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The RNA of the glutamate transporter EAAT2 is variably spliced in amyotrophic lateral sclerosis and normal individuals

Thomas Meyer[a,*], Andrea Fromm[a], Christoph Münch[a], Birgit Schwalenstöcker[a], Anne E. Fray[b], Paul G. Ince[c], Stefan Stamm[d], Georg Grön[e], Albert C. Ludolph[a], Pamela J. Shaw[b]

[a]*Department of Neurology, University of Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Federal Republic of Germany*
[b]*Department of Neurology, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK*
[c]*Department of Neuropathology, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK*
[d]*Max Planck Institute of Neurobiology, 82152 Martinsried, Germany*
[e]*Department of Psychiatry, University of Ulm, Ulm, Federal Republic of Germany*

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Abstract

Impaired re-uptake of synaptic glutamate, and a reduced expression of the glutamate transporter EAAT2 have been found in the motor cortex of patients with amyotrophic lateral sclerosis (ALS). Two splice forms of the EAAT2 RNA resulting from retention of intronic sequences (EAAT2/Int) and deletion of one protein coding exon (EAAT2/C1) have been reported to account for the EAAT2 protein loss in ALS. In this study we investigated the presence of two known (EAAT2/C1; EAAT2/Int) and three novel (EAAT2/C2-4) EAAT2 RNA in motor cortex of 17 ALS cases and 11 controls. Reverse transcription and PCR were carried out to amplify the complementary DNA of the complete and variably spliced EAAT2 transcripts. Nested PCR was followed to generate amplicons specific for EAAT2/C1-4 and EAAT2/Int. EAAT2/Int was detected in 59% of ALS specimens as compared to 36% of controls showing a trend but no statistical significance of a more frequent expression in ALS (Type I error 24.6%). EAAT2/C1-4 were found to be equally expressed in ALS patients and controls. Our results indicate that the involvement of EAAT2 transcripts in ALS is unlikely to be primary, and more complex than previously recognized. Alterations of quantitative expression of distinct EAAT2 splice forms in ALS cannot be excluded from this study and remain to be investigated.

Keywords: Amyotrophic lateral sclerosis; Glutamate; Excitatory amino acid transporter; Splicing; RNA; EAAT2

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset and fatal degenerative disorder of motor neurons that is characterized by progressive muscle wasting, weakness, and spasticity [1]. About 15% of familial ALS cases, i.e. 1 to 2% of all ALS patients, are related to mutations in the Cu/Zn superoxide dismutase gene [2]. For the majority of patients the cause of disease is unknown. Glutamate-mediated excitotoxicity has been suggested as a contributing factor to the pathogenesis of motor neuron degeneration in ALS. Interestingly, an alteration of the AMPA receptor subunit GluR-B in a mouse model has been shown to result in a late-onset motor neuron disease [3]. Impaired re-uptake of synaptic glutamate [4], and a substantial reduction in the expression of the glial glutamate carrier EAAT2, have been identified in the motor cortex and spinal cord of some ALS patients [5]. The screening of a complementary DNA library constructed from the motor cortex of a single ALS patient with a very low EAAT2 protein level resulted in the detection of abnormal EAAT2 transcripts [6]. Two of these transcripts have been reported to be specifically associated with ALS in 60% of studied patients and to account for the loss of

*Corresponding author. Tel.: +49-731-502-4569; fax: +49-731-502-4559.

E-mail address: thomas.meyer@medizin.uni-ulm.de (T. Meyer)

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the EAAT2 protein in this disease. One transcript originated from the deletion of a protein coding exon, and has been named 'exon-skipping' RNA [6]. A second transcript was characterized by retained intronic sequences which would result in truncation of the putative EAAT2 polypeptide. This transcript has been named 'intron-retention' RNA [6]. Previously, we cloned from normal human brain four novel EAAT2 RNA species, named EAAT2/C1-4, that showed a deletion of complete or partial exon sequences [7,8]. Interestingly, EAAT2/C1 was identical with the 'exon-skipping' RNA reported by Lin et al. [6]. This correlation was not recognized immediately, because two different preliminary nomenclatures of the EAAT2 genomic structure are in use [9,10]. Since the EAAT2/C1-4 transcripts were cloned from normal human brain the association of distinct EAAT2 RNA with ALS has remained uncertain. In this study we analyzed the presence of the EAAT2/C1-4 and the 'intron-retention' RNA of EAAT2 in ALS patients and controls.

2. Materials and methods

2.1. Patient characteristics

Post mortem tissue samples of motor cortex from 17 ALS cases and 11 controls was studied. The ALS patients all met the El Escorial criteria for definite or probable ALS [11]. The causes of death in the control group were ischemic heart disease (seven), emphysema (one), chronic obstructive airways disease (one), bronchopneumonia (one), and cancer of the uterus (one). The mean age in the ALS and control group was 70.0 (±18.0) and 67.3 (±21.3) years, respectively. The mean post mortem delay for the ALS and control group was 23.5 (±38.5) and 20.3 (±22.7) h, respectively. In all ALS and control patients immunohistochemistry analysis of the expression of the EAAT2 protein was performed as reported in full elsewhere [12].

2.2. Molecular methods

The guanidinium thiocyanate method was used for total RNA isolation from each of the tissue specimens [13]. The determination of the amount of total RNA was achieved by photometric spectroscopy (Biolumin 960, Molecular Dynamics). Total RNA (500 ng) was aliquoted for subsequent reverse transcription. For the reverse transcription and PCR amplification of the EAAT2/C1-4 transcripts, primers at the 3′ and 5′-end of the EAAT2 RNA were constructed (Table 1). Reverse transcription was carried out for 1 h at 42°C using 200 U reverse transcriptase (SuperScript II, GIBCO-BRL). The reverse transcription product was passed through 30 cycles of PCR amplification under conditions as described previously [7,14]. PCR was performed using a three-step program consisting of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min. From the final reaction volume of 50 μl an equal amount of 1 μl was aliquoted for nested PCR amplification. The second PCR amplification was performed using the transcript-specific primers as depicted in Fig. 1. Nested PCR amplifications were reproduced in separate reactions for each of the ALS and control individuals. PCR products were analyzed by electrophoresis through a 5% non-denaturing polyacrylamide gel, followed by fixation and silver staining (Fig. 2).

2.3. Molecular characteristics of EAAT2 RNA

Five of several known EAAT2 RNA species were included in this study. The molecular characteristics have been reported in detail elsewhere [6–8]. In this report, the EAAT2 transcripts were related to the genomic structure of the EAAT2 gene as described previously [10]. EAAT2/C1 and EAAT2/C3 resulted from the deletion of the complete exons CDS 8 and CDS 6, respectively. EAAT2/Int is the name we used for the 'intron-retention' RNA. This transcript was not spliced at the exon–intron boundary but 1008 nucleotides downstream in the intron following exon CDS 6. For primer construction in EAAT2/Int the sequence information reported by Aoki et al. was used [9]. EAAT2/C1 ('exon-skipping' RNA) and EAAT2/Int ('intron-retention' RNA) have been reported to be specifically detected in the motor cortex and spinal cord of ALS patients [6]. The expression of the other transcripts EAAT2/C2, EAAT2/C3 and EAAT2/C4 have not been examined in relation to ALS.

2.4. Statistical methods

The observed presence of EAAT2-transcripts were tested for group independence by means of χ^2-statistics. In cases of cell frequencies lower than five, one-tailed *P*-values of Fisher's exact test are reported. As nominal level of significance an alpha of $P<0.05$ was accepted. Post-hoc analyses were computed to test whether the variable age significantly differed between those groups showing presence of PCR products for distinct EAAT2-transcripts by means of a one-way analysis of variance.

3. Results

The reverse transcription and primary PCR of EAAT2 resulted in a weak signal of 1.7 kb corresponding to the full length complementary DNA [15], and signals of lower size and intensity in all 17 ALS patients and 11 controls. Subsequent nested PCR resulted in transcript-specific products of expected length in each of the reactions (Table 1; Fig. 2). The primary and nested PCR products of EAAT2/Int were in agreement with the expected DNA

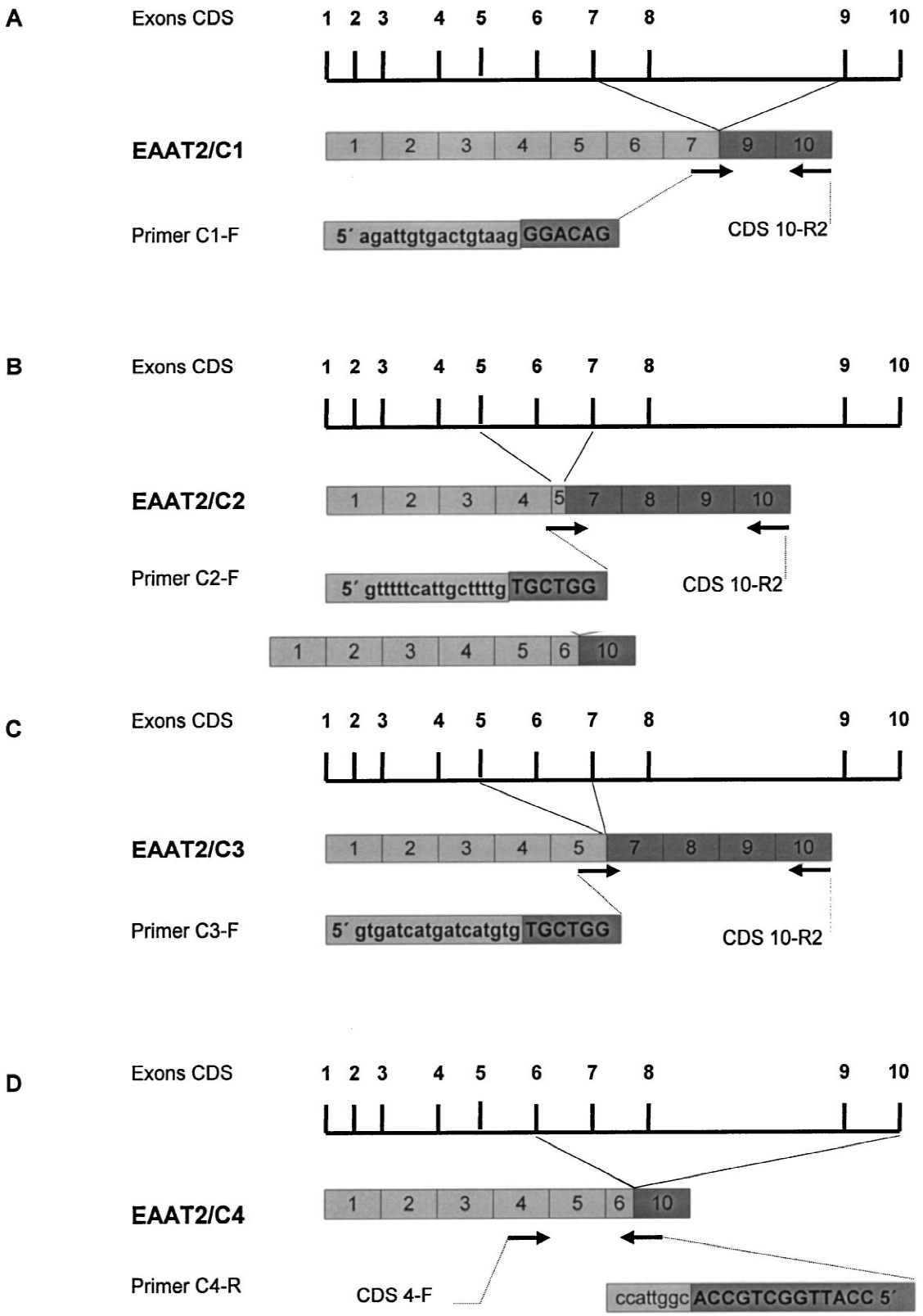

Fig. 1. Structure of glutamate transporter mRNA EAAT2/C1-4 and presentation of transcript-specific primer construction. Schematic presentation of variable EAAT2 genomic structure [8]. Numbered lines represent individual protein coding exons. The corresponding mRNA sequences are depicted as shaded and numbered boxes. The arrowheads indicate the primer binding region used for PCR. The sequence of transcript-specific primers is displayed in connection to the arrowheads. Transcript-specific priming is achieved by bridging novel sequence boundaries as symbolized by light and dark grey boxes.

Table 1
Primer used for reverse transcription and PCR[a]

Transcript	Reverse transcription	PCR	Nested PCR	Nested PCR product (nucleotides)
EAAT2/C1	CDS 10-R1	CDS 10-R1/CDS 1F	CDS 10-R2/C1-F	375
EAAT2/C2	CDS 10-R1	CDS 10-R1/CDS 1F	CDS 10-R2/C2-F	721
EAAT2/C3	CDS 10-R1	CDS 10-R1/CDS 1F	CDS 10-R2/C3-F	707
EAAT2/C4	CDS 10-R1	CDS 10-R1/CDS 1F	C4-R/CDS 4F	418
EAAT2/Int	Int-R1	Int-R1/CDS 6F1	Int-R2/CDS	688

[a] Primer pairing for reverse transcription and PCR amplification of partial sequences of distinct EAAT2 transcripts. Primer design was based on the sequence information of the EAAT2 complementary DNA [8,15]. Primer sequences: CDS 1-F: 5′ CAACAATATGCCCAAGCAGGTG; CDS 4-F 5′ TTCAACAGATTCAAACAGTGACG; CDS 6-F1: 5′ GATGAACGTCTTAGGTCTGATAG; CDS 6-F2: 5′ CACCGCTTCCAGGTAGAGAAC; CDS 10-R1: 5′ CAGTTACCATAGGATACGCTGG; CDS 10-R2: 5′ AAGAATTTGCTGAGACTCATATCC; EAAT2/C1: 5′ AGATTGTGACTGTAAGGGACAG; EAAT2/C2: 5′ GTTTTTCATTGCTTTTGTGCTGG; EAAT2/C3: 5′ GTGATCATGATCATGTGTGCTGG; EAAT2/C4R: 5′ CCATTGGCTGCCACTGTTACC; Int-R1: 5′ TGCTGGGATTACAAGCATGAGC; Int-R2: 5′ GATTCAGTCCAAGATGATAGTCC.

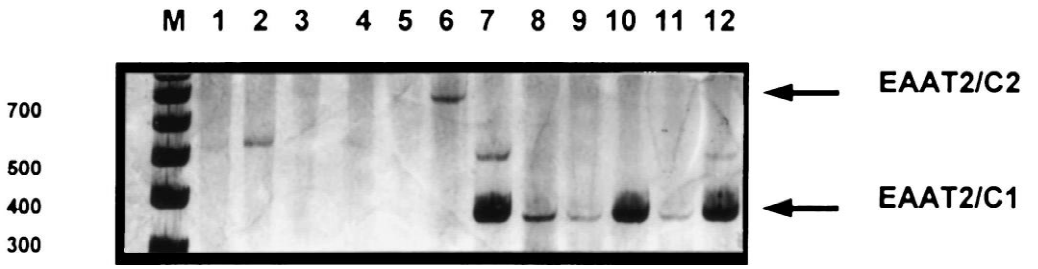

Fig. 2. PCR amplification of complementary DNA fragments specific for EAAT2/C1 and EAAT2/C2. Lanes 1–6 show PCR amplification products of EAAT2/C2 in motor cortex specimens of control individuals (lanes 1, 3, 5) and ALS patients (lanes 2, 4, 6). In one ALS patient (lane 6) a EAAT2/C2-specific PCR product is found whereas in other individuals (lanes 1–5) this transcript is not identified. In lanes 7–12 PCR amplicons are analyzed showing specific bands for EAAT2/C1 in control (lanes 7, 9, 11) and ALS (lanes 8, 10, 12) patients. Amplification products of unexpected size are regarded as being unspecific (lanes 2, 7, 12). M, molecular weight marker (number of base pairs).

fragments of 911 and 688 nucleotides, respectively. The presence of individual EAAT2 transcripts in ALS and controls as shown by PCR is summarized in Table 2. None of the EAAT2 transcripts were shown to be exclusively expressed in the ALS or control group. EAAT2/Int was detected in half of all motor cortex samples with a more frequent expression in ALS (59%) as compared to controls (36%; Table 2). In contrast, EAAT2/C1-4 were found to be equally expressed in ALS patients and controls. EAAT2/C1 was present in most of the motor cortex samples (89%) whereas EAAT2/C3 and EAAT2/C4 were detected at lower rates (46 and 32%). EAAT2/C2 was only rarely found in the studied samples (11%). To test whether the presence of EAAT2 transcripts were group dependent, statistical analyses revealed no significant difference in the frequency distributions ($P>0.05$). However, comparing the various P-levels the presence of EAAT2/Int (Table 2) indicated a trend towards a more frequent expression of this transcript in the ALS group (Type I error probability=24.6%). Comparing the expression rates of EAAT2/C1-4 and EAAT2/Int with respect to the age of patients, one-way analysis of variance revealed no significant age differences between the various groups ($F(4,57)=0.49$; $P=0.74$), thus ruling out an effect of age on the expression pattern of studied EAAT2 transcripts.

To investigate EAAT2/C1-4 further, we analyzed their splicing sites using a scoring system that expresses the coincidence of a splice site to the mammalian splice site consensus sequence [16]. In EAAT2/C1 and EAAT2/C3, the 5′ and 3′ splice scores were found to be 4.5 and 8.8 for exon CDS 6, and 6.0 and 7.2 for CDS 8, respectively. Comparison with the average mammalian 5′ and 3′ scores

Table 2
Distribution of variable EAAT2 transcripts in motor cortex of ALS patients and controls as shown by reverse transcription and PCR.

EAAT2 RNA Species	ALS		Control			
	Present	Absent	Present	Absent	Chi^2	P Value
EAAT2/C1	15 (0.88)	2 (0.12)	10 (0.91)	1 (0.09)	0.049	0.823
EAAT2/C2	2 (0.12)	15 (0.88)	1 (0.08)	11 (0.92)	0.049	0.823
EAAT2/C3	8 (0.47)	9 (0.53)	5 (0.45)	6 (0.55)	0.007	0.934
EAAT2/C4	5 (0.29)	12 (0.71)	4 (0.36)	7 (0.64)	0.148)	0.700
EAAT2/Int	10 (0.59)	7 (0.41)	4 (0.36)	7 (0.64)	1.348	0.246

of 8.0 and 9.4 indicates that both exon CDS 6 and 8 are surrounded by weak splice sites and might be subject to regulation by alternative splicing. In contrast, in EAAT2/C2 and EAAT2/C4 the deleted sequences were not flanked by consensus splice sites, and may thus be derived from allelic variation, or a yet unknown form of RNA processing.

4. Discussion

Aberrant splicing of the EAAT2 RNA has been reportedly associated with about 60% of studied ALS patients [6]. For two EAAT2 transcripts a dominant negative effect on wild-type EAAT2 expression has been described which accounted for the reported EAAT2 protein loss in a group of ALS patients [6]. The results of our study indicate that the expression of variably spliced EAAT2 RNA species in ALS is more complex than previously recognized. None of the analyzed EAAT2 transcripts were found to be ALS-specific. All studied transcripts were amplified from both diseased and normal motor cortex. Therefore, a primary involvement of EAAT2/C1-4 and EAAT2/Int in ALS is unlikely. Interestingly, EAAT2/Int showed a trend towards a more frequent amplification from ALS tissue suggesting that an altered expression of this transcript may be related to the disease process. However, the results of EAAT2/Int expression did not reach statistical significance making the investigation of an extended number of ALS and control samples desirable. EAAT2/C1-4 were detected in different frequencies in the human motor cortex. The findings are in correspondence with the primary reports on these splice forms showing an abundant expression of EAAT2/C1 and EAAT2/C3, whereas EAAT2/C2 and EAAT2/C4 were found less frequently [7,8]. The expression analysis of EAAT2 RNA species was normalized at the level of reverse transcription and nested PCR amplification. However, this study was not designed to meet the criteria of quantitative PCR analysis. Differences in the expression level of this and other transcripts in ALS cannot be excluded from this study. A quantitative expression analysis of the various EAAT2 splice variants remains to be undertaken but is important to further determine the pathogenetic significance of EAAT2 splicing for the reported glutamate transport defect in ALS [4–6]. Similarly, there is a need to perform expression studies of EAAT2 splice forms in the spinal cord where loss of EAAT2 is also reported [12]. Sequence analysis and splice score calculation suggested that EAAT2/C1 and EAAT2/C3 resulted from alternative splicing of cassette exons [16,17]. In EAAT2/C2 and EAAT2/C4 splicing occurred independently from the splice consensus sequence and may represent an abnormal or unknown form of RNA splicing [18]. Based on two immunochemical studies using different antibodies against EAAT2, there is no evidence that variably spliced EAAT2 are translated into novel EAAT2 polypeptides [5,12]. It is currently more likely, that alternatively spliced RNA species are part of the post-transcriptional gene regulation of EAAT2. However, more detailed molecular characteristics such as RNA half-life, differential expression patterns, and the functional role of variable EAAT2 transcripts are currently not fully understood. Much further work is required to obtain a comprehensive picture of EAAT2 RNA processing under normal conditions and in motor neuron degeneration.

Acknowledgements

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References

[1] Tandan R. Clinical features and differential diagnosis of classical motor neuron disease. In: Williams AC, editor, Motor neuron disease, London: Chapman and Hall Medical, 1994, pp. 1–27.
[2] Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993;362:59–62.
[3] Feldmeyer D, Kask K, Brusa R, Kornau HC, Kolhekar R, Rozov A, Burnashev N, Jensen V, Hvalby O, Sprengel R, Seeburg P. Neurological dysfunctions in mice expressing different levels of the Q/R site-unedited AMPAR subunit GluR-B. Nat Neurosci 1999;2:57–64.
[4] Rothstein JD, Martin LJ, Kuncl RW. Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. N Engl J Med 1992;326:1464–8.
[5] Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Ann Neurol 1995;38:73–84.
[6] Lin CL, Bristol LA, Jin L, Dykes-Hoberg M, Crawford T, Clawson L, Rothstein JD. Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. Neuron 1998;20:589–602.
[7] Meyer T, Munch C, Knappenberger B, Liebau S, Volkel H, Ludolph AC. Alternative splicing of the glutamate transporter EAAT2 (GLT-1). Neurosci Lett 1998;241:68–70.
[8] Meyer T, Munch C, Liebau S, Fromm A, Schwalenstöcker B, Völkel H, Ludolph AC. Splicing of the glutamate transporter EAAT2: a candidate gene of amyotrophic lateral sclerosis. J Neurol Neurosurg Psychiatry 1998;65:954.
[9] Aoki M, Lin CL, Rothstein JD, Geller BA, Hosler BA, Munsat TL, Horvitz HR, Brown Jr. RH. Mutations in the glutamate transporter EAAT2 gene do not cause abnormal EAAT2 transcripts in amyotrophic lateral sclerosis. Ann Neurol 1998;43:645–53.
[10] Meyer T, Ludolph AC, Morkel M, Hagemeier C, Speer A. Genomic organization of the human excitatory amino acid transporter gene GLT-1. Neuroreport 1997;8:775–7.
[11] Brooks BR. El Escorial World Federation of Neurology criteria for the diagnosis of amyotrophic lateral sclerosis. Subcommittee on Motor Neuron Diseases/Amyotrophic Lateral Sclerosis of the world Federation of Neurology Research Group on Neuromuscular Diseases and the El Escorial 'Clinical limits of amyotrophic lateral sclerosis' workshop contributors. J Neurol Sci 1994;124(Suppl):96–107.

[12] Fray AE, Ince PG, Banner SJ, Milton ID, Usher PA, Cookson MR, Shaw PJ. The expression of the glial glutamate transporter protein EAAT2 in motor neuron disease: an immunohistochemical study. Eur J Neurosci 1998;10:2481–9.
[13] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem 1987;162:156–9.
[14] Meyer T, Speer A, Meyer B, Sitte W, Kuther G, Ludolph AC. The glial glutamate transporter complementary DNA in patients with amyotrophic lateral sclerosis. Ann Neurol 1996;40:456–9.
[15] Arriza JL, Fairmann WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. J Neurosci 1994;14:5559–69.
[16] Stamm S, Zhang MQ, Marr TG, Helfman DM. A sequence compilation and comparison of exons that are alternatively spliced in neurons. Nucl Acids Res 1994;22:1515–26.
[17] Smith CW, Patton JG, Nadal-Ginard B. Alternative splicing in the control of gene expression. Annu Rev Genet 1989;23:527–77.
[18] Moore JM, Query CC, Sharp PA. Splicing of precursors to mRNA by the spliceosome. In: Gesteland RF, Atkins JF, editors, The RNA world, New York: Cold Spring Harbour Laboratory Press, 1993, pp. 303–57.