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Aberrant RNA Processing in a Neurodegenerative Disease: the Cause for Absent EAAT2, a Glutamate Transporter, in Amyotrophic Lateral Sclerosis

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Summary

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is characterized by selective upper and lower motor neuron degeneration, the pathogenesis of which is unknown. About 60%–70% of sporadic ALS patients have a 30%–95% loss of the astroglial glutamate transporter EAAT2 (excitatory amino acid transporter 2) protein in motor cortex and spinal cord. Loss of EAAT2 leads to increased extracellular glutamate and excitotoxic neuronal degeneration. Multiple abnormal *EAAT2* mRNAs, including intron-retention and exon-skipping, have now been identified from the affected areas of ALS patients. The aberrant mRNAs were highly abundant and were found only in neuropathologically affected areas of ALS patients but not in other brain regions. They were found in 65% of sporadic ALS patients but were not found in nonneurologic disease or other disease controls. They were also detectable in the cerebrospinal fluid (CSF) of living ALS patients, early in the disease. In vitro expression studies suggest that proteins translated from these aberrant mRNAs may undergo rapid degradation and/or produce a dominant negative effect on normal EAAT2 resulting in loss of protein and activity. These findings suggest that the loss of EAAT2 in ALS is due to aberrant mRNA and that these aberrant mRNAs could result from RNA processing errors. Aberrant RNA processing could be important in the pathophysiology of neurodegenerative disease and in excitotoxicity. The presence of these mRNA species in ALS CSF may have diagnostic utility.

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset chronic neuromuscular disorder that is clinically characterized by muscle wasting, weakness, and spasticity, reflecting the relatively selective degeneration of cortical motor neurons and spinal/bulbar motor neurons (Kuncl et al., 1992). ALS is uniformly fatal due to respiratory impairment. The disease is present worldwide; 95% of all cases are sporadic, with many of the remaining 5% of cases showing an autosomal dominant inheritance (Brown, 1996; Siddique and Deng, 1996). About 15%–25% of familial ALS, reflecting 1% of all ALS patients, is due to mutations of Cu^{2+}/Zn^{+} superoxide dismutase (Brown, 1996; Siddique and Deng, 1996). These mutations are not present in the sporadic ALS population (Brown, 1996; Siddique and Deng, 1996). Consequently, multiple mechanisms have been postulated to be the cause of this form of the disease, including excitotoxicity (Shaw, 1994; Rothstein, 1996), oxidative injury (Bowling et al., 1995; Borthwick et al., 1996), cytoskeletal abnormalities (Lee et al., 1994; Wong et al., 1995; Bruijn and Cleveland, 1996), and autoimmunity (Smith et al., 1992; Appel et al., 1994). Glutamate-mediated excitotoxicity has been suspected to contribute to motor neuron degeneration in ALS because of: (1) loss of high affinity glutamate transport (Rothstein et al., 1992; Shaw et al., 1994), (2) other abnormalities in glutamate metabolism (Rothstein et al., 1990; Couratier et al., 1993, 1994; Shaw et al., 1995), (3) susceptibility of motor neurons to glutamate toxicity (Ince et al., 1993; Rothstein et al., 1993; Carriedo et al., 1995; Ikonomidou et al., 1996), and (4) clinical efficacy of anti-glutamate agents (Bensimon et al., 1994; Lacomblez et al., 1996; Miller et al., 1996).

High affinity sodium-dependent glutamate transport is the primary means of inactivating neurotransmitter glutamate. EAAT1 and EAAT2 are glutamate transporters specific for astrocytes (Rothstein et al., 1994, 1995; Lehre et al., 1995), while neuronal glutamate transport is mediated by EAAT3 and EAAT4 (Arriza et al., 1994; Rothstein et al., 1994; Fairman et al., 1995; Furuta et al., 1997). Antisense knockdown and other studies suggest that EAAT2 is the predominant glutamate transporter (Haugeto et al., 1996; Rothstein et al., 1996). Detailed studies of ALS and other neurodegenerative diseases have shown that impaired glutamate transport in ALS appears to be due to a loss of only the EAAT2 protein. Up to 60%–70% of sporadic ALS patients have a 30%–95% loss of EAAT2 protein (Rothstein et al., 1992, 1995; Shaw et al., 1994).

What could account for the selective loss of EAAT2 protein in ALS? It does not appear to be due to decreased overall transcription of the *EAAT2* mRNA in ALS (Bristol and Rothstein, 1996), nor is there evidence for genomic mutations of *EAAT2* in familial or sporadic ALS (Aoki et al., 1998). We now report that aberrant *EAAT2* mRNA species appear to account for the regional selective loss of EAAT2 and occur commonly in the sporadic form of ALS.

Results

Identification of Aberrant *EAAT2* Transcripts in ALS Affected Areas

A male ALS patient (66 years old, 5 hr postmortem delay) without SOD1 mutations was initially investigated. This patient was chosen because immunoblot studies revealed extremely low levels of EAAT2 protein (5% of control) in motor cortex (Rothstein et al., 1995). The Northern blotting revealed that the quantity and size of *EAAT2* mRNA were normal (Bristol and Rothstein, 1996). To investigate whether there were aberrant *EAAT2* mRNA species, a cDNA library was constructed from a poly(A)$^{+}$ mRNA prepared from the motor cortex of this patient. The cDNA library was then screened, and an

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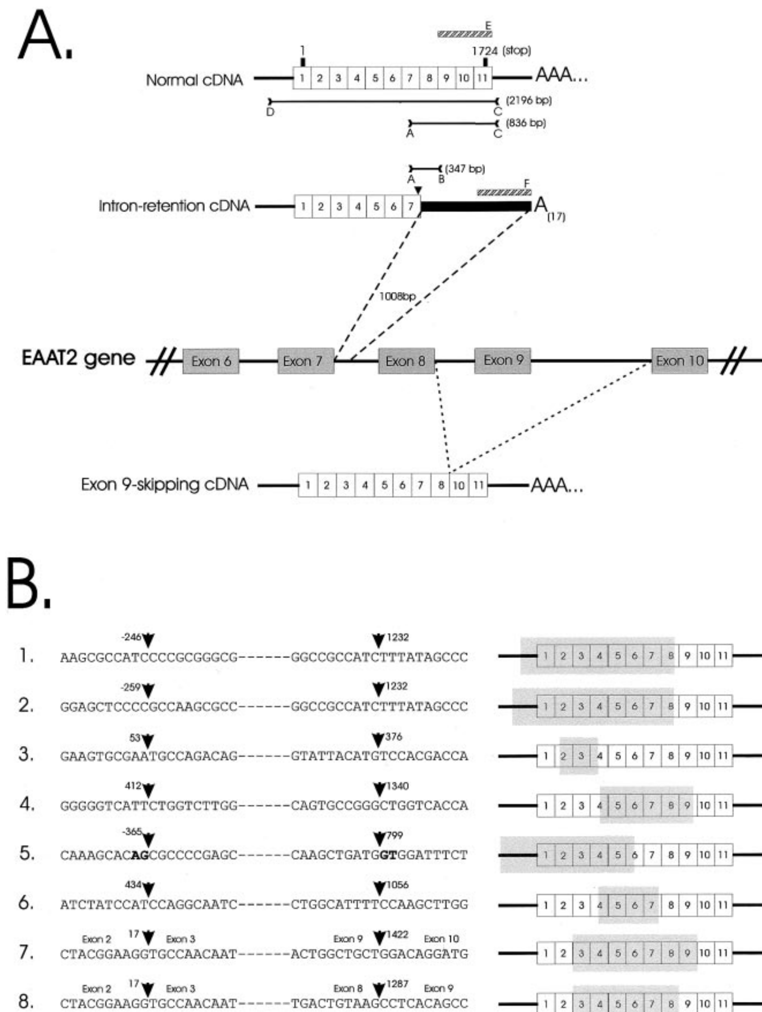


Figure 1. Structure of Aberrant *EAAT2* mRNA in ALS

(A) Schematic presentation of abnormally processed partial intron 7–retention and exon 9–skipping species in relation to the normal *EAAT2* gene. Numbered boxes represent individual exons. The connected arrow heads indicate the primer regions used for RT-PCR. The hatched bars indicate the regions used to prepare probes for in situ hybridization. Probe E corresponds to position 1687–2210 from the 5′ translation region. Probe F corresponds to position 537–1008 from the exon 7 intron junction. The triangle indicates a Hind III site used as an insertion site for constructing an internal control plasmid pE2F.

(B) Sequencing analysis of truncated *EAAT2* transcripts present in ALS. In the right panel, twenty nucleotides on both sides of each internal deletion transcript are displayed. Arrows indicate splicing junctions. Sequences between the arrows are spliced out. Dashed lines indicate nucleotides within the deleted sequences. A schematic representation of the deleted regions is presented in the right panel. Gray boxes represent regions of missing RNA sequence.

abnormal cDNA clone was subsequently identified from positive clones by restriction analysis. Sequencing of this clone revealed that it contained 1091 bp of the *EAAT2* coding region from exon 1 to exon 7, followed by 1008 bp of intron region, corresponding to partial sequence of intron 7, ending with a poly A tail (Aoki et al., 1998; Figure 1A). A stop codon was found immediately after this exon and intron junction. Thus, the presumed translation product would be EAAT2 protein truncated after codon 364.

To ensure that this was not a cloning artifact, reverse-transcription polymerase chain reaction (RT-PCR), with the use of a primer pair that included normal exonic and intronic sequences (Figure 1A, primers A and B), was performed on equal amounts of poly(A)$^+$ mRNA (DNase I treated) prepared from motor cortex of the ALS patient and normal controls. As shown in Figure 2A (at intron-retention EAAT2; ALS patient 3), a strong signal was observed in the ALS specimen but not in the control specimens.

As another approach, we also used RT-PCR to amplify *EAAT2* cDNA fragments from poly(A)$^+$ mRNA prepared from the spinal cord or motor cortex of the patient and from normal controls. A shorter PCR fragment was subsequently identified, using primers A and C (Figure 1A), which was amplified along with the normal-size PCR fragment in the patient but was not present in normal controls (Figure 2A; at exon 9–skipping EAAT2; ALS patient 2). The shorter PCR fragment was cloned. DNA sequencing revealed it to be a 135 bp in-frame deletion in which exon 8 is linked directly to exon 10, demonstrating that it arises from skipping exon 9 (Aoki et al., 1998; Figure 1A).

Furthermore, when primers D and C (Figure 1A) were used to amplify the full-length EAAT2 coding region, a large amount of shorter PCR fragments was observed in motor cortex of ALS but not in normal controls (Figure 2C). All of the shorter PCR fragments from ALS patient 3 (Figure 2C) were then cloned. Twenty clones were randomly selected and analyzed by restriction digestion. All of the these clones were found to have different internal deletions. In addition, a single band from the gel (Patient 3, Figure 2C) was cloned and was found to consist of multiple internally deleted cDNA, all similar in size. These results indicate that there are many different truncated *EAAT2* transcripts in ALS motor cortex.

Eight of the truncated cDNAs were then sequenced at the junction of the deletion regions (Figure 1B). Two cDNAs were found to be truncated due to exon-skipping (Figures $1B_7$ and $1B_8$). One cDNA was truncated at the

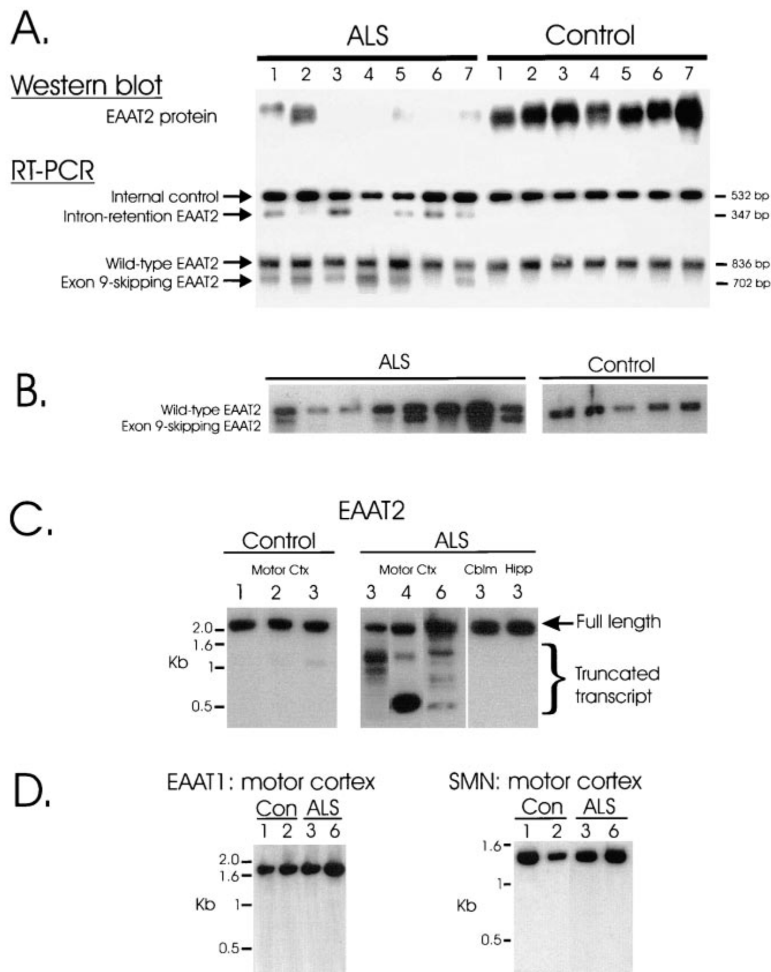


Figure 2. Aberrant mRNA Species Are Commonly Present in ALS but Not Control Motor Cortex

(A) Analysis of EAAT2 protein (Western blot) and aberrant *EAAT2* mRNA species (RT-PCR/Southern blot) in ALS and control motor cortex. Aliquots of tissue homogenates from control or ALS motor cortex were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with affinity-purified polyclonal carboxy-terminal antibody to EAAT2. Controls included nonneurological and neurodegenerative disease specimens. Individual lanes are numbered for each patient. RT-PCR was performed on parallel tissue samples from each control or ALS motor cortex specimen. An internal control was designed for comparative analysis of the partial intron 7–retention mRNA between samples.

(B) Exon 9–skipping *EAAT2* mRNA is present in some ALS CSF specimens.

(C) RT-PCR for full-length EAAT2 reveals multiple truncated transcripts in ALS but not in controls. The aberrant species were present in motor cortex but not cerebellum (Cblm) or hippocampus (Hipp). Lane numbers are identical to patients labeled in (A).

(D) No aberrant *EAAT1* or *SMN* transcripts were found in ALS motor cortex. Lane numbers are identical to patients labeled in (A).

appropriate splicing donor and acceptor sites (Figure $1B_5$). Other truncated cDNAs were identified, although the deletions did not occur at common splicing sites (Figures $1B_1$–$1B_4$ and $1B_6$). The presence of multiple precise exon-skipping deletions (Figures 1A and 1B) suggests that it is very unlikely that they could be caused by somatic DNA deletions. The abundance of multiple truncated transcripts may be the result of aberrant RNA splicing.

To examine the specificity of these defects for brain region and transporter subtypes, ALS and control tissue from several brain regions in the same patient were examined. As shown in Figure 2C, multiple truncated *EAAT2* mRNAs were found in ALS motor cortex but not cerebellum or hippocampus from the same patient (3). Similar results were obtained from every ALS patient examined ($n = 20$). Other cell-specific and general cellular mRNA species were examined. Truncated transcripts were not found for the astroglial glutamate transporter *EAAT1* (Figure 2D), the neuronal protein *EAAT3*, or the constitutive spliceosomal assembly protein (the survival motor neuron [*SMN*]) (Fischer et al., 1997; Liu et al., 1997; Figure 2D). EAAT1 and EAAT3 protein levels were previously shown to be normal in ALS (Rothstein et al., 1995).

Thus, several observations suggest that these aberrant RNA species were specific for ALS and were not an artifact. They were not observed in other neurodegenerative disorders, including Huntington's disease, Alzheimer's disease, and spinal muscular atrophy; they were found only in neuropathologically affected brain regions, while unaffected brain regions in the same patients had normal *EAAT2* RNA. Other transporter proteins, as well as constitutive RNA species, were not affected.

The Aberrant *EAAT2* Transcripts Are Present In Vivo

To verify that these aberrant *EAAT2* transcripts were present in vivo and not a cloning or PCR artifact, in situ hybridization was performed to detect partial intron 7–retention mRNA using a riboprobe to the intronic region (Figure 1A, probe F). Figures 3A and 3B show that this partial intron 7–retention mRNA was present only in ALS motor cortex, but not in control specimens, in agreement with the RT-PCR results. In addition, signals were uniformly localized to small cells, presumably astrocytes, present throughout the motor cortex (EAAT2 is an astrocyte-specific protein) (Figure 3D, asterisk). As a control, signals for actin (Figures 3C and 3F) were

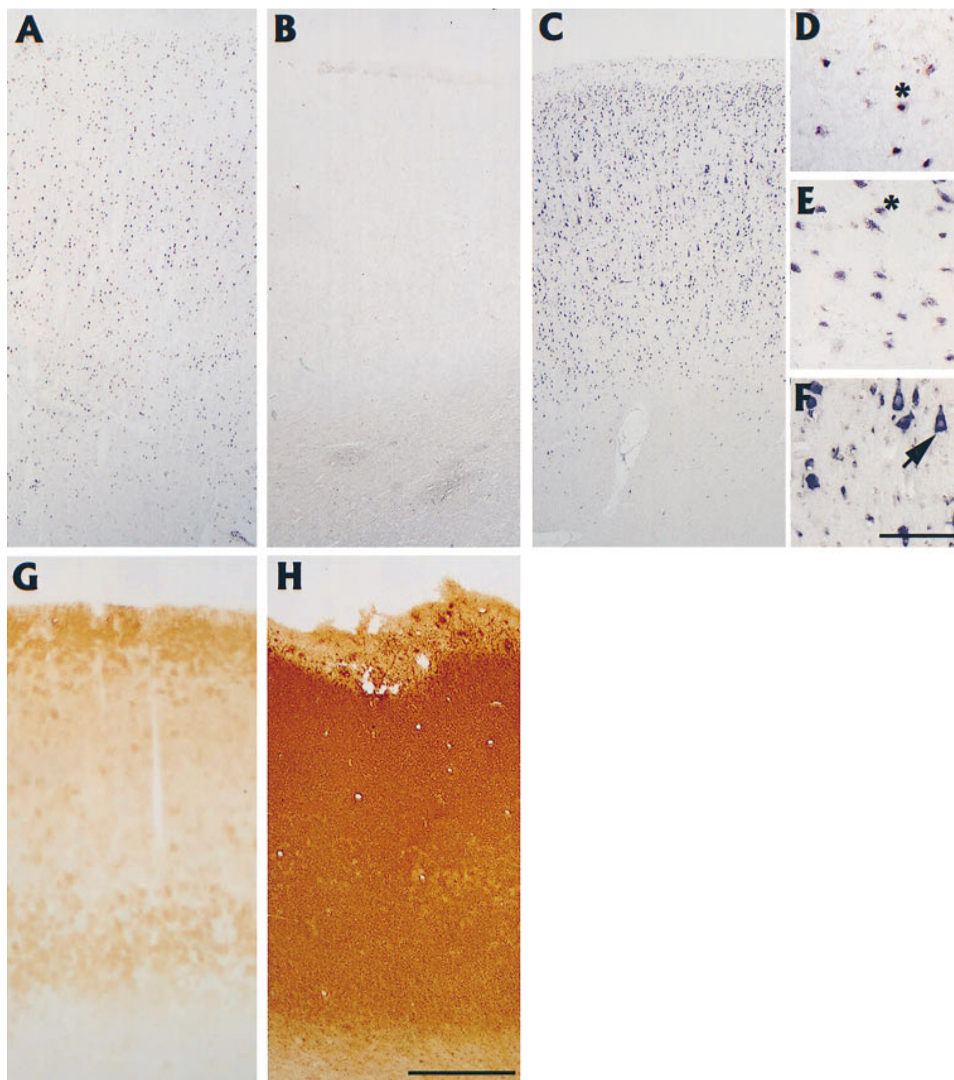


Figure 3. In Situ Hybridization of Partial Intron 7-Retention mRNA in Motor Cortex

The partial intron 7–retention mRNA was localized to small cells throughout the motor cortex in ALS (A) but was not detected in control motor cortex (B). When compared to mRNA for the general cellular protein actin (C), the localization appeared to be consistent with astrocytes. Higher power analysis ([D], [E], and [F]) of ALS motor cortex confirmed that aberrant partial intron 7–retention mRNA was localized to small cortical cells ([D], asterisk), identical to the localization of wild-type *EAAT2* mRNA in ALS cortical astrocytes (E), whereas actin was localized in both astrocytes and neurons ([F], arrow) in ALS motor cortex. The localization of the partial intron 7–retention *EAAT2* mRNA (A) correlated with the decreased expression of EAAT2 protein (G) in ALS compared with expression of EAAT2 protein in control motor (H) cortex. Immunohistochemistry and in situ hybridization studies were performed on three different specimens. Scale bar, 0.1 mm for (A), (B), (C), (G), and (H); 0.01 for (D), (E), and (F).

found to be expressed by both neurons (arrowhead) and astroglia. Wild-type *EAAT2* mRNA in ALS (Figure 3E) and in control tissue (data not shown) was expressed in a distribution similar to the partial intron 7–retention mRNA. No staining was seen with sense controls (data not shown). Furthermore, the dramatic loss of EAAT2 protein (Rothstein et al., 1995; Figures 3G and 3H) in ALS motor cortex appears to be associated with the presence of the partial intron 7–retention transcripts, which was specific to ALS and was not detected in motor cortex from control specimens (Figure 3B) or in specimens of motor cortex from ALS patients that were not RT-PCR–positive for the intron-retention transcripts (data not shown). Finally, S1 nuclease protection analysis of *EAAT2* mRNA (Figure 4C; described below) also provides evidence that these aberrant splicing *EAAT2* transcripts were present in vivo. Northern analysis of *EAAT2* mRNA could not be used for this purpose because the size of normal *EAAT2* mRNA is about 11 kb and the truncated transcript would only be ~1–2 kb smaller. Therefore, it would be difficult to identify these truncated transcripts from full length transcript using this method.

Up to 70% of Total *EAAT2* Transcripts Are Aberrant RNA Processing Products

To determine the abundance of the aberrant mRNA species relative to wild-type *EAAT2*, we performed quantitative RT-PCR using mRNA from ALS patient 4. This patient was chosen because RT-PCR studies revealed only

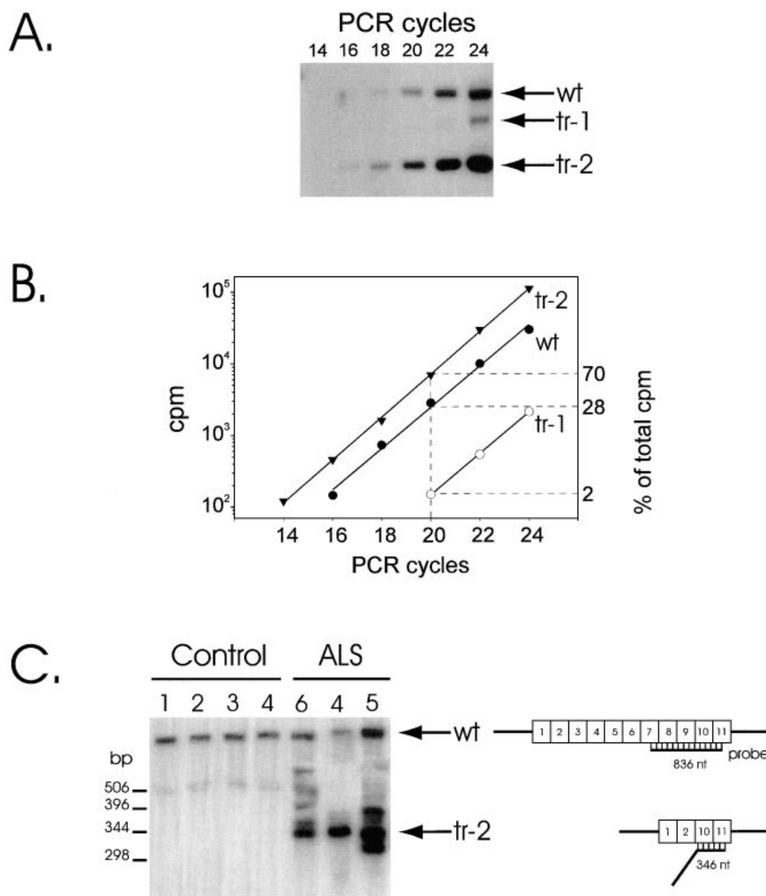


Figure 4. Quantitative Analysis of Aberrant *EAAT2* mRNA in ALS Motor Cortex

(A) Quantitative RT-PCR analysis of *EAAT2* mRNA from ALS patient 4. PCR reaction products from different amplification cycles were subjected to Southern blot analysis using an oligonucleotide probe located at the 3′ end of the PCR fragment. Abbreviations: wt, wild-type EAAT2; tr-1 and tr-2, two truncated aberrant species.

(B) The amounts of radioactivity recovered from the excised membrane bands in (A) were plotted as a function of the number of cycles. The rates of amplification were exponential between 14 and 24 cycles for full length and truncated templates. More than 70% of total *EAAT2* mRNA is in the form of aberrant transcripts.

(C) S1 nuclease protection analysis of *EAAT2* mRNA using a 836 base single strand DNA probe, located at the 3′ end of the coding region. A schematic representation of the probe, the wild-type mRNA, and the aberrantly spliced mRNA (tr-2) are shown on the right. The protection fragments were subjected to Southern blot analysis using an oligonucleotide probe located at the 3′ end of the protection fragment. As with quantitative RT-PCR, the abnormal species were abundant in the ALS specimens compared to normal EAAT2.

two types of truncated transcripts (Figure 2C). PCR reaction products from different amplification cycles were resolved by gel electrophoresis and transferred to membrane for Southern blot analysis (Figure 4A). The amount of radioactivity recovered from the excised membrane bands was plotted as a function of the number of cycles (Figure 4B). The rates of amplification were exponential between 14 and 24 cycles for full-length and truncated templates. The results revealed that full-length (wild-type) and truncated transcripts tr-1 and tr-2 represent 28%, 2%, and 70% of total *EAAT2* transcript, respectively. It is notable that the truncated transcript tr-2 from this patient is the same as the transcript in Figure $1B_7$. As described below, this truncated transcript produced downregulation of normal EAAT2 in vitro.

We also performed S1 nuclease protection assays to evaluate the relative abundance of the aberrant mRNA species. A 836 base single-strand DNA probe located at the 3′ end of the coding region was used. The S1 nuclease-resistant fragments were resolved by gel electrophoresis and transferred to membrane. The blot was probed with an oligonucleotide probe located at the 3′ end of the protection fragment. As shown in Figure 4C, large amounts of $<$836 bp S1 nuclease-resistant fragments were observed in ALS specimens but were not seen in the control specimens. The mRNA sample from ALS patient 4 used in this experiment was the same one used for quantitative RT-PCR described above. The 346 bp S1 nuclease-resistant fragment, corresponding to the truncated transcript tr-2, represents 73% of the total protection fragments, which is in agreement with the quantitative RT-PCR results (Figure 4B).

Aberrant *EAAT2* Transcripts Are Commonly Present in Sporadic ALS and They Are Correlated with Loss of EAAT2 Protein

To evaluate the prevalence of these aberrant *EAAT2* transcripts in ALS, 30 ALS specimens were screened, including spinal cord, motor cortex, and cerebellum, for partial intron 7–retention and exon 9–skipping transcripts by competitive RT-PCR (Figure 2A). An internal control was designed to amplify along with the partial intron 7–retention mRNA (Figure 1A; see Experimental Procedures). Since the exon 9–skipping mRNA was 135 bp shorter than the wild-type *EAAT2* mRNA, the wild-type *EAAT2* mRNA was used as an internal control for the comparative analysis of the exon 9–skipping mRNA between patients. All RT-PCR experiments were repeated 2–5 times for each specimen to verify the presence of aberrant mRNA species. Both aberrant transcripts were commonly found; 20 of the 30 ALS patients had the partial intron 7–retention mRNA and 11 of the 30 ALS patients had the exon 9–skipping mRNA. These aberrant mRNA species were found only in ALS motor cortex, or spinal cord, or both, but not in cerebellum. To determine the disease specificity of these aberrant mRNA species, other nonneurological and neurological disease control tissues were examined (Table 1), including tissue from neuropathologically affected and unaffected regions. The aberrant mRNA species were not

Table 1. ALS and Control Populations Used for the Analysis of EAAT2 Mutations

Group	Number of Patients	M/F	Age at Death (mean year ± SEM)	Postmortem Delay (hr) (mean ± SEM)
ALS	30	23/7	60.4 ± 2.1	7.3 ± 0.8
Nonneurological disease	25	15/19	65.2 ± 2.2	10.4 ± 0.6
SMA*	8	3/5	6.6 ± 1.0 (month)	9.3 ± 1.8
Alzheimer's disease	7	4/3	74.8 ± 2.1	8.2 ± 0.6
Huntington's disease	6	2/4	59.3 ± 4.9	11.9 ± 0.7

* SMA = spinal muscular atrophy, type 1.

found in any of the neural tissues from a total of 48 controls, including 27 nonneurological disease control specimens (12 spinal cord and 27 motor cortex), 8 spinal muscular atrophy specimens (SMA-type 1) (spinal cord), 7 Alzheimer's disease specimens (7 hippocampus and 7 motor cortex), and 6 Huntington's disease specimens (6 caudate and 6 motor cortex). The control tissues were matched for age and postmortem delay (Table 1).

The presence of abnormal mRNA was significantly ($p < 0.001$) correlated with the loss of EAAT2 protein. ALS specimens from motor cortex or spinal cord that had very low levels of EAAT2 protein (<30% of control) contained both partial intron 7–retention and exon 9–skipping transcripts (Figure 5A). Eight out of 30 ALS patients had both partial intron 7–retention and exon 9–skipping mRNA. An intermediate loss of EAAT2 protein in neural tissue, 30%–80% of control levels, was associated with the presence of the partial intron 7–retention mRNA alone. In addition, the loss of EAAT2 protein was inversely correlated with the amounts of either the exon 9–skipping ($rs = 0.81$, $p < 0.001$) or the partial intron 7–retention mRNA ($rs = 0.62$, $p < 0.001$; Figure 5B). Although the presence of aberrant mRNA species appeared to correlate with a loss of protein, rarely did patients with a loss of EAAT2 not have either of these aberrant mRNA. In those cases, the loss of protein could be due to other as yet unidentified variants in *EAAT2* mRNA. Alternatively, other mechanisms known to lead to degradation of glutamate transporter proteins, e.g., oxidative stress, could have resulted in a loss of EAAT2 (Volterra et al., 1994; Trotti et al., 1996).

The presence of the aberrant mRNA species did not correlate with the clinical presentation (spinal versus bulbar onset) of the disease in ALS patients. ALS patients with altered *EAAT2* mRNA species had a tendency toward shorter survival (17 ± 2.9 [SEM] months) when compared to the overall average for ALS of 2–5 years (Kuncl et al., 1992) but not when compared to the ALS patients in this study without aberrant mRNA species (23 ± 4.8 [SEM] months).

Detection of Aberrant EAAT2 Transcripts in Cerebrospinal Fluid

Cerebrospinal fluid (CSF) was also examined for the presence of the exon 9–skipping transcript in ALS and control specimens (Figure 2B). All specimens were processed identically. Control CSF was obtained from patients with neurological diagnoses that included multiple sclerosis, normal pressure hydrocephalus, hepatic encephalopathy, migraine headache, subarachnoid hemorrhage, stroke, epilepsy, ataxia, CNS vasculitis, and dementia. The exon 9–skipping mRNA was not detected in the 38 control specimens but was detected in 12 out of 18 (66%) ALS specimens. In addition, in four ALS cases (Table 2), CSF obtained during diagnostic evaluation and brain tissue obtained at autopsy were available from the same patients. In those patients, CSF was collected 25.5 ± 6 (SEM) months prior to death. In all cases, the presence of the exon 9–skipping mRNA was detected in both antemortem CSF and postmortem brain or spinal cord tissue.

Proteins Translated from These Aberrant Transcripts Are Unstable and/or Cause Downregulation of Normal EAAT2 In Vitro

To determine if proteins translated from these aberrant *EAAT2* transcripts could be identified in the ALS postmortem tissue, we analyzed ALS motor cortex for a carboxy-terminal truncated EAAT2 protein (the presumed protein product of the partial intron 7–retention transcript) by Western blotting with amino-terminal oligopeptide antibodies to human EAAT2 protein (Rothstein et al., 1995). Previous studies have shown that the amino-terminal antibody can recognize wild-type EAAT2 in human tissue. However, using this antibody, no immunoreactive band with the expected size of the truncated EAAT2 protein was detected. The protein from the exon 9–skipping transcript was also investigated in ALS tissue, using oligopeptide antibodies to the amino acid sequence spanning the deletion site. Again, no immunoreactive band was detected (data not shown). The inability to detect the aberrant EAAT2 proteins could be due to several possibilities: (1) the aberrant EAAT2 proteins could be very unstable or rapidly degraded in postmortem tissue, or both, or (2) the aberrant proteins may not be detectable with the available antibodies, or (3) the aberrant proteins may also affect normal EAAT2 function.

To evaluate the properties of these abnormal transcripts, *EAAT2* cDNA was transiently expressed in COS7 cells. EAAT2 protein was quantitated 72 hr posttransfection by Western blotting, and activity of the transporter was measured by a sodium-dependent ^{3}H-glutamate transport assay (Rothstein et al., 1992, 1995). Wild-type EAAT2 protein was detected by a carboxy-terminal EAAT2 antibody (Figure 6A, lane 1) as well as evidence of glutamate transport. Wild-type EAAT2 was not detected by the available amino-terminal antibody. As expected, predicted truncated protein synthesized from the partial intron 7–retention cDNA was not detected by this carboxy-terminal antibody (Figure 6A, lane 5). Nevertheless, there was evidence that the partial intron 7–retention *EAAT2* cDNA was expressed by the

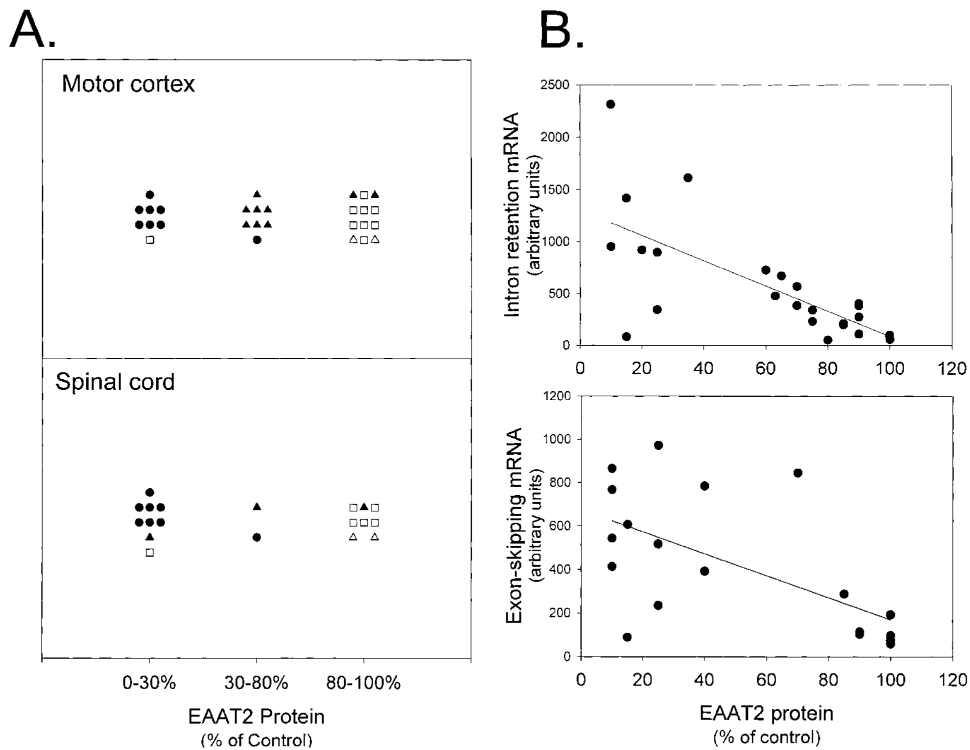


Figure 5. Aberrant *EAAT2* mRNA Species Correlate with the Loss of EAAT2 Protein

(A) Identification of both (closed circle) partial intron 7–retention and exon 9–skipping mRNA species in the same tissue samples was typically associated with a large loss of EAAT2 protein, while the presence of partial intron 7–retention alone (closed triangle) or trace amounts of partial intron 7–retention mRNA sequence (open triangle) was associated with moderate to little loss of EAAT2 protein. Absence of the abnormally processed mRNA (open square) was associated with normal levels of EAAT2 protein.

(B) The amounts of partial intron 7–retention and exon 9–skipping mRNA species in spinal cord and motor cortex were inversely correlated with the amount of EAAT2 protein.

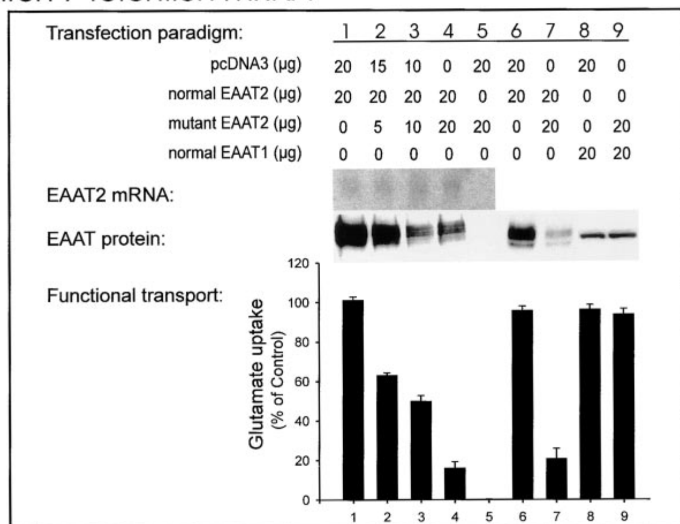
COS7 cells, because the partial intron 7–retention mRNA was detected in the transfected cells by Northern blotting and RT-PCR (data not shown). No glutamate transport was observed in COS7 cells transfected with the partial intron 7–retention cDNA.

When the partial intron 7–retention cDNA was cotransfected with the normal *EAAT2* cDNA into COS7 cells, there was a progressive decrease in the level of normal EAAT2 protein (Figure 6A, lanes 2–4). However, the levels of wild-type *EAAT2* mRNA, using Northern analysis, were not decreased by the partial intron 7–retention cDNA (Figure 6A, lanes 2–4). Glutamate transport also decreased in parallel with the loss of EAAT2 protein (Figure 6A, lanes 2–4). The same results were obtained when the partial intron 7–retention cDNA was coexpressed in HeLa cells (Figure 6A, lanes 6–7). These coexpression studies suggest that the partial intron 7–retention cDNA could dominantly downregulate EAAT2 protein. This effect was specific for EAAT2; coexpression of the intron-retention cDNA with wild-type *EAAT1*, *EAAT3*, or *EAAT4* cDNA had no effect on either the astroglial subtype EAAT1 (Figure 6A, lanes 8 and 9) or the neuronal subtypes EAAT3 and EAAT4 (data not shown) protein or glutamate transport. This effect is not due to the promoter competition between wild-type and partial intron 7–retention cDNA because (1) the levels of wild-type

Table 2. Exon 9–skipping EAAT2 Species Are Present in ALS CSF Years Prior to Death: Examination of CSF and Postmortem Brain Specimens

Patient	Age (at Death)	Disease Duration	CSF Collection: Time before Death	Exon 9-Skipping EAAT2 (RT-PCR)	
		(months)	(months)	CSF	Brain
1	44	39	27	+	+
2	52	46	41	+	+
3	62	15	12	+	+
4	63	22	22	−	−

+ Exon 9–skipping EAAT2 present in sample; − exon 9–skipping species not detectable.



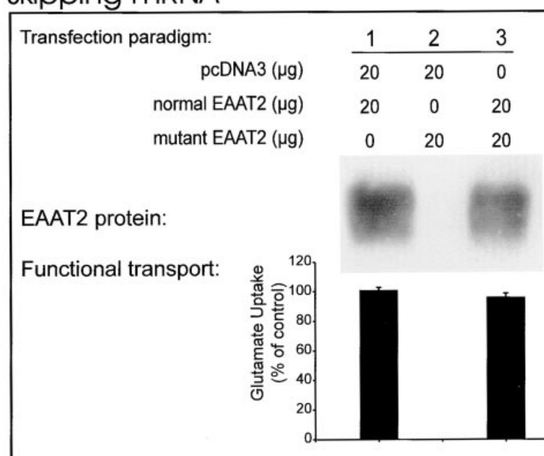


Figure 6. Transient Expression of the Partial Intron 7-Retention and Exon 9–Skipping *EAAT2* cDNA in COS7 Cells and in HeLa Cells

Different combinations of indicated plasmids were transfected into COS7 cells ([A], paradigms 1–5 and 8–9; [B], paradigms 1–3) or HeLa cells ([A], paradigms 6 and 7) by electroporation. After 72 hr, some transfected cells were used to quantitate expressed EAAT2 or EAAT1 protein by immunoblotting using carboxyterminal oligopeptide antibodies to EAAT2 or EAAT1 protein, or *EAAT2* mRNA expression by Northern blot. Others were used to measure functional EAAT2 or EAAT1 by sodium-dependent, ^{3}H-glutamate transport assay.

(A) The partial intron 7–retention *EAAT2* cDNA produced a negative effect on the normal EAAT2 protein level and glutamate transport (paradigms 1–7) but had no effect on *EAAT2* mRNA levels (paradigms 1–5), EAAT1 protein levels (paradigms 8 and 9), or EAAT1 glutamate transport (paradigms 8 and 9).

(B) The exon 9–skipping EAAT2 species had no effect on EAAT2 protein expression (paradigm 3) or on wild-type glutamate transport (paradigm 3). All aberrant or wild-type *EAAT2* (*EAAT1*) cDNAs were in the same vector (pcDNA3).

EAAT2 mRNA were not decreased and (2) this effect was not observed in other subtypes of glutamate transporters with the same vector and transfection conditions.

The exon 9–skipping *EAAT2* cDNA was also expressed in COS7 cells. Although the protein was not detected by Western blotting (Figure 6B, lane 2), the mRNA was detected in the transfected COS7 cells by RT-PCR. However, no glutamate transport was observed in the transfected COS7 cells by sodium-dependent ^{3}H-glutamate transport assay. Notably, in this paradigm, the exon 9–skipping cDNA had no effect on the expression of wild-type EAAT2 protein or glutamate transport (Figure 6B, lane 3).

To confirm that the aberrant *EAAT2* cDNAs were expressed and translated, we constructed chimeric genes to encode C-terminal fusions of wild-type or mutant EAAT2 to green-fluorescent protein (GFP). These constructs were expressed in COS7 cells and all produced similar levels of mRNA by Northern blotting (data not shown). The wild-type EAAT2-GFP–transfected cells had normal ^{3}H-glutamate transport activity (data not shown) when compared to non-GFP EAAT2-transfected cells. Expression of fusion proteins was examined by Western blotting using anti-GFP antibodies (Figure 7A). Both the partial intron 7–retention (Figure 7A, lane 3) and the exon 9–skipping (Figure 7A, lane 4) EAAT2-GFP proteins were expressed at the expected lower molecular weights. Furthermore, the mutant EAAT2-GFPs appeared to be unstable compared to the wild-type EAAT2-GFP (Figure 7A, lane 2), as judged by the presence of less protein and multiple lower molecular weight species (Figure 7A, lanes 3 and 4).

The cellular localization of the fusion proteins was then examined by fluorescent microscopy. Wild-type EAAT2-GFP was preferentially localized to the cell surface (Figure 7B). However, both the partial intron 7–retention and the exon 9–skipping EAAT2-GFP fusion proteins were restricted to the cytoplasm, were often peri-nuclear (Figure 7B), and were found occasionally in lysosomes (data not shown). When wild-type EAAT2-GFP was coexpressed with the partial intron 7–retention EAAT2 (not GFP-labeled), the wild-type protein was found largely confined to the cytoplasm, with occasional cell membrane staining (Figure 7B). The same results were observed from coexpression of partial intron 7–retention

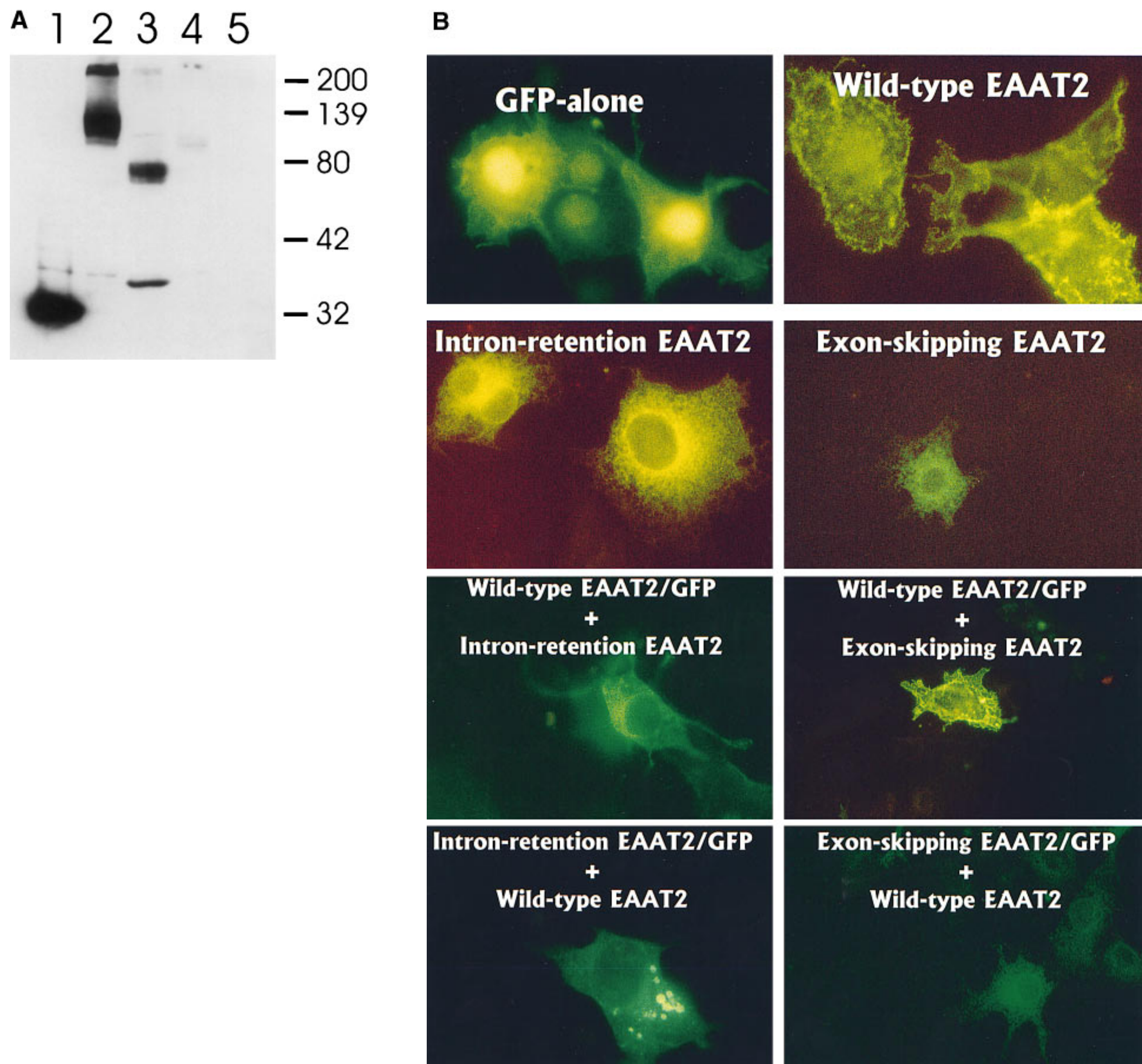


Figure 7. Expression of Normal and Aberrant EAAT2 Fused with Green-Fluorescent Protein (GFP) in COS7 Cells

(A) Immunoblot of COS7 cells expressing EAAT2-GFP fusion protein using anti-GFP antibody. Lane 1, pEGFP vector; lane 2, wild-type EAAT2-GFP; Lane 3, partial intron 7–retention EAAT2-GFP; Lane 4, exon 9–skipping EAAT2-GFP; and lane 5, COS7 cells alone. The partial intron 7–retention and exon 9–skipping mRNA produced truncated, unstable proteins.

(B) Visualization of normal and aberrant EAAT2 protein in COS7 cells. COS7 cells expressing GFP, wild-type EAAT2-GFP, partial intron 7–retention EAAT2-GFP, or exon 9–skipping EAAT2-GFP proteins were examined by fluorescent microscopy. Aberrant proteins (exon-9 skipping-GFP, partial intron 7–retention-GFP expressed alone or with wild-type EAAT2) were largely confined to the cytoplasm, compared to normal surface membrane localization of EAAT2. A coexpression of the GFP-labeled EAAT2 with the unlabeled partial-intron 7–retention EAAT2 revealed that the aberrant protein interfered with the localization of wild-type protein in the surface membrane. Magnification, 400×. All transfection experiments were repeated five times in triplicate. ^{3}H-glutamate transport is expressed as mean transport ± SEM compared to control.

EAAT2-GFP with wild-type EAAT2 (not GFP-labeled) (Figure 7B). These results suggest that the partial intron 7–retention EAAT2 protein may interfere with normal EAAT2 protein complex assembly or trafficking. These results were not seen with the exon-9 skipping EAAT2, which did not interfere with wild-type EAAT2 localization (Figure 7B). Importantly, the abnormal protein localization is probably not due to the influence of the GFP fusion because (1) wild-type EAAT2-GFP–transfected cells had normal glutamate uptake, (2) the wild-type GFP protein was localized on the cell surface when expressed alone or with the exon-9 skipping EAAT2 (Figure 7B), and (3) the partial intron 7–retention EAAT2 (not GFP-labeled) still interfered with the wild-type EAAT2 protein trafficking to the cell membrane.

Eight other aberrant cDNAs were also expressed (described in Figure 1B) in COS7 cells. Two of them, shown at Figures $1B_4$ and $1B_7$, also showed a dominant down-regulation effect on normal EAAT2 activity and protein expression like the partial intron 7–retention *EAAT2*

cDNA, whereas the remaining cDNAs were unstable and inactive like the exon 9–skipping *EAAT2* cDNA.

Discussion

Loss of EAAT2 Protein in ALS Is Due to Aberrant *EAAT2* Transcripts

Functional glutamate transport is deficient in ALS, and a substantial proportion of patients with sporadic disease have a large loss of the EAAT2 glutamate transporter protein. In this study, our results provide evidence that the loss of EAAT2 protein in ALS may be due to aberrant *EAAT2* mRNA, possibly as a consequence of abnormal RNA splicing. The evidence for this conclusion includes the following. (1) The aberrant *EAAT2* transcripts were found only in ALS motor cortex and spinal cord—the only regions where there was a concomitant loss of EAAT2 protein (Figure 2). (2) In situ hybridization studies indicated that loss of EAAT2 protein (Rothstein et al., 1995) in ALS motor cortex appeared to be associated with the presence of the aberrant *EAAT2* transcripts in vivo (Figure 3). (3) S1 nuclease protection assay and quantitative RT-PCR revealed that, in ALS specimens with low EAAT2 protein, the majority of *EAAT2* mRNA was in the form of aberrant splicing products (Figure 4). (4) Screening studies revealed that the aberrant *EAAT2* mRNA is commonly present in sporadic ALS patients. About 65% of all postmortem specimens have one or both of the partial intron 7–retention and exon 9–skipping transcripts. The relative quantity of these abnormal *EAAT2* mRNAs correlated with the degree of EAAT2 protein loss in ALS tissue (Figure 5). (5) Studies of CSF from living patients also independently confirmed their specific association with ALS (Figure 2B). (6) And finally, in vitro expression studies suggested that proteins translated from these aberrant *EAAT2* mRNAs may undergo rapid degradation and produce, in vitro, a dominant negative effect on normal EAAT2 resulting in loss of protein and activity (Figures 6 and 7). Taken together, these studies suggest that the presence of the aberrant *EAAT2* mRNA may directly relate to the loss of EAAT2 protein commonly found in sporadic ALS.

How Does Mutant Protein Dominantly Downregulate Normal EAAT2 Protein?

The mechanism by which the mutant proteins interfere with wild-type EAAT2 activity is not known. Little is known about glutamate transporter membrane organization or trafficking. Limited data suggests that under normal conditions, EAAT2 monomers may self-associate to form homomeric multimers in vivo (Haugeto et al., 1996). It may be that the heteromers composed of mutant truncated EAAT2 and wild-type EAAT2 protein subunits undergo rapid degradation, as suggested by our studies. This could explain the loss of EAAT2 in COS7 cell expression experiments and in ALS, although other processes may be responsible. Examples of such dominant-negative interactions between multimeric proteins is not uncommon. For example, a retained intron for the vitamin D receptor leads to a truncated protein in mice. This protein subsequently forms heterodimers with retinoic acid receptors, ultimately dominantly downregulating vitamin D signaling (Ebihara et al., 1996).

Can Aberrant *EAAT2* mRNA Contribute to Motor Neuron Degeneration?

Interestingly, the aberrant *EAAT2* mRNAs were restricted to motor cortex and spinal cord, the primary regions of neurodegeneration. Because ALS is characterized by the death of motor neurons, it is possible that these aberrant *EAAT2* transcripts are secondary to a motor neuron degeneration, or an astrocyte response to neuronal degeneration, or both. We did not find a loss of EAAT2 or the presence of aberrant *EAAT2* mRNA species in a subset of ALS patients, all of whom had pathological confirmation of motor neuron loss. Neither were they found in other neurodegenerative diseases, such as SMA, Alzheimer's, or Huntington's diseases, which are also characterized by slow loss of neurons (including motor neurons) and astrogliosis. Similarly, the partial intron 7–retention and the exon-9 skipping *EAAT2* mRNA were not found in transgenic mice carrying a familial ALS *SOD1* mutation (G37R) (unpublished data). The aberrant mRNA species were also detected in patient CSF up to 4 years prior to death. Thus, the aberrant mRNA species are not likely to be simply a result of motor neuron loss or postmortem artifact.

Antisense knockdown studies demonstrate that loss of EAAT2 can lead to excitotoxic neuronal degeneration and a progressive syndrome of motor impairment (Rothstein et al., 1996). *EAAT2* null mice also develop excitotoxic neuronal degeneration (Tanaka et al., 1997). Thus, even if aberrant *EAAT2* transcripts are due to an astrocyte response to neuronal degeneration or other factors, the subsequent loss of EAAT2 could act to propagate motor neuron degeneration in ALS. The clinical efficacy of anti-glutamate drugs in ALS and transgenic models of ALS (Bensimon et al., 1994; Gurney et al., 1996; Lacomblez et al., 1996) support the notion that glutamate-mediated toxicity, in part due to the loss of EAAT2, could contribute to the pathogenesis of ALS.

What Is the Origin of the Aberrant *EAAT2* Transcripts?

The origin of the abnormal mRNA has not been determined, but one can speculate on several possible mechanisms. It could result from relaxation of RNA processing fidelity or an abnormality in the pathway for degradation of mutant mRNA. In *C. elegans*, *smg* genes appear to participate in the degradation of some, but not all, mutant mRNAs (Morrison et al., 1997). The presence of multiple aberrant *EAAT2* mRNAs strongly suggests potential defects in RNA processing. Oxidative stress could damage DNA encoding RNA processing proteins or could damage the proteins responsible for RNA processing. Damaged RNA processing proteins could lead to relaxation of RNA processing fidelity, which may contribute to abnormal gene expression. Interestingly, *SMN*, the gene responsible for spinal muscular atrophy, another motor neuron disease, has been found to code for a protein that is a newly described component of the RNA processing complex (Fischer et al., 1997; Liu et al., 1997). SMN protein appears to part of a protein

complex responsible for the assembly of common small nuclear ribonucleoproteins, and as such may participate in the actions of RNA processing. Loss of SMN in spinal muscular atrophy can be lethal and causes motor neuron degeneration, perhaps because of interference with RNA processing. There may be acquired damage to RNA processing proteins, such as SMN protein, which subsequently leads to aberrant splicing of EAAT2 in affected ALS brain regions. Because abnormal pre-RNA processing may be a cause of spinal muscular atrophy, a future evaluation of *EAAT2* RNA in SMA would be interesting.

If these aberrant mRNA species are due to a region- or cell-specific defect in RNA processing, then other aberrantly processed RNA species may be discovered in future studies. EAAT1 is the other astroglial glutamate transporter and has a genomic structure similar to EAAT2 (Hagiwara et al., 1996; Stoffel et al., 1996; Meyer et al., 1997; Aoki et al., 1998). Protein levels for EAAT1 are normal in ALS (Rothstein et al., 1995), and aberrant mRNA species were not seen in the current study.

Alternatively, aberrant EAAT2 could be the result of somatic mutations of the *EAAT2* gene. Mutations at the intron–exon boundaries are often the cause of aberrant RNA splicing. Mutations within intronic regions, although less common, can also produce altered RNA processing. Single mutations in intronic or exonic DNA have been found to result in exon-skipping and intron-retention (Kuivenhoven et al., 1996; Sakai et al., 1996; Wang et al., 1996). In some disorders, such as cholesteryl ester transfer protein deficiency (Sakai et al., 1996), intronic mutations can be distant from the intronic retention, or the exon-skipping regions of the processed RNA, or both. However, a recent extensive study of *EAAT2* exon–intron splice sites in sporadic and familial ALS did not reveal the presence of mutations, ruling out intragenic DNA mutations of *EAAT2* as a cause of the aberrant mRNA species in our study (Aoki et al., 1998). The fact that aberrant *EAAT2* transcripts were found in neuropathologically affected areas in ALS suggests that there could be somatic alterations of the *EAAT2* gene restricted to motor cortex and spinal cord. Previous studies have documented evidence for both oxidative protein damage and DNA damage in sporadic ALS (Bowling et al., 1993; Borthwick et al., 1996; Beal et al., 1997; Ferrante et al., 1997a, 1997b). Oxidative stress could damage DNA encoding EAAT2 or could damage the proteins responsible for DNA repair and eventually lead to somatic alterations of EAAT2. Significantly, loss of EAAT2 can cause excitotoxic neuronal (including motor neuron) degeneration (Rothstein et al., 1996), and excitotoxicity can induce generation of free radicals and DNA damage (Campagne et al., 1995; Didier et al., 1996).

Accumulating evidence suggests that ALS, the prototypical motor neuron disease, may have multiple different pathogeneses. Furthermore, primary defects (e.g., *SOD1* mutations) may initiate a cascade of secondary, but potent, toxic events in the nervous system that are ultimately responsible for motor neuron degeneration. Loss of EAAT2 protein is a potent toxic event because it can lead to excitotoxic neuron degeneration (Rothstein et al., 1996; Tanaka et al., 1997). Our study provides evidence that the loss of EAAT2 protein in ALS could be due to aberrant *EAAT2* transcripts. Disorders of RNA processing, either as a primary defect as in spinal muscular atrophy or secondary to other cellular defects, could be important mechanisms for neurodegenerative disease. Ultimately, identification of the events that lead to aberrant RNA processing could provide an opportunity for the development of specific therapies designed to prevent the loss of glutamate transport in ALS. The observation that these aberrant *EAAT2* mRNA species are commonly present in sporadic ALS and can be detected in the CSF of ALS patients may also provide an important tool for development of a sensitive test for early diagnosis of the fatal disease, allowing for early initiation of treatments designed to prolong survival.

Experimental Procedures

Tissue and CSF Specimens

ALS brain and spinal cord tissue was obtained from the Johns Hopkins ALS Brain Bank and the Alzheimer's Disease Research Center Brain Bank, with institution-approved informed consent. Postmortem delays for autopsy were generally less than 12 hr. ALS and some control tissue was rapidly dissected by either one of us (J.D.R) or by Neuropathology faculty at Johns Hopkins. Dissected regions were rapidly frozen using isopentane/dry ice, and then stored at −75°C until use. Additional specimens were fixed in 4% paraformaldehyde. Pathological confirmation of ALS was made on all specimens by standard histological evaluation of spinal cord and motor cortex, with use of hematoxylin and eosin to evaluate motor neuron loss, and with myelin stains (Luxol-fast blue) to establish corticospinal tract degeneration. *SOD1* mutation analysis was performed by R. H. Brown, Harvard University. Pathologically confirmed Alzheimer's disease and Huntington's disease brain tissue was provided by the Johns Hopkins Alzheimer's Disease Research Center Brain Bank. SMA spinal tissue was obtained at autopsy from patients diagnosed by one of us (T.C.). CSF was obtained at the time of diagnostic evaluation for ALS and control patients with institution-approved informed consent. CSF was rapidly frozen and stored at −20°C to −70°C for up to 6 years.

The clinical diagnosis of ALS was made by the El Escorial criteria (Brooks, 1994), including the presence of both upper and lower motor neuron signs, a definite history of progression, absence of sensory abnormalities, normal nerve conduction velocities and late responses, and electromyographic evidence of diffuse denervation. Patients typically were initially classified as having either probable or definite ALS based on these criteria. Patients were excluded if they had unexplained bowel or bladder changes or anatomic, metabolic, or toxic disorders that could mimic ALS, e.g., endocrine abnormalities, hexosaminidase A deficiency, lead intoxication, myelopathy, or peripheral neuropathy.

Immunoblot

Immunoblots were performed as described previously (Rothstein et al., 1995) on 4–25 μg of crude homogenate from motor cortex or from homogenized transfected COS7 cells grown on 75 mm plastic culture plates (CoStar), with affinity-purified polyclonal antibodies to the carboxyl-terminal region of EAAT2 (Rothstein et al., 1995) or to green fluorescent protein (GFP) (1:2000; Clontech). Glutamate transport was measured as described (Rothstein et al., 1992) except using intact COS7 cells grown on 30 mm, six-well plates (CoStar).

EAAT2 Cloning

Poly (A) mRNA was isolated using the FastTrack 2.0 mRNA isolation kit (Invitrogen). cDNA was synthesized from the isolated mRNA by using ZAP express cDNA synthesis kit (Stratagene). The cDNA was size fractionated into three fractions, >6 kb, 3–6 kb, and <3 kb, by Sephacryl S-500 spin column. Each cDNA fraction was ligated into the ZAP express vector arms and packaged into Gigapack III Gold packing extract (Stratagene). The cDNA library was subsequently screened by a DNA probe prepared from full length *EAAT2* cDNA

by random priming. The positive clones in the pBK-CMV phagemid were obtained after in vivo excision from the ZAP express vector with ExAssist helper phage.

RT-PCR

Poly(A) mRNA was isolated from 50-100 mg of specimens (motor cortex, frontal cortex, hippocampus, caudate, cerebellum, and lumbar or cervical spinal cord) by using Micro-Fast Track Kit (Invitrogen). The isolated mRNA was treated with DNase I for 20 min, heated to 65°C for 5 min, and then reverse transcribed into cDNA. A 25 μl reverse-transcription reaction mixture containing 50 ng of mRNA, 1 mM dithiothreitol, 0.5 mM dNTPs, 20 units of RNasin, 200 units of M-MLV reverse transcriptase (BRL), 1× buffer, and 0.5 μM primer (primer B for the intron-retention and primer C for the exon 9–skipping and the full-length cDNA) was incubated at 42°C for 60 min, heated to 65°C for 5 min, and quick-chilled on ice. PCR was performed at a final concentration of 1× PCR buffer/0.2 mM dNTPs/ 0.5 μM each 5′ and 3′ primers (primers A and B for the intron-retention; primers A and C for the exon 9–skipping; primers D and C for the full length; Figure 1)/2 U of Taq polymerase (Boehringer Mannheim)/2.5 μl of reverse transcription reaction mixture in a total volume of 50 μl. The mixture was overlaid with mineral oil and then amplified with the Delta Cycler II thermal cycler (Ericomp) for 25 or 30 cycles. The amplification profile involved denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min. PCR products (10 μl) were resolved by 1.2% agarose gel electrophoresis and transferred to membrane. The blot was probed with a ^{32}P-labeled *EAAT2* cDNA probe prepared by the random priming method (Figures 2A and 6) or a ^{32}P-end labeled oligonucleotide probe (Figures 2B, 2C, 4A, and 4C) and then subjected to autoradiography. The same RT-PCR conditions were used to analyze *EAAT1* mRNA.

To screen for intron-retention mRNA, a plasmid (pE2F) was constructed for use as an internal control for the comparative analysis of the intron-retention mRNA between patients. A 185 bp DNA fragment with HindIII on both ends was inserted into the intronic mutant cDNA at a HindIII site (indicated in Figure 1, triangle). This plasmid, pE2F, was used as a template for transcription by the T3 polymerase. The resulting pE2F complementary RNA (cRNA) product was purified by oligo(dT) chromatography. Fifty nanograms of mRNA isolated from human tissue and 5 pg of cRNA were combined and then reverse transcribed into cDNA by using primer B (Figure 1). The cDNA mixture was then amplified for 25 cycles. Under these conditions, the reaction rates of pE2F cRNA and intron-retention mRNA were identical within an exponential phase of the PCR reaction. Because the exon-skipping mRNA was 135 bp shorter than the wild-type *EAAT2* mRNA, the wild-type *EAAT2* mRNA was used as an internal control for the comparative analysis of the exon-skipping mRNA between patients. Using the internal controls as standards, the relative amounts of intron-retention and exon 9–skipping mRNA were calculated and compared, between patient samples, by densitometric analysis of Southern blots.

For CSF studies, 400 μl CSF was centrifuged (1500 × g) for 10 min to pellet blood cell debris. Total RNA was isolated from the supernatant using QIAamp kit (Qiagen). Poly(A) mRNA was further isolated and subjected to RT-PCR procedures as described above. EAAT2 is only expressed by CNS tissue. No wild-type or exon 9–skipping *EAAT2* mRNA was detected from the pelleted blood cell debris.

Cloning of PCR Products and Sequencing

PCR products were isolated from agarose gel by using QIAquick Gel Extraction Kit (Qiagen). They were then cloned into a pCR 2.1 vector (TA Cloning Kit, Invitrogen). Plasmid DNA was isolated from transformed cells by using PERFECT prep Plasmid DNA kit (5 prime → 3 prime, Inc.). Sequencing of the DNA was carried out on an Automated DNA Sequencer (Applied Biosystem).

Oligonucleotides

Primers used for RT-PCR of EAAT2 were as follows: Primer A (5′-GGCAACTGGGGATGTACA-3′) corresponding to position 933 from 5′ translation region; Primer B (5′-CCAGAAGGCTCAAGAAGT-3′) corresponding to position 170 from exon 7-intron junction; Primer C (5′-ACGCTGGGGAGTTTATTCAAGAAT-3′) corresponding to position 1768 from the 5′ translation region; Primer D (5′-ACCGTCCTCT GCCACCACTCT-3′) corresponding to position −428. Primers used for RT-PCR of EAAT1 were as follows: 5′-AGGAGGTTTGGCTTTCTG TGG-3′ corresponding to position −73; 5′-GGTTTTTAACACCTGGT GCTCAA-3′ corresponding to position 1655 from the 5′ translation region. Primers used for RT-RCR of SMN were as follows: 5′-GCGGGTTTGCTATGGCGATGA-3′ corresponding to position −11; 5′-CAGCCATGTCCACCAGTTAGA-3′ corresponding to position 1398 from the 5′ trasnslation region. Oligonucleotides used for preparing probes were as follows: 5′-CGGCTGACTTTCCATTGGCTG-3′ corresponding to position 1664 from the 5′ translation region of EAAT2; 5′-CCTGGTGCTCAAGAAAGTGTTTC-3′ corresponding to position 1664 from the 5′ translation region of EAAT1; 5′-TTTTGCCACATAC GCCTCACA-3′ corresponding to position 1369 from the 5′translation region of SMN.

In Situ Hybridization

In situ hybridization was performed with digoxigenin-UTP–labeled RNA probes generated from 1 μg of DNA template using the Maxiscript in vitro transcription kit (Ambion, CA). The probes for detecting wild-type *EAAT2* mRNA and the partial intron 7–retention mRNA are indicated in Figure 1 as probes E and F, respectively. Specificity controls included sense RNA probes and pretreatment of tissue with RNase A (10 μg/ml) in 2× SSC at 37°C for 30 min. An actin control provided with the kit contained ∼250 bp of β-actin fragment in the antisense orientation. Frozen tissue was sectioned (8 μm), fixed with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4), rinsed with PBS, treated with 0.2% Triton X-100, rinsed again with PBS followed by proteinase K^+ (1 μg/ml) in TE buffer (100 mM Tris-HCl, 50 mM EDTA [pH 8.0]) at 37°C for 30 min, then rinsed with 0.1 M glycine in PBS, and again with 4% paraformaldehyde in PBS. The sections were acetylated with 0.25% acetic anhydride in TE with 0.1 triethanolamine, rinsed with DEPC water, air dried, and then hybridized overnight with probe (2 ng/ml) in hybridization buffer (50% deionized formamide, 5× SSC, 10% dextran sulfate, 5× Denhardt solution, 2% SDS, 100 μg/ml denatured salmon sperm DNA). The single-stranded RNA probe was removed with RNase A (10 μg/ml) at 37°C for 30 min. The immunological detection of the digoxigenin-labeled RNA probe was performed using alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) at 1:200 and standard reagents for alkaline phosphatase.

In Vitro Expression and Construction of *EAAT2-GFP* Fusion Gene

Wild-type as well as aberrant cDNA fragments were cloned into a eukaryotic expression vector (pcDNA3). The constructed plasmids were then transfected into COS7 cells or HeLa cells by electroporation. Electroporation (Bio Rad "Gene Pulser II", 300 V at 500 μF) was performed on 1 × 10^7 cells in a solution of DMEM containing 500 μg of salmon sperm DNA along with the pcDNA3 plasmid containing the transporter subtype.

For construction of the *EAAT2-GFP* fusion gene, wild-type or aberrant *EAAT2* cDNA in pcDNA3 was amplified by PCR using (+) strand T7 primer and (−) strand primer (for wild-type and exon-skipping mutant using 5′-GGATCCCGGGCCCTTTCTCACGTTTCCA AGG-3′; for partial intron 7–retention species using 5′-GGATCCCG GGCCCCCTGGAAGCGGTGCCCAG-3′). To create a restriction site for cloning purposes, we added a 13 sequence (shown underlined) at the 5′ ends of the primer to create ApaI. The resultant PCR products were digested with EcoRI and ApaI and then cloned into the N terminus of the *GFP* gene in *pEGFP-N2* vector (Novagen). Sequences of the chimeric genes were checked at their fusion sites.

S1 Nuclease Protection Assay

A 836 base single strand DNA probe was generated by asymmetry PCR using primers A and C. PCR conditions are the same as described above, except 0.05 μM primer A and 0.5 μM primer C were used. The expected single strand DNA was isolated from the gel. For hybridization and S1 nuclease analysis, 0.5 pmol of single strand DNA probe was mixed with 1 μg of the mRNA sample and hybridized

in a solution containing 80% formamide, 0.4 M NaCl, 40 mM piperazine-N,N′-bis (2-ethanesulfonic acid) (PIPES; pH 6.4), and 1 mM EDTA for 16 hr at 55°C. Samples were then diluted 15-fold with ice-cold S1 nuclease buffer (BRL) and treated with 0.5 U of S1 nuclease per microliter at 25°C for 1 hr. The protected fragments were resolved by 1.5% agarose gel electrophoresis and transferred to membrane. The blot was probed with an oligonucleotide probe located at the 3′ end of the protection fragment (described above) and then subjected to autoradiography.

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