DECREASED GLUTAMATE TRANSPORT BY THE BRAIN AND SPINAL CORD IN AMYOTROPHIC LATERAL SCLEROSIS

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Abstract *Background.* Amyotrophic lateral sclerosis (ALS) is a chronic degenerative neurologic disorder characterized by the death of motor neurons in the cerebral cortex and spinal cord. Recent studies have suggested that the metabolism of glutamate, a potentially neurotoxic amino acid, is abnormal in patients with ALS. We hypothesized that the high-affinity glutamate transporter is the site of the defect.

Methods. We measured high-affinity, sodium-dependent glutamate transport in synaptosomes from neural tissue obtained from 13 patients with ALS, 17 patients with no neurologic disease, and 27 patients with other neurodegenerative diseases (Alzheimer's disease in 15 patients and Huntington's disease in 12 patients). The groups were comparable with respect to age and the interval between death and autopsy. Synaptosomes were prepared from spinal cord, motor cortex, sensory cortex, visual cortex, striatum, and hippocampus. We also measured sodium-dependent transport of γ-aminobutyric acid and phenylalanine in the synaptosomal preparations.

Results. In patients with ALS, there was a marked decrease in the maximal velocity of transport for high-affinity glutamate uptake in synaptosomes from spinal cord (−59 percent, $P<0.001$), motor cortex (−70 percent, $P<0.001$), and somatosensory cortex (−39 percent, $P<0.05$), but not in those from visual cortex, striatum, or hippocampus. The affinity of the transporter for glutamate was not altered. No abnormalities in glutamate transport were found in synaptosomes from patients with other chronic neurodegenerative disorders. The transport of γ-aminobutyric acid and phenylalanine was normal in patients with ALS.

Conclusions. ALS is associated with a defect in high-affinity glutamate transport that has disease, region, and chemical specificity. Defects in the clearance of extracellular glutamate because of a faulty transporter could lead to neurotoxic levels of extracellular glutamate and thus be pathogenic in ALS. (N Engl J Med 1992;326: 1464-8.)

AMYOTROPHIC lateral sclerosis (ALS) is a chronic progressive disease of unknown pathogenesis that is characterized by the selective degeneration of upper and lower motor neurons. It is the cause of death in 1 in 1000 people. As a result of recent reports of abnormal glutamate metabolism in ALS, we[1] and others[2] have postulated a role for this amino acid in the pathophysiologic process of the disease. Glutamate, the primary excitatory neurotransmitter in the brain, can exert specific neurotoxic effects through a well-described cascade of cationic and second-messenger events and can induce neuronal degeneration in vivo and in vitro.[3,4] Abnormalities of glutamate metabolism are thought to have a role in the pathophysiologic processes of two chronic degenerative diseases, olivopontocerebellar atrophy[5,6] and Huntington's disease.[7,8] Analogous exogenous excitotoxins may also cause neuronal degeneration. The glutamate analogue β-*N*-oxalyl-L-α,β-diaminopropionate, which is derived from the chickling pea *Lathyrus sativus*, is probably responsible for the degeneration of upper motor neurons in lathyrism.[9] A related toxin, β-*N*-methylamino-L-alanine, found in the cycad seed, has neurotoxic properties in vitro.[3,10] On the basis of both such examples of neurotoxicity and earlier, limited studies of amino acids in specimens of brain and spinal cord from patients with ALS,[2,11-13] we hypothesized that the metabolism of the excitatory amino acids might be altered in patients with ALS. In support of this hypothesis are the increased cerebrospinal fluid concentrations of glutamate and aspartate in patients with ALS early in their disease.[1,14] Another finding of interest is that the administration of the branched-chain amino acids leucine, isoleucine, and valine, which can increase the oxidation rate of synaptosomal glutamate,[15] slowed the progression of weakness in patients with ALS in one trial,[16] but not in a second trial.[17]

The primary mechanism for the inactivation of glutamate and aspartate is their removal from the extracellular space by a sodium-dependent transport system in astrocytes and neurons.[18,19] This transport system has both high-affinity and low-affinity carriers for glutamate and aspartate. The low-affinity glutamate carrier subserves general metabolic activities. The high-affinity carrier is a component of the glutamate neurotransmitter system and is responsible for the clearance of neurotransmitter glutamate from the synaptic cleft.[18,19] This carrier cannot distinguish between glutamate and aspartate. The inhibition of glutamate transport has been shown experimentally to be toxic to neurons, probably because of the persistent elevation of extracellular glutamate.[20] One possible mechanism for the elevated cerebrospinal fluid concentrations of glutamate and aspartate in patients with ALS could be deficient transport into cells. To test this possibility, we studied the glutamate-transport system in brain and spinal-cord tissue obtained post mortem from patients with ALS, control patients with no neurologic disease, and patients with the neurodegenerative disorders Alzheimer's disease and Huntington's disease.

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METHODS

Tissue Specimens

Specimens of spinal cord and brain were obtained at autopsy from 13 patients with strictly defined sporadic ALS,[1] 17 patients with no neurologic disease, and two groups of patients with other

types of degenerative neurologic disease — 15 patients with Alzheimer's disease and 12 patients with Huntington's disease. Tissue from all regions of the central nervous system was not available for every patient. The criteria for the diagnosis of ALS were the presence of both upper- and lower-motor-neuron signs, a definite history of progression, the absence of sensory abnormalities, normal nerve-conduction velocities, and electromyographic evidence of diffuse denervation.[1] Patients were excluded if they had unexplained changes in bowel or bladder function, or anatomical, metabolic, or toxic disorders that could mimic ALS, such as endocrine abnormalities, hexosaminidase A deficiency, lead intoxication, myelopathy, or peripheral neuropathy. The patients with no neurologic disease all died of acute myocardial infarction. The diagnosis of Alzheimer's disease or Huntington's disease was made on the basis of standard clinical and neuropathological criteria.

The specimens from the patients with ALS were obtained from the Brain Resource Center, Neuropathology Laboratory, Johns Hopkins Hospital. The specimens from patients with diseases other than ALS were obtained from the same laboratory, the National Disease Research Interchange in Philadelphia, or the National Neurological Research Specimen Bank in Los Angeles (kindly provided by Dr. W. Tourtellotte). All diagnoses were confirmed by neuropathological examination of tissue. All specimens were dissected according to established anatomical criteria,[21] rapidly frozen on dry ice, and stored at −70°C until assay.

Amino Acid Transport

High-affinity sodium-dependent glutamate transport was measured[22,23] in specimens of brain and spinal cord obtained at autopsy and stored at −70°C until use. Tissue (0.5 to 1 g) was homogenized in 10 to 20 μl of a solution of 0.32 mol of buffered sucrose per liter (0.05 mol of TRIS buffer per liter at pH 7.4) with a Teflon–glass homogenizer and then centrifuged at 1000×*g* for 10 minutes. The supernatant was collected and centrifuged at 20,000×*g* for 30 minutes. This process was repeated twice. The crude synaptosomal pellet was finally resuspended in Krebs buffer (pH 7.4) at a final concentration of 1.5 mg of protein per milliliter. Duplicate assays were performed in a final assay volume of 1 ml, with approximately 150 μg of protein, 0.2 μCi of [^{3}H]glutamate (54.7 Ci per millimole, New England Nuclear), and 10 μmol of glutamate per liter, or various concentrations of glutamate (1 to 50 μmol per liter) for kinetic analysis. Low-affinity transport was measured with 50 to 1000 μmol of glutamate per liter. The concentration of endogenous glutamate remaining in the washed synaptosomes was less than 0.5 μmol per liter, as measured by reverse-phase high-performance liquid chromatography with fluorometric detection.[24] The assay mixtures were incubated for four minutes at 37°C, and transport was terminated by placing the mixtures on ice and then centrifuging them at 14,000×*g* for five minutes at 4°C. The synaptosomal-tissue pellets were dissolved (Solvable, New England Nuclear), and the trapped radioactivity was quantified by scintillation spectroscopy. The assay was linear under these conditions, and less than 5 percent of added glutamate was metabolized. Insufficient tissue was available for kinetic studies from some patients. To correct for sodium-independent binding or transport, sodium-free blanks were used. The uptake under sodium-free conditions was less than 10 percent of that in the presence of sodium. In all uptake assays, specimens from patients in each group (those with ALS, those with other degenerative neurologic diseases, and those without neurologic disease) were assayed simultaneously. Results from tissue that did not exhibit sodium-dependent transport were not included (thus, specimens from 1 of 13 patients with ALS and from 2 of the 44 control patients were excluded); most of the tissues excluded had been stored for more than five years. Kinetic analysis revealed linear plots, consistent with the measurement of a high-affinity carrier at the substrate concentrations tested.

In samples of motor cortex from three patients with ALS, inhibition constants for dihydrokainate and DL-threo-3-hydroxyaspartate, which inhibit the uptake of glutamate, were determined by measuring glutamate uptake in the presence of 10 μmol of inhibitor per liter. The inhibition constants were calculated as described by Cheng and Prusoff.[25]

The uptake of γ-aminobutyric acid was measured as described by Hardy et al.[26] with slight modifications. Synaptosomes were prepared as described above, and aliquots (150 μg of protein) were added to microfuge tubes along with 0.1 μCi of [^{3}H]γ-aminobutyric acid (200 Ci per millimole, New England Nuclear) in Krebs buffer, for a final assay volume of 1 ml. The final concentration of γ-aminobutyric acid was 1 μmol per liter. The mixtures were incubated for 10 minutes at 37°C, and transport was terminated by centrifugation (14,000×*g* for 5 minutes at 4°C). Active, sodium-dependent uptake was calculated after the values obtained from matched samples assayed with sodium-free Krebs buffer had been subtracted.

The uptake of phenylalanine was measured as described by Benjamin et al.[27] Aliquots of synaptosomes (150 μg) were added to microfuge tubes containing 0.2 μCi of [^{14}C]phenylalanine (499 mCi per millimole, New England Nuclear) and incubated for 15 minutes at 37°C. The uptake was terminated by centrifugation (14,000×*g* for five minutes at 4°C).

Kinetic and Statistical Analysis

The kinetic constants were estimated by a nonlinear iterative computer program (SigmaPlot 4.1, Jandel Scientific, Corta Madera, Calif.) designed to fit and graph the equation $v = (V_{max} \times s)/(K_m + s)$, where v is the velocity of transport, V_{max} is the maximal velocity of transport, s is the substrate concentration, and K_m is the Michaelis constant. Inhibition constants were calculated with a computer program.[28] Statistical analysis of data was performed by analysis of variance and t-tests with two-tailed comparisons. All P values of less than 0.05 were considered to indicate significance.

Results

The characteristics of the patients whose tissue was studied and the intervals between death and autopsy and between autopsy and analysis are shown in Table 1. There were no significant differences in these intervals between the groups.

We were able to measure high-affinity, sodium-dependent glutamate transport in synaptosomal preparations obtained at autopsy up to 15 hours after death, in agreement with previous studies.[23] The rate of sodium-dependent transport was markedly depressed in patients with ALS (Fig. 1) when measured at subsaturating concentrations of substrate. As compared with patients with no neurologic disease, in patients with ALS the uptake of glutamate was decreased by 66 percent in cervical spinal cord ($P<0.01$), 47 percent in motor cortex ($P<0.01$), and 38 percent in somatosensory cortex ($P<0.05$). By contrast, there was no significant difference in high-affinity glutamate transport in the visual cortex between patients with ALS and patients with no neurologic disease.

Kinetic analysis of the high-affinity carrier was performed to determine whether the reductions in uptake were attributable to decreases in the affinity of the

Table 1. Clinical Characteristics of the Patients with ALS and Control Patients.*

Group	Men/Women	Age at Death	Time from Death to Autopsy	Time from Autopsy to Analysis
		yr	*hr*	*mo*
ALS	11/2	62±8	7.5±5.5	30±26
No neurologic disease	12/5	67±16	10.4±4.4	41±25
Alzheimer's disease	9/6	76±8	9.2±2.8	37±34
Huntington's disease	7/5	57±12	10.3±3.0	31±21

*Plus–minus values are means ±SD.

carrier (K_t) or in the maximal velocity of transport (V_{max}), a measure whose value is indicative of the relative density of carriers. Lineweaver–Burk analysis of glutamate-transport data from specimens of brain and spinal-cord tissue from each patient revealed that the abnormal transport was due primarily to a change in the V_{max} for high-affinity glutamate uptake. In kinetic experiments in which paired samples of spinal-cord tissue were assayed, the analysis always revealed distinctly different saturation kinetics between the patients with ALS and control patients; in the example shown in Figure 2, the control patient had a K_t of 29 μmol per liter and a V_{max} of 1.7 nmol per minute per milligram of protein, and the patient with ALS had a K_t of 35 μmol per liter and a V_{max} of 0.11 nmol per minute per milligram of protein. The mean V_{max} and K_t values for all groups are shown in Figure 3. In the patients with ALS, as compared with the patients with no neurologic disease, the mean V_{max} for glutamate uptake was decreased by 59 percent in the spinal cord ($P<0.001$), 70 percent in the motor cortex ($P<0.001$), and 39 percent in somatosensory cortex ($P<0.05$). In paired comparisons of different regions of the central nervous system in patients with ALS, the high-affinity transport in motor cortex was disproportionately lower than that in somatosensory cortex ($P<0.05$). Glutamate transport was not decreased in samples of striatum and hippocampus from patients with ALS. There was no change in the K_t in any region examined. There was also no relation between abnormalities in transport and the interval between death and autopsy, the length of time the specimens were stored, the age at death, or the duration of disease. Low-affinity glutamate transport was similar in motor cortex from both the patients with ALS and the

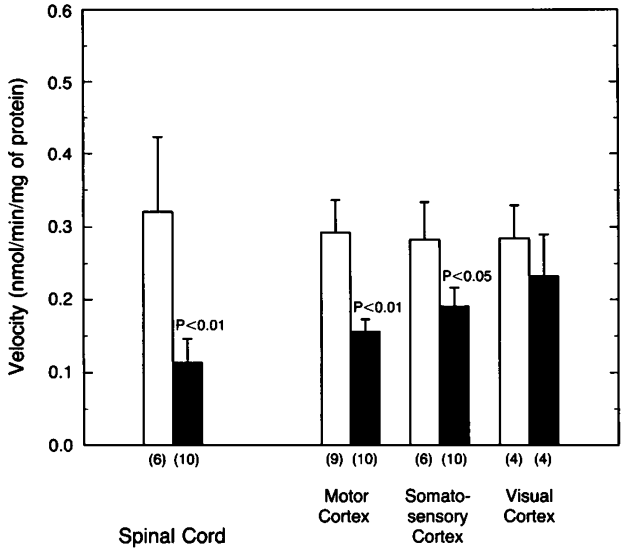

Figure 1. Mean (±SE) Uptake of [^{3}H]Glutamate by Synaptosomes Obtained from Specimens of Spinal Cord and Cortex from Patients with ALS (Solid Bars) and Patients with No Neurologic Disease (Open Bars).

These studies used subsaturating concentrations of glutamate (10 μmol per liter). Values in parentheses are the numbers of individual tissue specimens.

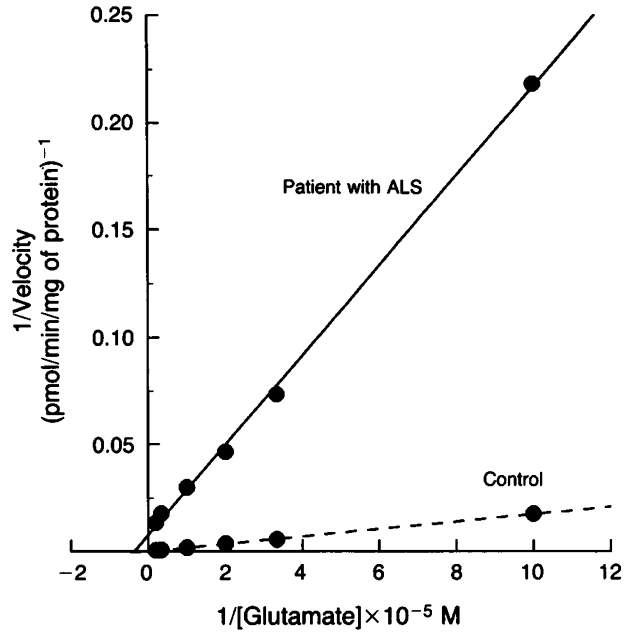

Figure 2. Lineweaver–Burk Plot of High-Affinity Glutamate Transport into Synaptosomes Obtained from Spinal-Cord Tissue from a Control Patient with No Neurologic Disease and a Patient with ALS.

The two studies were performed at the same time.

patients with no neurologic disease: in five patients with ALS, the mean (±SE) K_t was 100±28 μmol per liter and the mean (±SE) V_{max} was 2.0±0.2 nmol per minute per milligram of protein, and in five patients with no neurologic disease, the K_t was 80±16 μmol per liter and the V_{max} was 2.3±0.4 nmol per minute per milligram of protein.

To assess the specificity of abnormal glutamate transport in ALS, we examined high-affinity transport in samples of motor cortex and additional regions of the central nervous system from patients with two other chronic degenerative neurologic disorders — Alzheimer's disease and Huntington's disease. As shown in Figure 3, the results in spinal cord, cortical, striatal, and hippocampal tissue from the patients were similar to those in patients with no neurologic disease.

To assess whether the transport defect in ALS was selective for glutamate, we examined two other sodium-dependent transport systems: phenylalanine and γ-aminobutyric acid. The transport of γ-aminobutyric acid and phenylalanine in motor cortex was similar in tissues from patients with ALS and patients with no neurologic disease (Fig. 4).

Studies in animals have suggested that there are at least two pharmacologically distinct high-affinity sodium-dependent glutamate transporters, as suggested by the different effects of dihydrokainate, a competitive inhibitor of glutamate transport,[29] in different regions of the brain. To assess pharmacologically the nature of the high-affinity glutamate carrier, we studied the inhibitory effects of both dihydrokainate and DL-threo-3-hydroxyaspartate (another competitive inhibitor)[30] in motor cortex. We found that DL-threo-3-hydroxyaspartate inhibited high-affinity glutamate

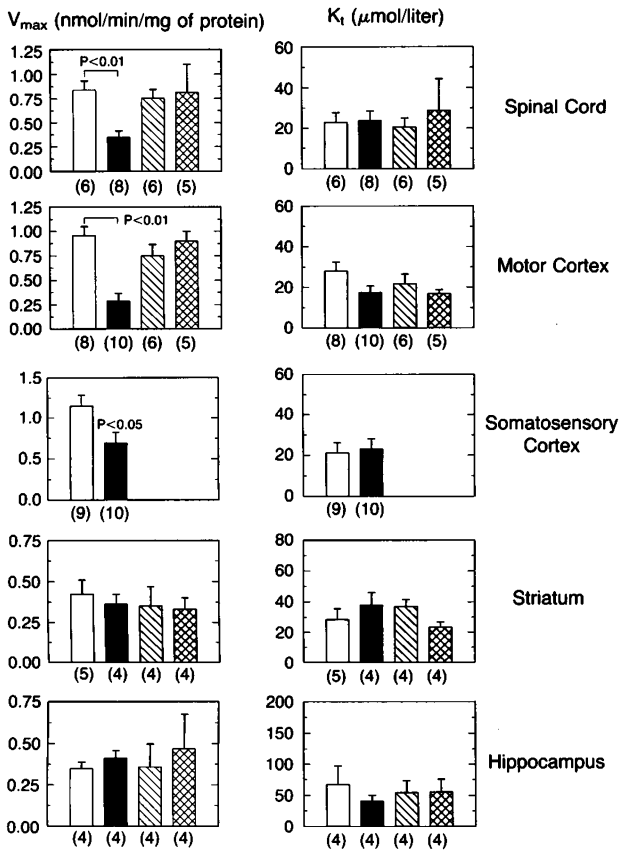

Figure 3. Mean (±SE) V_{max} and K_t Values of High-Affinity Glutamate Transport in Tissue from Patients with No Neurologic Disease (Open Bars), Patients with ALS (Solid Bars), Patients with Alzheimer's Disease (Hatched Bars), and Patients with Huntington's Disease (Crosshatched Bars).

In these experiments, high-affinity glutamate uptake was measured over a range of substrate concentrations (1 to 50 μmol per liter) for each tissue sample. Values in parentheses are the number of patients from whom tissue samples were obtained for testing.

transport to the same extent in motor cortex from both patients with no neurologic disease (inhibitor constant, 30 μmol per liter) and patients with ALS (inhibitor constant, 50 μmol per liter). Similar results were found with dihydrokainate.

Discussion

The decreased glutamate transport in spinal cord and brain tissue from patients with ALS is a neurophysiologic abnormality that has important pathophysiologic implications. Numerous studies have demonstrated that glutamate and its structural analogues can have short-term or long-term toxic effects on cortical neurons and motor neurons.[3,10,31,32] Previous studies that demonstrated elevated concentrations of glutamate and aspartate in the cerebrospinal fluid[1,14] led to our hypothesis of inefficient clearance of glutamate from the extracellular space by the high-affinity glutamate transporter. Defective clearance of glutamate could result in the exposure of neurons to abnormally high concentrations of glutamate. The reason cells die after exposure to glutamate and glutamate analogues has not been ascertained, but it probably involves the entry of calcium into the cells and calcium-mediated events.[3,4] The metabolic events initiated by increases in intracellular calcium levels can kill cells by activating a variety of intracellular enzymes (protein kinase C, calpain I, xanthine oxidase, and phospholipase) as well as by triggering further release of glutamate.[3]

The defect in glutamate transport appears to be restricted to the V_{max}, or density, of carrier proteins and is regionally selective. Since the affinity of the high-affinity carrier was normal in patients with ALS, and since the competitive inhibitors had the same effects in tissues from both patients with ALS and control patients, it is unlikely that the transport protein itself is altered in ALS. The regional defect in glutamate transport tends to parallel the known pathological changes in ALS: prominent loss of alpha motor neurons in the spinal cord and loss of upper motor neurons in the motor cortex, but a limited loss of cells in the somatosensory cortex.[33] It is notable that there are no reported morphologic changes in the visual cortex, striatum, or hippocampus in patients with ALS.

The transport of glutamate was normal in spinal-cord tissue and tissue from multiple regions of the brain in patients with Alzheimer's disease and Huntington's disease. Although decreases in the uptake of glutamate, as measured indirectly by D-aspartate binding, have been reported in patients with Alzheimer's disease,[34] we found no abnormality of glutamate uptake in such patients on direct measurement. This finding suggests that in certain regions of the brain, the changes in glutamate-transport kinetics in ALS are relatively disease-specific. Furthermore, the function of other sodium-dependent transport systems

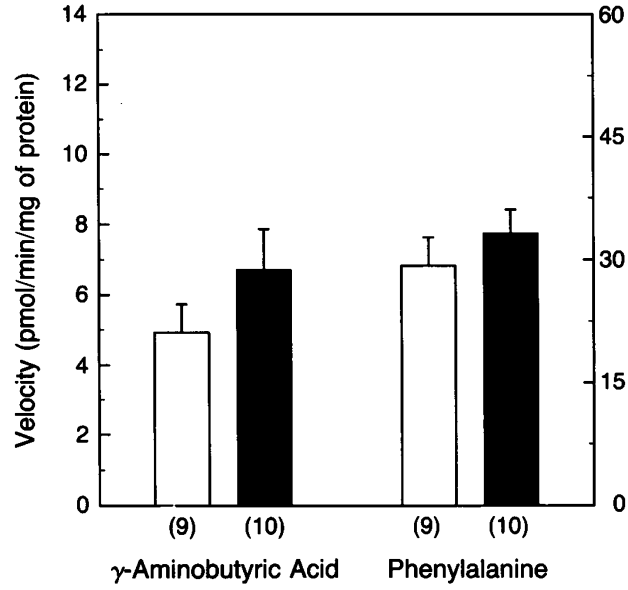

Figure 4. Mean (±SE) Velocity of Sodium-Dependent Transport of γ-Aminobutyric Acid and Phenylalanine in Motor Cortex from Patients with No Neurologic Disease (Open Bars) and Patients with ALS (Solid Bars).

Values in parentheses are the numbers of patients studied. None of the differences were statistically significant.

was normal in tissue from patients with ALS, suggesting that the defect is relatively specific for the glutamate carrier.

The cause of the defect in glutamate transport is not clear, but there are numerous possible explanations. One is the loss of intrinsic glutamatergic neurons in certain regions of the brain. The reduction in high-affinity uptake in the spinal cord could reflect the loss of corticospinal fibers. However, the large reduction in uptake in the motor cortex cannot be readily explained by the loss of pyramidal neurons, since they constitute a small minority of glutamatergic neurons in cortex and form presynaptic terminals primarily in the spinal cord or brain stem. Another possibility is selective abnormalities in astrocytes, which like neurons have a high-affinity glutamate-transport system and can take up glutamate from the synaptic cleft.[18,19] A third possibility is decreased production or increased degradation of transporter protein in either of these cell types. Once the transporter is cloned, some of these possibilities may be eliminated. Alternatively, the uptake defect could be due to some other abnormality, such as decreased intracellular glutamate metabolism, which can secondarily alter transporter kinetics,[22] or down-regulation of glutamate uptake in presynaptic terminals in response to death and loss of postsynaptic upper and lower motor neurons. However, down-regulation of glutamate uptake has not been found in animal models of excitotoxicity in the striatum.[35]

These results do not explain the selective loss of motor neurons. Motor neurons possess both *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors,[36-38] as do other neurons, so that abnormalities in synaptic glutamate concentrations cannot explain the selective loss of motor neurons. The proportion and subtypes of motor-neuron NMDA and non-NMDA glutamate receptors are not known, but cultured motor neurons are susceptible to toxic effects mediated selectively by non-NMDA glutamate receptors[38] (and unpublished observations). Whatever the mechanism for selective vulnerability, the results of this study provide important evidence for abnormal cellular metabolism of glutamate in patients with ALS.

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