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REGULAR PAPER

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Excitatory amino acid transporter 1 and 2 immunoreactivity in the spinal cord in amyotrophic lateral sclerosis

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Abstract The spinal cord of 20 patients with amyotrophic lateral sclerosis (ALS) and 5 patients with lower motor neuron disease (LMND) were investigated immunohistochemically using anti-human excitatory amino acid transporter 1 (EAAT1) and EAAT2 antibodies which are the astrocytic transporters. The purpose of the study was to examine relationships between EAAT1 and EAAT2 immunoreactivity and degeneration of anterior horn neurons. Specimens from 20 patients without any neurological disease served as controls. In controls, spinal cord gray matter was densely immunostained by antibodies, whereas the white matter was generally not immunostained. In motor neuron disease (MND) patients, EAAT1 immunoreactivity was relatively well preserved in the gray matter despite neuronal loss of anterior horn cells. On the other hand, EAAT2 immunoreactivity in anterior horns correlated with the degree of neuronal loss of anterior horn cells: in the patients with mild neuronal depletion, anterior horns were densely immunostained by the antibody, whereas in the patients with severe neuronal loss, EAAT2 expression was markedly reduced. Degenerated anterior horn cells frequently showed a much denser EAAT1 and EAAT2 immunoreactivity around the surface of the neurons and their neuronal processes than that observed in normal-appearing neurons. There was no difference in the expression of EAAT1 and EAAT2 immunoreactivity between LMND and ALS patients. These findings suggest that in the early stage of degeneration of anterior horn cells, EAAT1 and EAAT2 immunoreactivity is preserved in the astrocytic foot directly attached to normal-appearing neurons, whereas levels of EAAT1 and EAAT2 protein rather increase in the astrocytic foot directly attached to degenerated anterior horn neurons; the latter effect most probably reduces the elevated glutamate level, compensates for the reduced function of astroglial glutamate transporters, or represents a condensation of EAAT1 and EAAT2 immunoreactivity secondary to loss of neurites and greater condensation of astrocytic processes. Thus, we demonstrate a difference in EAAT1 and EAAT2 immunoreactivity in different stages of progression in ALS, as a feature of the pathomechanism of this disease.

Key words Amyotrophic lateral sclerosis · Motor neuron disease · Excitatory amino acid · Astroglial glutamate transporter · Spinal cord

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Introduction

Multiple pathogenic mechanisms have been proposed as possible causes of motor neuron degeneration in sporadic and familial amyotrophic lateral sclerosis (ALS). Neuronal excitotoxicity from excessive extracellular glutamate is one of these potential candidates [24, 25, 27–29]. Glutamate released from the presynaptic terminals is rapidly removed from the synaptic cleft by a sodium-dependent glutamate transporter in astrocytes and neurons to maintain the extracellular glutamate concentration below an excitotoxic level, and to protect neurons from glutamate excitotoxicity in normal circumstances [7, 8, 10, 17]. The reuptake of glutamate is critical, as an elevated synaptic glutamate level results in postsynaptic neuronal death through excitotoxic mechanisms [6]. Increased concentrations of glutamate in the cerebrospinal fluid early in the clinical course of ALS [21–23, 30] and decreased glutamate transport in patients with sporadic ALS [24, 25, 27–29] are possibly related to this excitotoxicity, although there is controversy as regards the levels of fasting plasma glutamate [18, 20] and excitatory amino acids in the CSF in motor neuron disease (MND) [8, 22, 23].

Recently, five different glutamate transporters have been identified and immunohistochemically localized to

distinct regions of the central nervous system [26]. Excitatory amino acid transporter 1 (EAAT1) (GLAST/GluT-1) [31] and EAAT2 (GLT-1) [19, 26] are astroglial-type transporters in the cerebrum and cerebellum, whereas EAAT3 (EAAC1) and EAAT4 are neuron-type transporters [11], and expression of EAAT5 is relatively retina specific [3]. Experimentally, the inhibition of glutamate transport has been shown to be neurotoxic, presumably due to the persistent elevation of extracellular glutamate [13]. Moreover, knockout of each glutamate transporter selectively and specifically reduces the protein expression and function of glutamate transporters; the loss of functional glutamate transport produces elevated extracellular glutamate levels, neurotoxicity, and progressive paralysis [28].

However, little is known about expression of EAAT1 and EAAT2 immunoreactivity in the human and ALS patient spinal cord [9, 15]. We carried out an immunocytochemical studies using anti-human EAAT1 and EAAT2 antibodies in the spinal cords of patients with MND to examine relationships between EAAT1 and EAAT2 immunoreactivity and degeneration of anterior horn neurons.

Materials and methods

We investigated the lumbar spinal cords (L1–5) of 20 patients with clinically and pathologically diagnosed sporadic ALS (ages 41–83 years, average 64.1 years) and 5 patients with clinically and pathologically confirmed lower MND (LMND) (ages 49–76 years, average 66.8 years) who had no upper motor neuron or corticospinal tract involvement. Lumbar spinal cords (L1–5) from 20 patients who had no neurological disease served as controls. Their ages ranged from 35 to 81 years (average 64.2 years). Autopsies of all 45 individuals were performed within 6 h after death. All the spinal cords were routinely fixed for 2 weeks with 10% buffered formalin and embedded in paraffin.

Antibody to EAAT1 and EAAT2

Monoclonal antibodies to the EAAT1 and EAAT2 have been used. The antibodies recognize a recombinant protein corresponding to the C-terminal domain of EAAT1 and EAAT2, respectively [2]. The generation of the EAAT2 antibody is described in full elsewhere [15]. Immunoblotting and immunocytochemical analyses have confirmed that the two primary antibodies used do not cross-react with any of the other four EAATs [3, 7, 27].

Immunocytochemistry for EAAT1 and EAAT2 protein

Alternating serial transverse sections (4 µm) of formalin-fixed, paraffin-embedded lumbar spinal cords (L1–5) were stained with hematoxylin and eosin (H&E) and immunostained for EAAT1 and EAAT2 expression. The latter was done with a mouse monoclonal antibody against human EAAT1 and EAAT2 (Novocastra, Newcastle, UK), and a polyclonal antibody to glial fibrillary acidic protein (GFAP; Dako) using the avidin-biotin-immunoperoxidase complex (ABC) method. Briefly, after deparaffinization and quenching of endogenous peroxidase with H_2O_2, the sections were incubated overnight (12 h for EAAT1 and 48 h for EAAT2) at 4 °C with primary antibody (diluted to 1:40 for EAAT1, 1:20 for EAAT2 and 1:1,000 for GFAP with a mixture of 50 mM TRIS buffer pH 7.6 and 150 mM NaCl). A microwave procedure was used for EAAT1 and EAAT2 immunostaining before overnight incubation. Normal horse serum served as the blocking reagent and sections from which the primary antibody was omitted served as reaction controls. Bound anti-EAAT1, -EAAT2, and -GFAP antibodies were visualized with the horse anti-mouse IgG Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.) following the manufacturer's recommendations. 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was the final chromogen.

The sections from patients and controls were processed in parallel to minimize technical variability. Assessment of the immunostaining intensities was performed by visual inspection. The evaluators were blinded to avoid possibility of observer bias.

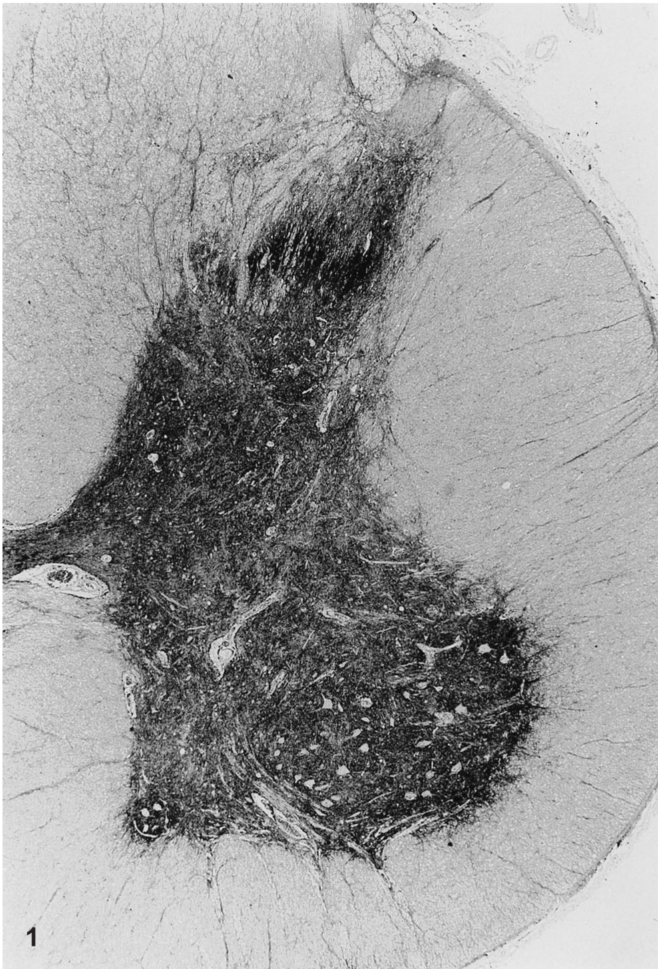
Semiquantitative analyses were performed on sections subjected to EAAT2 immunostaining using a computer-assisted image analysis system consisting of a microscope (Olympus BX 40), a CCD camera (KY-F55MD, Victor), a microcomputer (Power Macintosh 8500/120), and a software system (MacScope, Mitani Inc., Fukui, Japan). In three patients with mild neuronal loss of anterior horn cells, the entire anterior horn of the spinal cord at the fifth lumbar level (L5) was measured for EAAT2 immunoreactivity. In each patient, the light microscopic images of an entire anterior horn of the spinal cord in a high-power field were inputted through the CCD camera. The photoimages of EAAT2-like immunoreactive granules were digitized as binary images with a fixed cut-off value. A fraction (area ratio) of binary images in a targeted area was measured and a mean value of the fractions was obtained as the fractional area density of immunoreactive granules. The data obtained were compared with those of controls (n = 3) and assessed by Student's t-test. Semiquantitative analyses were not carried out in the patients with moderate or severe neuronal loss, because reduction of EAAT2 immunoreactivity was definite by visual inspection as compared to EAAT2 immunoreactivity of the controls.

Results

Controls

The gray matter of anterior horns, posterior horns, particularly Rexed layer II (substantia gelatinosa), and Clarke's columns displayed very dense immunoreactivity stained for both EAAT1 (Fig. 1) and EAAT2 (Fig. 2). Ependymal cells of the central canal and the gray matter surrounding the central canal (Rexed's lamina X) were immunostained for EAAT1, but not for EAAT2. In the gray matter, immunoreactivity of EAAT1 and EAAT2 was observed in the neuropil, on the surface of neurons and their neuronal processes, and around blood vessels. In the neuropil, there were numerous granular deposits of immunoreactivity of EAAT1 and EAAT2 (Fig. 3). The surface of anterior horn cells, in which lipofuscin had markedly accumulated, was only occasionally surrounded by denser immunoreactivity than that observed in normal-appearing neurons. EAAT1 and EAAT2 immunoreactivity varied between cases but was consistent at various levels of control spinal cord within the same case.

The white matter was not immunostained for EAAT1 or EAAT2, except for occasionally stained astroglial cells around the blood vessels, frequently stained astroglial cells closely adjacent to the gray matter, and positively stained heterotopic neurons which were almost always displayed on the surface of the cell bodies and their neuronal processes. There was no difference in the distribution of immunoreactivity of EAAT1 and EAAT2 among patients of different ages.



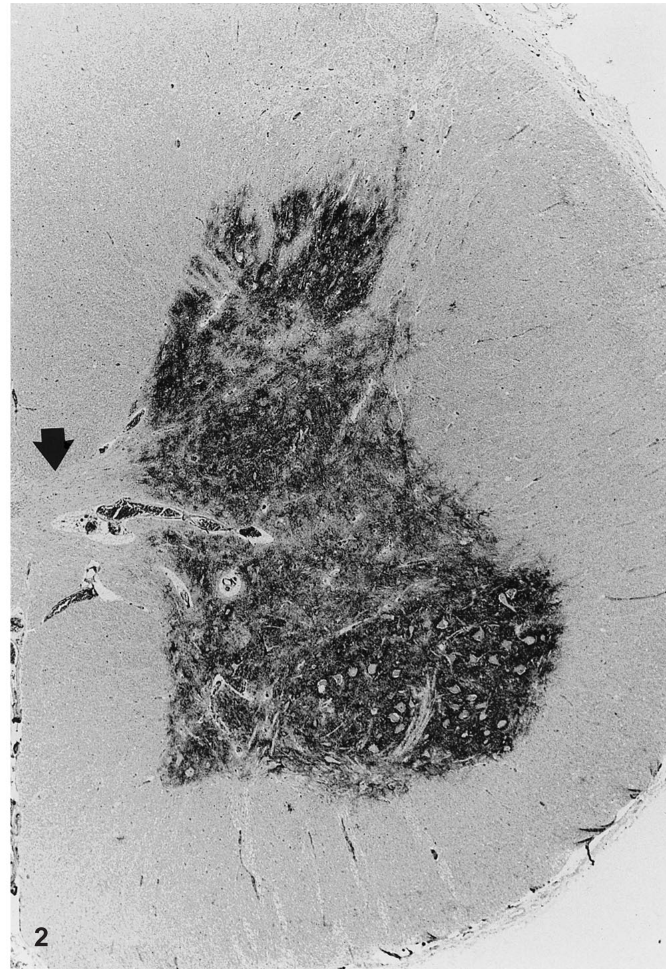


Fig. 1 EAAT1 immunoreactivity of the human spinal cord (L3). The gray matter of the anterior and posterior horns and Clarke's columns is intensely immunostained (*EAAT* excitatory amino acid transporter). × 17

Fig. 2 EAAT2 immunoreactivity of the human spinal cord (L3). The gray matter of the anterior and posterior horns and of Clarke's columns are intensely immunostained. Ependymal cells of the central canal and the gray matter surrounding the central canal (Rexed's lamina X) (*arrow*) show no immunoreactivity. × 17

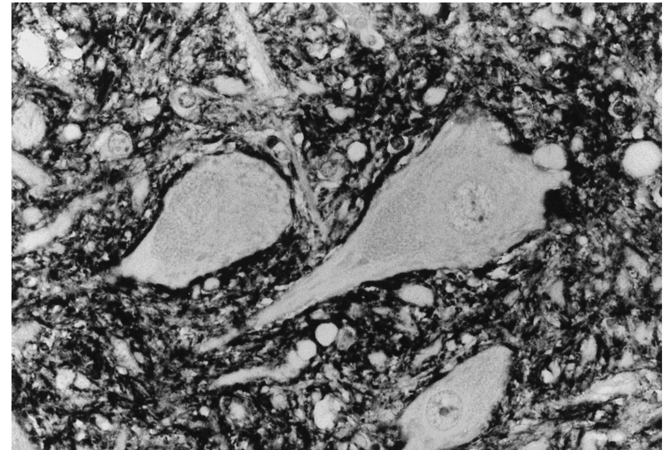


Fig. 3 Anterior horn immunostained for EAAT1. The surface of the anterior horn cells, and their neuronal processes, and the neuropil are densely immunostained for EAAT1. × 320

MND patients

We classified the degree of neuronal loss of anterior horn cells into three groups: mild depletion when more than 20 large anterior horn neurons were still present in each anterior horn at the L5 level; severe loss when less than 6 large anterior horn cells were found in each anterior horn; and moderate neuronal loss covering the neuronal depletion between mild and severe. In the patients with mild neuronal depletion of anterior horn cells, the gray matter of the anterior and posterior horns and of Clarke's columns was densely immunostained for EAAT1. The distribution and intensity of EAAT1 immunoreactivity were not different from those in controls. Even in the patients with moderate or severe depletion of anterior horn neurons, EAAT1 expression was relatively well preserved in the neuropil, on the surface of anterior horn neurons and their neuronal processes, and around the blood vessels in the anterior horns as well as in Clarke's columns and posterior horns (Fig. 4). Occasionally, clustered astrocytes were immunostained. Degenerated anterior horn cells showing pigmentary atrophy, central chromatolysis, or simple atrophy frequently showed much denser im-

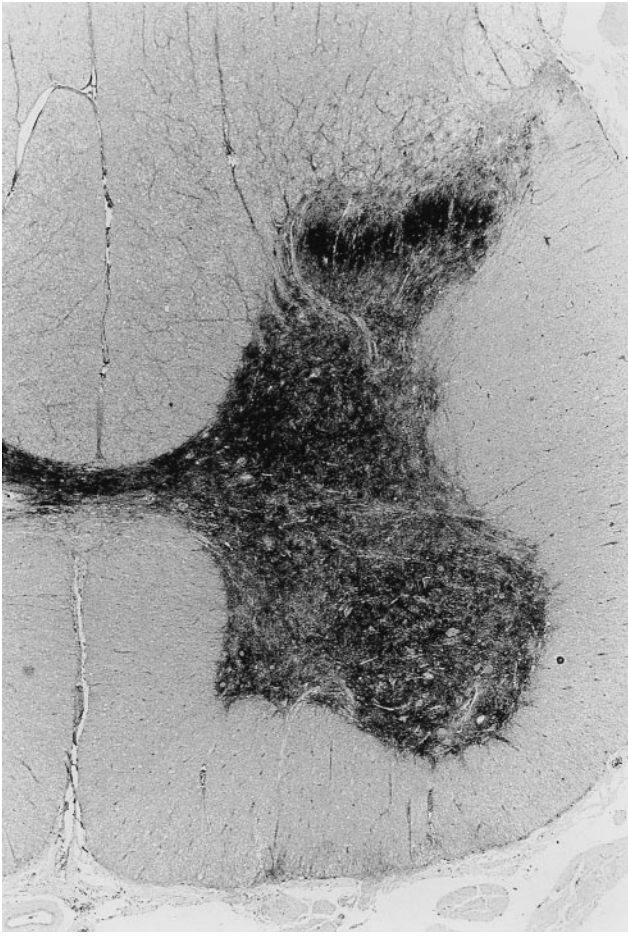


Fig. 4 In a patient with moderate depletion of the anterior horn neurons, EAAT1 immunoreactivity is well preserved in the anterior horn as well as in Clarke's column and the posterior horn (L3). × 17

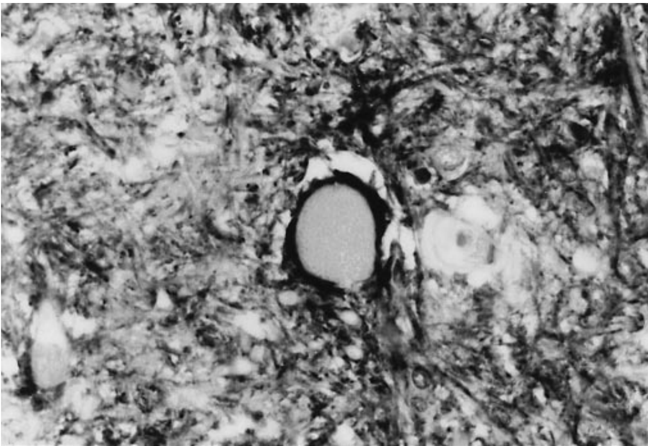


Fig. 5 A degenerated anterior horn cell shows much denser EAAT1 immunoreactivity around the surface of anterior horn neurons and their neuronal processes than that observed in normal-appearing neurons. × 480

munoreactivity of EAAT1 around the surface of anterior horn neurons and their neuronal processes than that appeared in normal-appearing neurons (Fig. 5).

On the other hand, the decrease of EAAT2 immunoreactivity in the neuropil of anterior horns correlated with the degree of neuronal loss or of degeneration of anterior horn cells: in the patients with mild neuronal depletion, the gray matter, including the anterior horns, was immunostained by the antibody (Fig. 6). The mean value of the fractions (area ratios) obtained as the fractional area density of EAAT2-positive granules in the anterior horn of the spinal cord in this group (17.8 ± 12.0) significantly increased as compared with that of the controls (8.3 ± 4.5) ($P < 0.0001$) (Student's *t*-test); in the patients with moderate neuronal depletion, EAAT2 immunoreactivity was diminished in the neuropil of the anterior horns, but the neuropil where the anterior horn neurons of lateral and medial nuclear groups still remained was intensely immunostained by the antibody (Fig. 7); in the patients with severe depletion of anterior horn neurons, EAAT2 expression was markedly reduced in the anterior horns in contrast with posterior horns and Clarke's columns (Fig. 8). Degenerated anterior horn cells having pigmentary atrophy or central chromatolysis were frequently encircled with much denser immunoreactivity of EAAT2 compared to normal-appearing neurons (Fig. 9). There was no difference in the expression of EAAT1 and EAAT2 immunoreactivity between LMND and ALS patients.

In the white matter, including the corticospinal tracts in both ALS and LMND patients, there was no EAAT1 or EAAT2 immunoreactivity, except for some positive immunoreactivity around the surface of heterotopic neurons and their neuronal processes, and occasionally around the blood vessels. In all ALS patients, increase of GFAP immunoreactivity in the neuropil of anterior horns was demonstrated and the intensity of the immunoreactivity varied from patient to patient. However, there was no close correlation in the intensity of immunoreactivity between GFAP and astroglial glutamate transporters.

Discussion

In the present study, we provided normative information on the localizations of EAAT1 and EAAT2 immunoreactivity in the human spinal cord. Previous immunohistochemical studies demonstrate that the cellular localization of EAAT1 and EAAT2 transporters is similar in the cerebral cortex of rat and human tissue [27]. In the normal human motor and frontal cortex, the EAAT2 protein is restricted to the gray matter, to regions in which the astroglial-immunoreactive protein is present [27]. EAAT1 shows a patchy distribution in the motor and frontal cortex, present only in astroglia at the light microscopic level [27]. In the rat spinal cord, EAAT2 immunoreactivity is enriched in spinal gray matter astroglial fibers and is particularly intense in the posterior horn (Rexed layers I~III) [27], whereas in human spinal cord the substantia gelatinosa shows mild to moderate diffuse immunoreactivity, whereas the ventral horn and the nucleus of Clark demonstrate strong immunoreactivity [9, 15]. EAAT1 immunoreactivity is enriched in the neuropil of the posterior horn

Fig. 6 **A** In a patient with mild neuronal depletion of the anterior horn cells, EAAT2 immunoreactivity in the anterior horns is relatively well preserved (L5). **B** For comparison, EAAT2 immunoreactivity of the spinal cord of a control subject (L5). **A**, **B** × 17

(Rexed layer II) and in some motor neurons, but is less intense in the neuropil of anterior horns [26]. In our study, EAAT1 and EAAT2 immunoreactivity in the human spinal cord showed a similar staining pattern to that observed in rats: it was restricted to and enriched in the spinal gray matter; it was also seen in the astroglial cells around the blood vessels of the white matter. However, strongly positive immunoreactivity of EAAT1 in anterior horns and negative immunostaining of EAAT2 in ependymal cells of the central canal and Rexed's lamina X of the human spinal cord, as were observed in this study, are in sharp contrast with less-enriched EAAT1 immunoreactivity in anterior horns and strongly positive immunoreactivity of EAAT2 in the gray substance surrounding the central canal of rats.

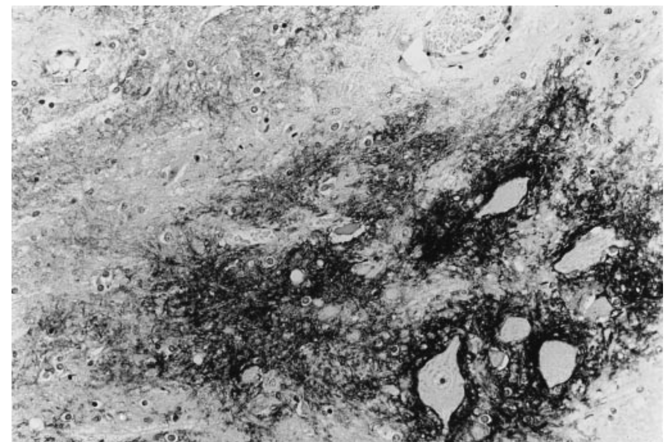


Fig. 7 In a patient with moderate neuronal depletion of the anterior horn cells, EAAT2 immunoreactivity is diminished in the neuropil of the anterior horn, but the neuropil where the anterior horn neurons of the medial nuclear group still remain is intensely immunostained for EAAT2. × 120

Deficiencies of glutamate transport activity were identified in synaptosomes from the motor and sensory cortex of ALS patients [24]. The reduction was subsequently ascribed to a selective loss of EAAT2 protein in ALS motor cortex and spinal cord, despite complete preservation of astroglia as assessed by immunoblot or immunohistochemical methods for each transporter subtype [27, 28]. However, Northern blotting revealed that the quantity and size of EAAT2 mRNA are normal in the ALS motor cortex, even in patients with a large loss of EAAT2 protein [4, 14]. Aoki et al. [1] reported that germ-line mutations

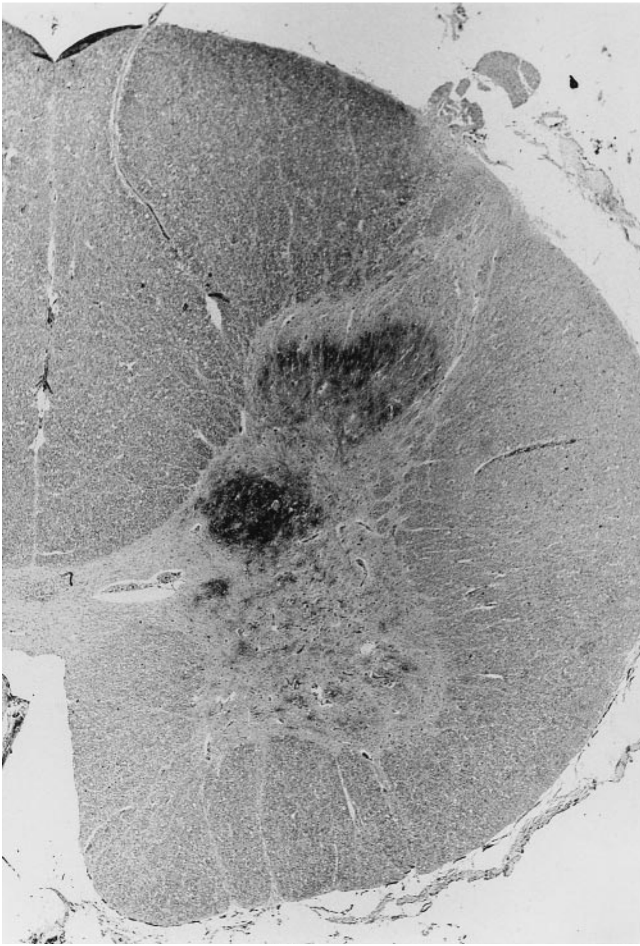


Fig. 8 In a patient with severe depletion of anterior horn neurons, EAAT2 expression is markedly reduced in the anterior horn, in contrast to intensely immunostained posterior horn and Clarke's column (L2). × 17

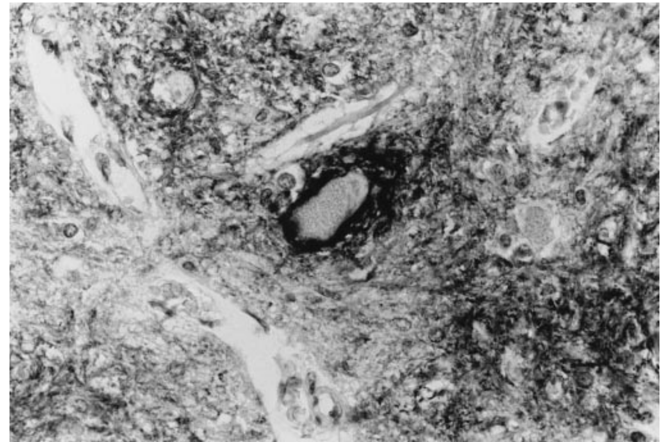


Fig. 9 A degenerated anterior horn cell is encircled by much denser EAAT2 immunoreactivity compared to normal-appearing neurons. × 320

in the EAAT2 gene do not cause abnormal EAAT2 transcripts in ALS. Thus, the pathomechanism of the selective loss of EAAT2 protein remains unknown. Recently, Lin et al. [12] identified multiple abnormal splicing of EAAT2 mRNA transcripts, including intron retention and exon skipping, in 65% of sporadic ALS patients, suggesting that proteins translated from aberrant mRNA may undergo rapid degeneration and/or produce a dominant negative effect on normal EAAT2, resulting in a loss of EAAT2 protein and its activity. However, the primary involvement of EAAT2 protein in the pathomechanism of ALS remains controversial. First, there is a selective decrease of the EAAT2 protein level in spinal cord extracts from end-stage ALS-linked SOD1 mutant G85R mice [5]. The selective loss of EAAT2 protein may be secondary to the consequence of SOD1 mutation in this mutant ALS model. Second, alternative splicing forms of EAAT2 mRNA were found even in control subjects, including those with other neurological diseases such as Alzheimer disease and cerebral infarction [16].

In the present study, we revealed that EAAT2 immunoreactivity in the spinal cord of ALS patients correlated with the degree of neuonal loss of anterior horn cells: in the patients with mild neuronal depletion, EAAT2 immunoreactivity was well preserved, whereas in the patients with severe neuronal loss, EAAT2 expression was markedly reduced. On the other hand, EAAT1 immunoreactivity was relatively well preserved in the anterior horns, despite the neuronal loss of anterior horn cells. These findings are consistent with those previously reported for ALS patients [27] and animal models [12, 28]: the major defect in glutamate transport in ALS may be due to a selective loss of EAAT2, whereas there is no appreciable change in EAAT1. The degeneration of anterior horn cells in ALS progresses from normal-appearing neurons to degenerated ones such as central chromatolytic neurons, and finally to neuronal loss. We demonstrated that EAAT1 and EAAT2 immunoreactivity was well preserved in normal-looking neurons, whereas degenerated anterior horn cells were frequently more densely immunostained. Thus, in the early stages of neuronal degeneration, when mild neuronal loss is present, the function of EAAT1 and EAAT2 is preserved in the astrocytic foot directly attached to normal-appearing neurons. The level of EAAT1 and EAAT2 protein increases in the astrocytic foot directly attached to degenerated anterior horn neurons, probably to reduce the elevated glutamate level, or to compensate for the reduced function of astroglial glutamate transporters; the increase may represent a condensation of EAAT1 and EAAT2 immunoreactivity secondary to loss of neurites and greater condensation of astrocytic processes. In the later stages, EAAT2 protein levels are definitely diminished in the anterior horns. Whether the loss of EAAT2 protein reflects a direct contribution to the pathogenesis of ALS or simply represents a secondary phenomenon still remains unclear, although there is a close relationship between the loss of EAAT2 immunoreactivity and degeneration of anterior horn neurons. Thus, we demonstrate a difference in EAAT1 and EAAT2 immunoreactivity, as a feature of the pathomechanism of ALS.

To our knowledge, this is the first comparative demonstration of EAAT1 and EAAT2 immunoreactivity in the spinal cord expressed in different stages of progression in ALS. Since the loss of EAAT2 protein is closely associated with the loss of motor neurons in this disease, agents capable of deterring the spread of degeneration of anterior horn cells caused by a loss of EAAT2 protein should be efficacious in neuroprotective therapeutic strategies intended to help ALS patients.

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