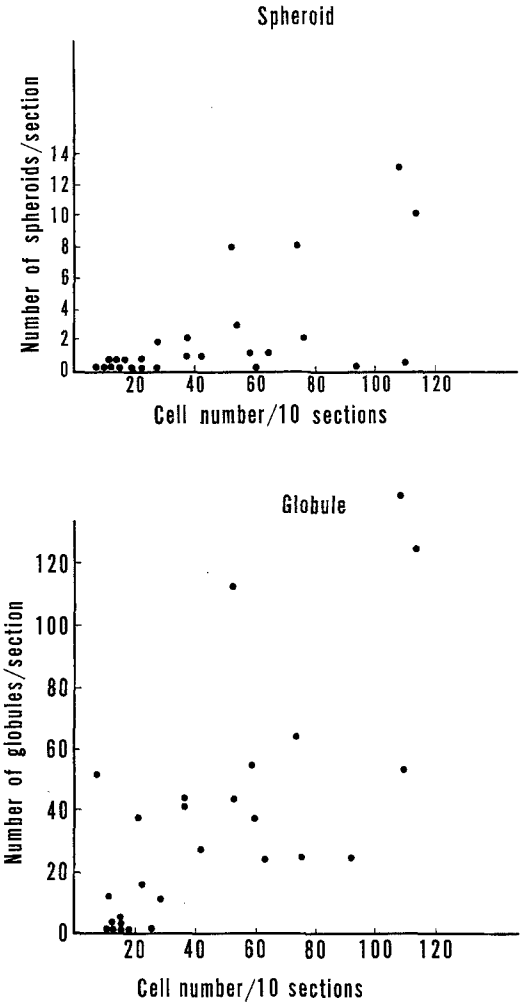

Fig. 7. Relationship between the population of spheroids and globules, and the number of the remaining large motor neurons in ALS showing a fairly good relationship. Relation coefficient was 0.54 ($P < 0.003$) and 0.699 ($P < 0.0001$), respectively

more prominently in the small neurons distributed in the intermedio-medial zone of the ventral horn which correspond mainly to the interneurons [17, 33, 38]. Although the nature of these interneurons, particularly their mode of NF metabolism and phosphorylation is not clear, NF-H phosphorylation of these neurons may possibly be different from that of large α-motor neurons. In addition, both X-BSNP and MSA take a long clinical course, suggesting that the process of neuron loss may be more chronic than ALS or WH, and the size of compartment of actively degenerating neurons may be small [32], which may influence the occurrence of intra-perikaryal phosphorylated NF-H.

According to the present concept of metabolism of the NF [20, 30, 31, 39], subunits of non-phosphorylated NF are produced in the neuronal perikarya and then the NF fragments migrate into the proximal axons and dendrites by axonal transport. In the course of these events, the assembly of NF subunits is completed and phosphorylation takes place. Recently, neurofilamentous accumulation in the motor neuron soma of ALS was morphologically documented [15, 16] and evidence was provided to suggest that axonal transport mechanisms are extensively impaired in the ALS motor neurons even in early stages [1, 3]. It can not be concluded here, but occurrence of phosphorylated NF in the motor neuron soma may be the consequence of impaired axonal transport, rather than increased abnormal phosphorylation or increased synthesis of NF in the perikarya.

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