Reactive Astrogliosis is Widespread in the Subcortical White Matter of Amyotrophic Lateral Sclerosis Brain

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Abstract. Widespread astrogliosis exists in the subcortical white matter in amyotrophic lateral sclerosis (ALS). As revealed by glial fibrillary acidic protein (GFAP) immunostaining, the gliosis has the morphological properties of an active process. It is present in the midfrontal, inferior parietal, temporal, cingulate, and occipital cortices, as well as in the motor cortex. Compared to matched regions from other neurological diseases, the gliosis in ALS does not appear to be the nonspecific result of a progressive, degenerative disease. In cell number and apparent cell size, the gliosis is comparable to that present in neurological diseases known to have white matter gliosis. Cytologically, the gliosis most closely resembles that present in cases of cerebral infarction. The basis for this similarity is unknown.

Key Words: Amyotrophic lateral sclerosis; Astrocytes; Gial fibrillary acidic protein; Motor neuron disease; Reactive gliosis; Subcortical white matter.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive paralysis of unknown etiology. While it is generally agreed that the motor neuron in the spinal cord, brainstem and the cortex is the affected cell in this disease, a few investigations have provided subtle evidence for more widespread metabolic (1, 2) and biochemical (3) changes in the brain. These latter data call for an investigation into the possibility that the pathophysiology of ALS extends beyond the motor neuron.

Glial cells have been generally ignored in investigations of ALS. Astrocytes are particularly important in view of the increasingly complex role they have recently been found to play in brain function. Astrocytes, usually thought to serve a supportive, nutritive and restorative role, may, in some neurological diseases, exacerbate or directly contribute to lesions. For example, astrocytes can convert the parkinsonism-inducing neurotoxin (MPTP) to its active metabolite (MPP+) (4, 5) and thus can contribute to the degeneration in parkinsonism. Astrocytes synthesize the endogenous excitotoxin quinolinic acid, which may contribute to the neuronal degeneration in Huntington's disease (6, 7). Astrocytes express β-amyloid precursor protein (8) and may contribute to the deposition of amyloid in Alzheimer's disease. Our own studies have uncovered a subtle and possibly specific alteration in astrocytes present in certain neurological conditions including ALS (9, 10). An extreme view generated by these examples is that changes in astrocytes precede certain neuronal dysfunctions. However, whatever the interpretation, the examples emphasize the intimate roles that astrocytes play in brain function and suggest that astrocytes should be carefully evaluated in all neurological disorders. In pursuing these issues, we used

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an antiserum to GFAP, the principal intermediate filament component of astrocytes, as a simple but sensitive monitor of the condition and distribution of these glial cells in the brain from cases of ALS. We determined that astrocytes in ALS subcortical white matter were more intensely stained for GFAP than astrocytes in matched regions from neurologically normal cortex, as judged visually (11). This study extends those observations.

MATERIALS AND METHODS

Tissue Sources: A total of 34 cases were examined, 13 cases of amyotrophic lateral sclerosis (ALS), six neurologically normal cases, and 15 cases with other neurological diseases. Two familial and eight sporadic ALS cases were obtained through the cooperation of Dr. F. H. Norris and the Pathology Department of the Pacific Presbyterian Medical Center (PPMC). Nine of these cases had clinical histories of both upper and lower motor neuron involvement. One of the cases had bulbar ALS. One sporadic case of ALS was obtained through the cooperation of Dr. H. Harrison, Humboldt Central Laboratory, Eureka, CA and one through Dr. R. Ciliak, Pathologists' Regional Laboratory, Clarksston, WA. Two additional ALS cases were generously provided by Dr. W. Tourtellotte, National Neurological Research Bank (NNRB). Veterans Administration Medical Center, Wadsworth Division, Los Angeles, CA. Six neurologically normal cases (three cases of heart disease, one case of liver disease, two cases of carcinoma) were obtained from PPMC. Neurologically diseased cases included one case each of focal cerebral infarction, seizures, meningitis and metastatic carcinoma (obtained from PPMC), four cases of Alzheimer's disease (AD) (kindly provided by Dr. R. Terry, University of California, San Diego) and cases of multiple sclerosis (MS) (two cases), cerebral infarction (multiple microinfarction, two cases), Parkinson's disease (two cases) and Pick's disease (one case) (provided by NNRB). Diagnosis was determined by clinical criteria and confirmed by pathological examinations at the source institution. For the ALS cases, ages ranged from 39-70 years (yr) (mean 59 yr), postmortem delay interval (PMDI) 2-23 hours (h) (mean eight h), time in fixative (10% formalin) <1-35 months (mean 19 months). For the neurologically normal cases, ages ranged from 50-86 yr (mean 64 yr), PMDI 5.5-58 h (mean 20 h), time in fixative <one to six months (mean five months). For the neurologically diseased cases, ages ranged from 59-94 yr (mean 78 yr), PMDI 3-24.5 h (mean 11 h), time in fixative <1-41 months (mean 14 months).

Regions Examined: Blocks of isocortex were excised from formalin-fixed brains as follows: Midfrontal cortex, approximately 10 cm caudal to the frontal pole and, from the superior temporal gyrus, halfway between the foot of the central sulcus and the temporal pole; inferior parietal cortex, 1 cm caudal and 2 cm posterior to the foot of the lateral fissure; motor cortex, 1 cm rostral to the central sulcus and 4 cm lateral to the longitudinal fissure; cingulate cortex, 1 cm superior to the corpus callosum and 6 cm caudal to the frontal pole; temporal cortex, 2 cm above the base of the temporal pole on the lateral hemisphere; occipital pole, 1 cm lateral to the occipital pole. The blocks of tissue did not include centrum semiovale or periventricular white matter.

Tissue Processing: Formalin-fixed blocks, stored at 4°C, were rinsed in a large volume of phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM Na2PO4, pH 7.4) and equilibrated through cold, aqueous graded sucrose solutions, 12% (24 h), 15% (24 h) and 18% (24-76 h) sucrose. These steps optimize tissue preservation and antigenicity (9; unpublished data). Blocks were mounted in gum tragacanth with the orientation that allowed an examination of a maximum area of gray and white matter within each tissue section. Mounted blocks were flash frozen in liquid nitrogen-cooled isopentane, stored at ~70°C and cryosectioned at 10 micrometers (µm) with three sequential sections thaw-mounted onto each slide.

Immunostaining: Gliarial fibrillary acidic protein (GFAP) was localized using an antiserum to GFAP (Biogenex Labs, San Ramon, CA). Cryosections were hydrated briefly in PBS and incubated overnight at 4°C in anti-GFAP. Sections were washed, blocked for five minutes (min) with 5% casein in PBS and stained with either fluoresceinated anti-rabbit immunoglobulins (Antibodies Inc., Davis, CA) diluted 1:75 in PBS (2 h, 22°C in the dark) or a
peroxidase anti-peroxidase system employing goat anti-rabbit immunoglobulins and peroxidase rabbit anti-peroxidase (Biogenex Labs), used according to the manufacturer's recommendations. Each tissue was stained with both peroxidase and fluorescence techniques. The chromogen used for development of the peroxidase reaction was 0.05% diaminobenzidine tetrahydrochloride in Tris buffer (pH 7.4) with 0.01% H₂O₂. Peroxidase-stained sections were dehydrated and mounted in synthetic mounting medium; fluorescein-stained sections were mounted in 70% glycerol. Slides were viewed on a Zeiss Ultraphot microscope equipped with epi-illumination and Nomarski optics.

The following stains were also performed in parallel: for cytoarchitecture, alternate slides were stained with cresyl violet, and slides from each case were stained with hematoxylin/eosin. As positive antibody controls, sections were stained with antibodies that display a binding pattern different from anti-GFAP. Two monoclonal antibodies were used: anti-neurofilament (anti-NF, which recognizes the 68 kDa and 200 kDa subunits, Biogenex Labs) and Tor 23 (hybridoma culture supernatant, diluted 1:10 in PBS), an antibody that recognizes a determinant of the neuronal cell surface (12, 13). As negative antibody controls, sections were incubated with no primary antibody; these sections verified the lack of non-specific binding of the secondary antibody. Both positive and negative controls were performed on sections adjacent or near-adjacent to the experimental section with every immunolocalization experiment. Immunostaining with anti-NF and Tor 23 employed fluorescein-conjugated anti-mouse immunoglobulins (Antibodies Inc.: 1:100 in PBS) and an avidin-biotin two step reaction for peroxidase localization of mouse immunoglobulins (ABC kit, Vector Labs, Burlingame, CA), used according to the manufacturer's specifications. The subsequent development and processing of these stained sections were as described above for anti-GFAP.

Quantitation of Stained Sections: A representative peroxidase-stained section from each of the different tissues (all cases) was scored in the subcortical white matter for the number of stained cell bodies and the apparent size of individually stained cells. This scoring was carried out by an observer who did not know the source of the tissue. For determination of cell number, the number of GFAP-stained cells in a 15 mm² area was counted in three separate fields and the average determined. For the cell size determination, GFAP-stained cells containing a visible nucleus were measured with an ocular micrometer along the longest axis of the cell and along the diameter perpendicular to that axis. The elliptical area was calculated for the apparent cell size and an average determined from three cell measurements. After these measurements were obtained, the slides were recored and the results grouped into disease categories: ALS cases, neurologically normal cases, neurologically diseased cases without known white matter gliosis, and neurologically diseased cases with known white matter gliosis. The number of cells and the cell body area were plotted relative to disease category. A statistical analysis of the means of the disease categories was performed by applying an independent, two-tailed t-test calculated with the FASTAT program on a Macintosh computer.

RESULTS

Formalin-fixed cortical specimens were processed according to a protocol devised for the detection of rare surface antigens of the human brain (9; unpublished data). Excised superficial blocks, measuring approximately 1 × 1 × 1.5 cm³, included both gray and subcortical white matter. After gradual sucrose equilibration, blocks were flash-frozen in liquid nitrogen-cooled isopentene, 10 µm cryosections cut and mounted on gelatin-coated slides, and sections stained for GFAP with immunofluorescence and immunoperoxidase techniques. Results are based on the examination of 260 slides made from a total of 34 cases, 13 ALS, six neurologically normal and 15 other neurologically diseased cases.

Glial fibrillary acidic protein staining in postmortem ALS isocortex revealed a distinctive cellular alteration within the subcortical white matter. Immunostained astrocytes were also observed in the subpial layer but did not differ from those seen in neurologically normal cortex; therefore, we focussed our investigations on the

subcortical white matter. In this region, in all ALS cases examined, there were numerous, large, intensely stained astrocytes within a background of GFAP-positive fibers (Fig. 1A). This staining profile was markedly different from that observed in matched cortical regions from neurologically normal cases where GFAP-stained astrocytes were sparse and faintly labeled in an unstained fiber background (Fig. 1B). Sections stained only with second labeling antibody alone were completely negative (Fig. 1C). Staining of near-adjacent sections with an antibody to neurofilaments revealed no obvious differences between control and ALS white matter (data not shown).

Control experiments were performed to exclude the potential contribution of confounding variables, such as tissue processing and case variables. First, by examining each tissue with both peroxidase and fluorescence techniques, we controlled for immunostaining variables caused by the peroxidase product, which relies on an enzymatic reaction. As a second control for variations in peroxidase development times, sections of ALS and each of the different conditions were stained in exact parallel on the same day. To evaluate the effects of fixation time, the same tissues from two cases were multiply processed after varying intervals (four and seven months) in fixative. To determine the potential contribution of the effects of freezer storage time, blocks were sectioned and stained and then resectioned and restained three, six and ten months later. To exclude the length of time from cryosectioning to staining as a variable, old (up to nine months) and newly cut cryosections from the same cortical block were stained. To evaluate case variables, first neurologically normal cases of varying ages (50 to 86 yr), sex and PMDI (5 to 58 h) were examined. In addition, ALS cases were matched to neurologically normal cases according to the PMDI and according to age and sex. Since ALS patients often die from respiratory insufficiency, the contribution of pulmonary dysfunction and possible hypoxia was especially considered. To control for this variable, one of the ALS cases examined had bulbar ALS and hence should have been more hypoxic than the others, two of the ALS cases had been maintained on a respirator to provide sufficient levels of oxygen for the period of time before death, and all ALS patients from PPMC in this study had been closely monitored for pulmonary function (by the pulmonary specialist at PPMC, Dr. R. J. Fallat) and had been determined not to have endured respiratory distress during most of their illness; nor did their brains at autopsy indicate changes described as typical of hypoxia (14). To evaluate hypoxia additionally, control cases were examined that had clinically diagnosed and pathologically confirmed pulmonary dysfunction. These included two cases of long-standing emphysema and four cases with end-stage cardiomyopathy, atherosclerosis, pulmonary infarction and edema. The results from testing these tissues, systematically, in exact parallel, suggested that the gliosis in ALS is not a result of any of these other variables and correlates only with the presence of the motor neuron disease.

Astrocytes stained with GFAP in ALS subcortical white matter displayed two

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Fig. 1. Glial fibrillary acidic protein (GFAP) is increased in the subcortical white matter of ALS cases (A) as compared to neurologically normal cases (B); micrographs are from immunofluorescence-stained sections of midfrontal cortex. A: Astrocytes in ALS appear enlarged, are intensely-stained and are among a stained feltwork of glial fibers. B: Immunopositive astrocytes from neurologically normal cases are relatively infrequent, small and faintly labeled, and no stained glial feltwork is apparent. C: ALS sections stained with omission of the GFAP antiserum show no specific immunostaining; bright deposits are autofluorescent lipofuscin. All sections were stained in parallel on the same day. Bar = 20 μm (A–C).
distinct morphologies in different areas. Within the middle region of the white matter, stained astrocytes had few processes and existed within a prominently stained glial fiber feltwork (Fig. 2A). In the white matter bordering the gray matter, the interface zone, astrocytes had extensive, elongated processes, and minimal glial feltwork staining (Fig. 2B). Tissue stained with no primary antibody displayed no detectable staining (Fig. 2C). These morphological features of the astrocyte profiles suggest an active process of gliosis (15) in the subcortical white matter in ALS.

To investigate the extent of the gliosis, we examined the distribution of GFAP in blocks from six different cortical areas. There was gliosis in all areas examined. Astrogliosis in the motor, midfrontal, inferior parietal, temporal and cingulate cortices was pronounced and qualitatively equal; in the occipital cortex, gliosis was somewhat less pronounced but still present (Fig. 3). The two morphological patterns of astrogliosis previously described were present in all regions.

Next, GFAP staining in ALS was compared to that in other neurological diseases. For these investigations, the midfrontal cortex was examined as a matched, non-motor cortical region; for all diseases, tissue examined did not include any visible lesion, i.e., sclerotic plaque, infarct or epileptic focus. Immunostained astrocytes were not prominent in the subcortical white matter of three of the four AD cases (Fig. 4A), although all cases did have prominent GFAP-stained clusters in the gray matter, similar to published descriptions of GFAP immunoreactivity surrounding senile plaques (16-19). In the case of Pick's disease, a disease where the white matter is known to be affected (20, 21), two different gyri from the midfrontal cortex were sampled. In the subcortical white matter of one gyrus, astrocytes were abundant at the perimeter, near the gray matter, but were rare more centrally (data not shown). In the other gyrus, approximately 2 cm away, GFAP staining revealed numerous, intensely stained astrocytes throughout the white matter (Fig. 4B). This difference in GFAP staining may correspond to the lobar and focal nature of the disease (20, 21). Moderately increased astrogliosis was observed in the white matter of two cases of multiple sclerosis (Fig. 4C). In all three of the cases of cerebral infarction examined, astrocytes were numerous, intensely stained and swollen; astrocytes with abundant processes were located in the interface zone nearer gray matter; astrocytes with fewer processes were located in the central area (Fig. 4D). White matter gliosis was not observed in cases of Parkinson's disease, meningoitis, metastatic carcinoma or seizures. Staining of GFAP within the subcortical white matter of ALS (Fig. 4E) was unlike that in the various different neurological diseases examined with the single exception of cerebral infarction, where the pattern of GFAP staining was virtually indistinguishable from that observed in ALS (compare Fig. 4D, E).

To confirm these observations, a representative set of peroxidase-stained slides from all of the cases was coded and a section from each scored for the number of stained astrocytes in the subcortical white matter and for apparent size. Figure 5

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Fig. 2. Astrocytes positive for GFAP in ALS subcortical white matter display two different morphologies; micrographs are from immunoperoxidase-stained sections of the midfrontal cortex. A: Within the more central portion of the subcortical white matter, astrocytes in ALS have few processes and lie in a background of prominently stained glial fibers. B: Within the subcortical white matter at the junction of the white and gray matter, GFAP-reactive astrocytes have numerous, extensive processes with minimal glial background staining. C: ALS cortex stained with second antibody alone displays no reaction product. Micrographs are from two ALS cases; both morphologies occur in all ALS cases and in all cortical regions examined. Bar = 20 μm (A–C).
shows that these measurements reveal a similarity in the extent of astrogliosis in ALS with that present in neurologically diseased cases with gliosis, i.e. MS, Pick's disease, and cerebral infarction. Statistically, in ALS frontal cortex the means of the number and the apparent size of astrocytes were different from those of neurologically normal cases (number, \( p = 0.02 \); size, \( p = 0.002 \)). Consistent with this result, there was no significant difference in the means between ALS cases and neurologically diseased cases with gliosis. Comparing astrocytes in the subcortical white matter of ALS frontal cortex to those in all cases grouped together without gliosis, i.e. neurologically normal cases plus neurologically diseased cases without gliosis, the means were highly significantly different (number, \( p = 0.001 \); size, \( p = 0.0001 \)).

DISCUSSION

The commonly described histopathological features of ALS are degeneration of upper and lower motor neurons, a pronounced axonal degeneration, demyelination and astrocitosis in the primary motor tracts, the latter feature comprising the "sclerosis" of ALS. We describe an astrogliosis within the subcortical white matter in all cases of ALS examined which appears distinctive for two reasons: 1) the GFAP-immunoreactive astrocytes have morphological features of a "reactive" process and 2) the astrogliosis is widespread.

Most pathological descriptions of ALS have focussed on the motor neuron. In those reports where astrocytes have been mentioned, reactive astrocytes have been described in association with degenerating motor neurons (22–26). Within the context of other findings, one report briefly noted the presence of reactive astrocytes within the subcortical white matter but only in the motor cortex (27). Reactive astrocytes have been described within the gray matter of the motor cortex of some ALS cases (27); this finding we have confirmed and extended to multiple cortical areas (11). Two studies reported neuropathological examinations outside the motor system; each study described widespread degenerations in ALS, although neither focussed on astrocytes. One of these reports showed extensive Marchi degeneration within the subcortical white matter (28). Unlike our observations, the degenerating areas were described as fanning out from the primary motor cortex, with little or no degeneration in other cortical areas, e.g. temporal cortex, where we found an astrocitotic response equal to that in motor cortex. The second study described extensive neuronal depletion and astrocitosis in the thalamus, globus pallidus and caudate nucleus and astrogliosis in the corpus callosum (29). Not all cases examined in that study displayed these changes. Some of the changes may, therefore, have been attributable to other complications in those patients. There is, therefore, little in neuropathological descriptions of ALS that hint at the widespread astrogliosis in the subcortical white matter that we have found.

In any slowly progressive brain disorder, one might expect secondary, or "non-specific" alterations, particularly among astrocytes, a plastic neuroglial cell. Since

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Fig. 3. Astrogliosis is present in the subcortical white matter of multiple cortical regions of ALS brain. A: Motor cortex; B: midfrontal cortex; C: cingulate cortex; D: temporal cortex; E: inferior parietal cortex; F: occipital cortex. All micrographs are from within the subcortical white matter of fluorescent-stained sections. Tissues are from the same ALS case processed and stained in parallel. The staining pattern was similar both in other ALS cases and in sections stained with peroxidase. Bar = 30 μm (A–F).
astrogliosis was widespread in the subcortical white matter in all of the ALS cases we examined, we tested the possibility that it represented a general response to neurodegeneration or axonal degeneration by comparing ALS cases to matched tissues from other neurologically diseased states. Where multiple cases of the same disorder were tested, there was a constancy of astrocytic staining pattern within disorders, especially within the subcortical white matter. While this was particularly obvious in the 13 ALS cases, it was also seen in three of the four cases of AD and the two cases each of MS, Parkinson’s disease and multiple microinfarction. This finding suggests that astrocytes may respond differently to specific disease conditions. By comparing ALS cases with cases of those disorders typified by a dramatic and widespread cortical atrophy, namely AD and Pick’s disease, we found that the pattern of gliosis in ALS appeared distinctive.

Of the eight different neurological disorders examined, the GFAP-staining profile in the subcortical white matter of ALS most resembles that present in cerebral infarction. The pathological similarities in astrocytes between ALS and cerebral infarction include the presence of astrocytes within the subcortical white matter, their two different morphologies and the regional distribution of each morphology. These two disorders are obviously very different clinically and, therefore, the basis for this similarity is not clear. One possibility is that since ALS patients often die with end stage, respiratory failure, hypoxia may produce an astrocytosis similar to that in cerebral infarction. This seems unlikely because a local rather than a generalized hypoxia often occurs in focal cerebral infarction. Also, many ALS patients have not been found to be hypoxic during the course of their illness as determined by arterial blood gas measurements (30). Finally, hypoxia also seems an unlikely explanation for the gliosis in the subcortical white matter because the control brains of the cases we examined with severe lung dysfunction did not display intense gliosis in this subcortical area.

Another possible explanation for our finding of subcortical astrogliosis in ALS may be related to the metabolic function of astrocytes. Among their housekeeping chores, astrocytes have a principal task of regulating glutamate metabolism within the brain. Like neurons, astrocytes are depolarized by glutamate via pharmacologically distinct glutamate receptors (31–34). In tissue culture (35) and in vivo (36) astrocytes have a higher affinity for glutamate than the neurons they surround. They actively convert glutamate to glutamine via two enzymatic pathways, glutamine synthetase (37) and glutamate dehydrogenase, found in many, but not all, astrocytes (38). How does a role in glutamate metabolism relate to neurological dysfunction? There is evidence in tissue culture studies that astrocytes reduce the potency of glutamate as an excitotoxin (39). In cerebral infarction glutamate-induced damage is strongly implicated (40, 41) and the proliferation of astrocytes may ameliorate

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Fig. 4. Glial fibrillary acidic protein staining in Alzheimer’s disease (AD) (A), Pick’s disease (B), multiple sclerosis (MS) (C), cerebral infarction (D) and ALS (E). A: AD displays little GFAP staining in the central white matter. B: In Pick’s disease, one of the two gyri examined reveals numerous astrocytes that are strongly GFAP positive. C: MS reveals moderate gliosis. D: Focal infarction shows extensive gliosis with intensely stained astrocytes that are enlarged and lie in a bed of stained fibers. The area examined was not the region of infarction. This profile matches that observed in the two cases of multiple microinfarction in this study. E: ALS gliosis is virtually indistinguishable from that present in cerebral infarction (D). All micrographs are from the subcortical white matter of the midfrontal cortex. Bar = 20 μm (A–E).

Fig. 5. The subcortical white matter of ALS is similar to that of neurological diseases with white matter gliosis in the number (A) and apparent size (B) of GFAP-stained astrocytic cell bodies. C, Neurologically normal cases (n = 5); NC, neurologically diseased cases (n = 9); NgC, neurologically diseased cases with white matter gliosis (n = 6); ALS (n = 8); ALSm, ALS motor cortex (n = 7); ALSt, ALS temporal cortex (n = 4). All values are from subcortical white matter of midfrontal cortex except as indicated. Bars: mean ± SEM. Mean values in the ALS category are significantly different from those in C (number, p = 0.02; size, p = 0.002). Mean values of ALS relative to those in C and NC grouped together are significantly different (number, p = 0.001; size, p = 0.0001). * Emphysema (from the inferior parietal lobe and not included in averages or statistics). Several values of the C population are higher than those obtained from the NC category, possibly due to the presence of cerebrovascular disease, confirmed in four of the five cases.
damage. In ALS, there is some suggestive evidence implicating glutamate as an excitotoxin (42-47). Our description of widespread subcortical white matter astrogliosis is morphological evidence in line with other data implying a global pathophysiology in ALS (1-3). If this pathophysiology is due to a specific insult as has recently been discussed (48, 49), it must be one to which the motor neuron is selectively vulnerable.

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