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Alternative splicing of the 5′-sequences of the mouse EAAT2 glutamate transporter and expression in a transgenic model for amyotrophic lateral sclerosis

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Abstract

Glutamate-mediated neurotoxicity and a reduced expression of the excitatory amino acid transporter 2 (EAAT2) have been described in the pathogenesis of several acute and chronic neurological conditions. EAAT2 is the major carrier of glutamate in the mammalian brain. However, the principles of EAAT2 expression regulation are not fully understood. For the human brain, extensive alternative splicing of the EAAT2 RNA has been shown. To delineate the complex RNA regulation of EAAT2 we investigated whether the murine species is a suitable model for the study of EAAT2 splicing events. We identified five splice variants (mEAAT2/5UT1–5) encoding different 5′-untranslated sequences and two distinct N-termini of the putative EAAT2 polypeptide. In the murine CNS we found a region-specific expression pattern of the novel 5′-variants of EAAT2 as shown by *in situ* hybridization, dot blotting and competitive reverse transcription polymerase chain reaction. Furthermore, we performed an expression analysis of the EAAT2 splice variants in the spinal cord of a transgenic model (SOD1G93A) of amyotrophic lateral sclerosis, a motor neurone disease for which altered splicing of EAAT2 has been discussed. We found an increased expression of mEAAT2/5UT4 and a reduction of mEAAT2/5UT5 in the early course of the disease. We conclude that alternative splicing of 5′-sequences may contribute to the regional expression of the EAAT2 RNA and was altered in the presymptomatic stage of the SOD1G93A-mouse model for amyotrophic lateral sclerosis.

Keywords: alternative splicing, excitatory amino acid transporter, glutamate, mouse, SOD1.

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The high-affinity glutamate transporter EAAT2 mediates rapid removal of the excitatory neurotransmitter glutamate from the synaptic cleft. This process modulates the termination of glutamatergic synaptic signalling and prevents excitotoxic effects of glutamate on post-synaptic neurones (Nicholls and Attwell 1990). Five members of the gene family of excitatory amino acid transporters have been cloned from the human, rat and mouse brain (EAAT1–5) showing distinct regional, cellular and subcellular expression patterns (Arriza *et al*. 1994, 1997; Fairman *et al*. 1995). The major carrier of synaptic glutamate is the excitatory amino acid transporter 2 (EAAT2) serving more than 90% of total glutamate uptake (Tanaka *et al*. 1997). EAAT2 is an astroglial protein of transmembrane localization which is predominantly expressed in the cerebral cortex, the hippocampus, the caudate nucleus, the nucleus basalis of Meynert and the spinal ventral horn and with lower levels of expression throughout the mammalian CNS (Milton *et al*. 1997).

A reduced EAAT2 protein expression has been found in acute and chronic neurological conditions such as cerebral ischaemia (Torp *et al*. 1995), Alzheimer's disease (Li *et al*. 1997), amyotrophic lateral sclerosis (ALS; Rothstein *et al*. 1995) and the transgenic mouse model of

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Abbreviations used: EAAT2, excitatory amino acid transporter 2.

the autosomal-dominant form of ALS (Bruijn *et al*. 1997). Moreover, in ALS, aberrant splicing of the EAAT2 RNA has been reportedly associated with the loss of EAAT2 protein in the motor cortex and spinal cord of ALS patients (Lin *et al*. 1998). We and other groups have cloned alternative and aberrant EAAT2 splice forms in both ALS and the normal human brain (Meyer *et al*. 1999; Honig *et al*. 2000). There is currently no evidence for disease-specific splice variants of EAAT2 in ALS. However, it is not known whether the complex regulation of normal EAAT2 splice forms is disturbed in the pathogenesis of ALS. We have already identified extensive alternative splicing of protein coding and untranslated exons and differential polyadenylation/cleavage of the 3′-sequences of the EAAT2 transcripts in the normal human brain (Meyer *et al*. 1998; Münch *et al*. 1998, 2000).

It was the aim of this study to identify alternative splicing events of the EAAT2 RNA in the mouse CNS and to determine whether the murine species is a suitable model to study alternative EAAT2 splicing under normal and pathogenetic conditions. Based on the molecular characterization of the murine EAAT2 splice variants, we studied alternative splicing of EAAT2 in a transgenic model of familial ALS harbouring a disease-associated mutation (G93A) in the superoxide dismutase 1 (SOD1; Gurney *et al*. 1994). Expression analysis of five EAAT2 5′-splice variants showed a pre-symptomatic alteration in the expression of two EAAT2 transcripts in the SOD1G93A-model. These findings suggest that the expression regulation of EAAT2 splice variants may be altered in the early course in the studied model of chronic motor neurone degeneration.

Materials and methods

5′-Rapid amplification of cDNA ends

For 5′-rapid amplification of cDNA ends (RACE) we used mouse brain cDNA ligated with a 5′-adaptor sequence (Clontech, Palo Alto, CA, USA). The 5′-ends of EAAT2 cDNA (accession number AB007810; Utsunomiya-Tate *et al.* 1997) were polymerase chain reaction (PCR)-amplified using an adaptor primer AP1 (Clontech, Palo Alto, CA, USA) and a 5′-gene specific primer GSP1 [5′-GAAGGCTATCAA CATGACCACATC-3′, nucleotides (nt) 876–899]. The PCR consisted of a two-step programme of five cycles at 94°C (45 s) and 70°C (4 min) followed by 25 cycles at 94°C (20 s) and 68°C (4 min), respectively. The resulting product was template of a second amplification using a nested adapter primer AP2 (Clontech) for the adaptor sequence and a 5′-gene-specific primer GSP2 (5′-CATGCGCATGCCCAGGTTTC-3′, nt 751–770). The PCR product was subcloned (pCR2.1, TA cloning kit, Invitrogen, Carlsbad, CA, USA) and insert-containing constructs were sequenced (ABI 377 DNA sequencer, Applied Biosystems, Foster City, CA, USA).

Oligodeoxynucleotides for competitive PCR

Primers for competitive PCR of the murine EAAT2 gene were synthesized as follows. Based on the published coding sequence (AB007810): CDS1 (5′-GCTGTGGGCCTGCCAACG-3′, nt 2022–2039), CDS2 (5′-GGTTCTTCCTCAACACTGCAG-3′, nt 2339–2359), CDS3 (5′-GATTTCTAATGAGATCCAGGAAG-3′, nt 1160–1182) and 5UT1 (5′-CATCAACAGAGGGTGCCAAC-3′, nt 664–683).

Based on the novel 5′-untranslated sequences of EAAT2 (Fig. 1) the following primers were synthesized: 5UT2 (5′-CCCTGGCATT-TAGGAGTTGC-3′, nt 163–182 based on mEAAT2/5UT2), 5UT3 (5′-GAGCCTTGATTCCGGATTCAG-3′, nt 306–326 based on

mEAAT2/5UT1

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1   ccccctcgag gtcgacggta tc(...)ggg gggtgcgagg gcggagagag gctgcccgtt
573 aaataccgct ctccgccgca ctccgggctc acccagctcg ccgccaccgt ctccagaccg
633 tgcccgggag agggggcgtt cccacgcatg gcatcaacag aggg|tgccaa caatatgccc
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mEAAT2/5UT2

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1                                              ccggg agaggggcg ttcccacgca
26  tggcatcaac agaggg|GAGG AGGTAAAATG TCGTTACTGT GCTGAGCAGT GCTCCTTGCT
86  GTTCCGTCTT ATGGGGAAGG GTCTGCAGGG ACCAGCCGTT CACCTTGGCT CCTCTTCGCT
146 GGCTAAGGGC CTTGTTCCCC TGGCATTTAG GAGTTGCATA AAAG|tgccaa caatatgccc
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mEAAT2/5UT3

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1                    TG ATGACCACAG GCCGGGCAAT GGGATTTTCC TGCCACTCTA
43  AAGCAAGAGC CAGAGGGTGA ATCGCTCTTT CCCAGTCCAG CACCAGCTGA CCCCTGGATT
103 TTAAAGAAGG AAAGGCCTGA TACTTTGATT TTCAAACATT CCAAGTTACC CTGAGTTGTC
163 AAATCAGCCA GAGATCCAGA GTTCTTCGGG GACTACCTTT TGCTGGGAGG AATTTGGCAG
223 TCTTGGTTCT TGTTACCCAT TTTGGCCCTA GATCCTCTCT CTCACGTTGT GCTCCTGGAT
283 TCTCCTCAGC CAAGTGCAAG AAGGAGCCTT GATTCCGGAT TCAG|tgccaa caatatgccc
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mEAAT2/5UT4

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1   GACGAGGGTC TCATACTGAA CTTGAAGTTC ATCCTTGGCT AGGCTGGGTG GCCGGCGAGC
61  TTCCGGAGAC TGCCTGCTCT CCCATGACAG TATTGGAGTC ACAG|tgccaa caatatgccc
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mEAAT2/5UT5

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1                              GTTG GACCAGTCAT CTGTTTCACG TCCCATTCTC
35  TTGAAGTCCA AACTTTGTCC ACTGACTTCA GCAAATCGGA CATGGACAAG GCTCAGGCCT
95  TTCTAAGGAC ATCATGTACA CCCAGAGGCC AGACTGAGGC CAGAACTTTG ATCTCTGTGC
155 CAAGGATGCT CTGCGTTGGA CCTGCTGTGC TGTGATGATC AGAG|tgccaa caatatgccc
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Fig. 1 Nucleotide sequence of the constitutively (mEAAT2/5UT1) and alternatively spliced (mEAAT2/5UT2–5) mouse EAAT2 RNA (5′ partial sequence). The exon boundaries are indicated by vertical lines. The upstream start codons (uATG) and the translational start codon (ATG) are shown in bold and the stop codons are underlined. Lower cases indicate the known 5′-sequences.

mEAAT2/5UT3), 5UT4 (5′-TCCCATGACAGTATTGGAGTCAC-3′, nt 80–102 based on mEAAT2/5UT4) and 5UT5 (5′-AGAACT-TTGATCTCTGTGCC-3′, nt 136–155 based on mEAAT2/5UT5). For the amplification of the murine ribosomal S12 gene (MMRPS12) the primers S12s (5′-TCGCATCCAACTGTGATGA-GCC-3′, nt 211–232) and S12as (5′-CCTGAGATTCTTTGCCA-TAGTC-3′, nt 393–414) were used.

Competitive RT–PCR

The quantitative expression of the EAAT2 splice variants mEAAT2/5UT1–5 (Fig. 2) was studied using competitive reverse transcription polymerase chain reaction (RT–PCR). Total RNA was isolated from mouse frontal cortex, hippocampus, cerebellum and spinal cord (mRNA isolation kit, Perkin-Elmer, Foster City, CA, USA). One milligram was used as template for reverse transcription with random primers at 42°C for 50 min (RNA PCR core kit, Perkin-Elmer). For competitive PCR we designed primers specific for each of the mEAAT2/5UT1–5 transcripts (5UT1–5). We performed a multiplex amplification of splice variant-specific and of two standard RNA sequences (mEAAT2/CDS, 40S ribosomal protein S12 RNA). mEAAT2/CDS represents a highly conserved part of the EAAT2 coding sequence comprising the total amount of all known splice forms of the transporter RNA and was amplified using the primers CDS1 and CDS2. As an additional standard RNA we co-amplified the 40S ribosomal protein S12, a murine housekeeping gene with the primers S12s and S12as. The PCR was carried out using 10 pmoles of each primer and a programme consisting of denaturation at 94°C (15 s), annealing at 60°C (30 s) and of extension at 72°C (45 s) (AmpliTaq, Perkin-Elmer). Then, 5 μL of PCR products were removed after 28, 30, 32, 34, 36, 38 and 40 cycles, respectively, separated on a 2.5% agarose gel and visualized by ethidium-bromide staining. Images were captured by UV-translumination (GelDoc1000, Bio-Rad, Hercules, CA, USA). The optical density values of PCR signals were quantified using an image analysis system (MultiAnalyst Software, Bio-Rad). Quantitative expression of EAAT2 transcripts was determined at cycle 38 during linear regression of amplification in relation to the internal standard S12 RNA. The values were expressed as a ratio of mEAAT2/5UT1–5 and S12.

In situ hybridization analysis

Animals were killed by rapid decapitation, the brain and spinal cord were removed immediately, frozen on dry ice, and stored at − 70°C. Sections (12-mm) were cut using a cryostat and also kept frozen at − 70°C until further processing. For *in situ* hybridization of the variable spliced 5′-untranslated sequences of EAAT2 radioactively labelled RNA probes were synthesized by *in vitro* transcription using SP6/T7 polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 35S-CTP (> 1000 Ci/mmol, Amersham, Uppsala, Sweden). As template we constructed transcript-specific oligonucleotides encompassing the target sequence (Fig. 1 and AB007810) and the SP6/T7 promoter sequence as follows: probe 1 complementary to nt 212–303 of mEAAT2/5UT1, probe 2 nt 91–182 of mEAAT2/5UT2, probe 3 nt 238–326 of mEAAT2/5UT3, probe 4 nt 9–102 of mEAAT2/5UT4, probe 5 nt 81–175 of mEAAT2/5UT5.

The probes were isolated by precipitation in ethanol and incorporation of the radioactive label was assessed by liquid scintillation counting. Slide-mounted sections were fixed in 4% paraformaldehyde for 10 min. The sections were washed in 0.1 M phosphate-buffered saline (PBS; 0.1 M, pH 7.4) three times (5 min each), acetylated (0.25% acetic anhydride in 0.1 M triethanolamine) for 10 min, rinsed in PBS (5 min), dehydrated in ethanol and delipidated in chloroform. Sections were then covered with 80 mL of hybridization mixture containing the specific 35S-labelled probe (150 000 cpm/80 mL/section), 50% formamide, 0.3 M NaCl, 10 mM Tris, pH 8.0, 5 mM EDTA, 10% dextran sulfate, 1 × Denhardt's solution, 100 mM dithiothreitol (DTT), 0.1% sodium dodecyl sulphate (SDS), 0.1% sodium thiosulfate, 100 mg/mL salmon sperm DNA and 250 mg/mL yeast tRNA for 4 h at 50°C. After hybridization the sections were washed in 2× saline–sodium citrate (SSC) at room temperature briefly, and than in 0.1 × SSC at 70°C for 30 min. They were then treated with RNase A, 100 mg/mL for 30 min at 37°C, rinsed in RNase buffer for 15 min, and washed twice in 0.1 × SSC at 70°C for 30 min each. Sections were rapidly dehydrated in ethanol, dried, apposed to Kodak XAR-5 film and developed after 3 days (mEAAT2/5UT1–3) and 1 week (mEAAT2/5UT4–5). The specificity of the *in situ* hybridization was verified by using antisense probes that showed no specific hybridization signal.

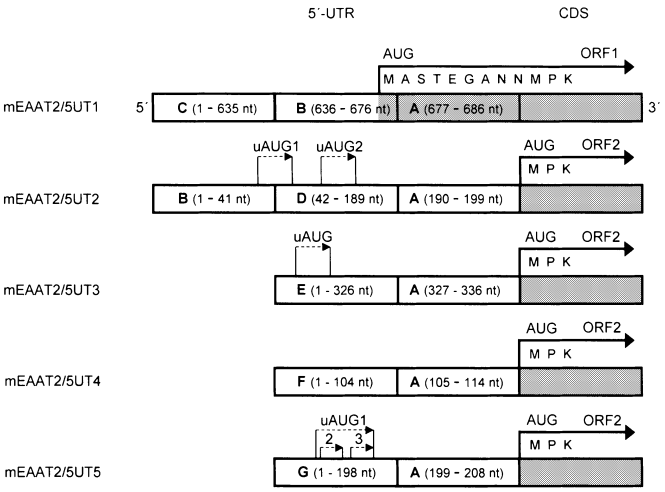

Fig. 2 Schematic presentation of mEAAT2/5UT1–5. The blank boxes labelled A–G represent putative 5′-exons. Open reading frames (ORF1, ORF2) are shown as grey shaded boxes. The truncation of N-terminal residues (MASTEGANN) resulting in ORF2 (mEAAT2/5UT2–5) is indicated. The nucleotide (nt) positions are based on GenBank AB007810 (mEAAT2/5UT1) and Fig. 1 (mEAAT2/5UT2–5). Upstream open reading frames are displayed by dashed lines and upstream start (uAUG) and stop signals by vertical lines. CDS, coding sequence.

Dot blotting

To study the quantitative RNA expression of mEAAT2/5UT1–5 we hybridized an expression array of different mouse tissues (Multiple Tissue Expression Array, Clontech). The RNA amount per dot has been normalized based on the expression of various housekeeping genes and allowed the comparative analysis of regional gene expression. The RNA probes used (probe 1–5) were synthesized by *in vitro* transcription as described above for *in situ* hybridization. Blots were hybridized at 60°C for 1 h (ExpressHyb Solution, Clontech), washed for 40 min in 2 × SSC and 0.05% SDS at room temperature followed by 40 min at 50°C in 0.1 × SSC and 0.1% SDS. Membranes were exposed to Kodak XAR-5 film with intensifying screens at − 70°C during 24 h.

Animal studies

The animal experimental protocols performed in this study conformed to the guidelines of the German Animal Care Act and were approved by the regional council of Tübingen and the ethics committee of the University of Ulm. Brain and spinal cord tissue of 18 mice that express the human SOD1 gene harbouring the ALS-associated mutation G93A and 18 non-transgenic control animals were used in this study. The transgenic colony was started from mice provided by Iffa Credo, France, and originally generated by Gurney *et al*. (1994; TgN[SOD1-G93A]1Gur. To confirm the presence of the SOD1 transgene, all mice were genotyped using primers for the human SOD1. At the age of 30, 120 and 180 days mutants and controls were killed by rapid decapitation. For the investigation of the late symptomatic disease stage, mutants were killed when they presented their first symptoms, at around day 180. The corresponding control group was investigated at day 180. Brain and spinal cord were removed immediately and stored at − 70°C until further use.

Competitive RT–PCR of the five murine EAAT2 splice variants was performed for tissue samples of the spinal cord, the cerebral cortex, the hippocampus and the cerebellum of 18 mice at days 30, 120 and 180, respectively. Statistical analysis of the data of six animals per time point was performed using Student's *t*-test and ANOVA with Fisher's LSD protected *t*-test. A value of $p < 0.05$ was considered significant. The quantitative expression analysis was based on the optical density values of PCR signals as described in the Competitive PCR section (MultiAnalyst Software, Bio-Rad).

Results

5′-Sequences

RACE-analysis of a mouse whole-brain cDNA library yielded 53 EAAT2 subclones containing the 5′-end of the transporter RNA. Sequence analysis revealed five 5′-variants of the murine EAAT2 RNA, named mEAAT2/5UT1–5 (Fig. 1). mEAAT2/UT1 has been reported before, whereas mEAAT2/5UT2–5 were previously unknown. Among the 53 EAAT2 clones, mEAAT2/5UT1 was predominantly present ($n = 31$), while the novel EAAT2 RNA splice forms were found in a smaller number of clones (Table 1). Sequence analysis revealed variable 5′-sequences of mEAAT2/5UT1–5 coding for diverse N-termini of the putative transporter polypeptide (Figs 1 and 2), whereas the remaining coding region showed complete homology. Within the 5′-regions of mEAAT2/5UT1–5 we identified sequence motifs that were assembled at highly conserved sequence boundaries and were likely to present 5′-exons. mEAAT2/5UT1 was regarded to be the constitutively spliced form of the murine EAAT2 RNA. The transporter transcripts mEAAT2/5UT2-5 were in principle generated by the alternative use of 5′-exons resulting in distinct 5′-sequences (Fig. 2). mEAAT/5UT2 was characterized by the insertion of a 148 nucleotide sequence motif (exon D) leading to the disruption of the N-terminal open reading frame (ORF). As a result, mEAAT2/5UT2 presented a shortened ORF beginning at a downstream translation start codon at position 687–689 nt (the nucleotide positions are referred to GenBank AB007810 throughout this paper; Utsunomiya-Tate *et al*. 1997). This transcript encoded a putative EAAT2 polypeptide lacking nine N-terminal amino acids (MASTEGANN) while the 5′-untranslated region was expanded by 148 nucleotides. The 5′-untranslated sequences upstream of position 41 are homologous to mEAAT2/5UT1. mEAAT2/5UT3 originated from the alternative use of exon E. This RNA encoded a N-terminally truncated EAAT2 polypeptide by nine amino acids as in mEAAT2/5UT2 and included a novel 5′-untranslated sequence motif of 326 nt. In mEAAT2/5UT4–5, the exons B and C were replaced by previously undescribed sequences (F, G). Also, the transcripts mEAAT2/5UT4-5 were lacking the nine amino terminal amino acids. Upstream of the truncated protein encoding sequences we identified novel 5′-untranslated sequences showing low homology to the known and novel upstream sequences. In mEAAT2/5UT2 and mEAAT2/5UT3 we identified three upstream open reading frames (uORF) located 175, 147 and 334 nucleotides upstream of the authentic translation start codon consisting of 7, 4 and 18 nucleotides, respectively. mEAAT2/5UT5 was

Table 1 Overview of EAAT2 subclones isolated from murine whole-brain 5′-RACE

Name of clone	No. of clones	Length of UTR (nt)	N-terminus (aa)	GenBank
mEAAT2/5UT1	31	659	MASTEGANN	AB007810
mEAAT2/5UT2	9	199	MPK	–
mEAAT2/5UT3	9	336	MPK	–
mEAAT2/5UT4	3	114	MPK	–
mEAAT2/5UT5	1	208	MPK	–

aa, amino acid.

characterized by three uORF of variable lengths (6–36 nucleotides) located within the exon G. The AUG codons preceding the upstream ORF were surrounded by sequences which variably matched the Kozak (1999) consensus sequence suggesting a differential regulatory control by the means of uAUG in each of the transcripts. Thus we found uAUG with complete (uAUG1 in mEAAT2/5UT2), incomplete (uAUG1 and uAUG2 in mEAAT2/5UT3; uAUG1 and uAUG3 in mEAAT2/5UT5) and no match (uAUG2 in mEAAT2/5UT2; uAUG2 in mEAAT2/5UT5) of the Kozak sequence motif, respectively.

In situ hybridization

The gross anatomical distribution of the EAAT2 5′-splice variants was examined by *in situ* hybridization. For the constitutively spliced isoform mEAAT2/5UT1, the *in situ* hybridization signal was most intense and widely distributed throughout the mouse brain (Fig. 3). The highest expression level of mEAAT2/5UT1 was found in the neocortical areas followed by the hippocampus > cerebellum > caudatus nucleus, whereas in other brain regions only weak signals were detected (Fig. 3). The broad tissue distribution in the brain was found to be similar in all splice variants although in mEAAT2/5UT4 and mEAAT2/5UT5 longer exposure was required to achieve sufficient intensity of the hybridization signal. Furthermore, mEAAT2/5UT4–5 showed a reduced expression in the cerebellar cortex as compared to mEAAT2/5UT1 (Fig. 3). A more detailed analysis of selected anatomical structures revealed other differential expression patterns of individual EAAT2 splice forms (Fig. 4). In the hippocampal formation the constitutive EAAT2 RNA (mEAAT2/5UT1) was found prominently in the dentate gyrus and the CA3 pyramidal cell layer and moderately in the CA2 and CA1 regions. In contrast, EAAT2 splice forms were strongly (mEAAT2/5UT2), weakly (mEAAT2/5UT3-4) or not (mEAAT2/5UT5) expressed in the dentate granular cell layer. Furthermore, a differential expression was also found in the CA2 and CA1 areas demonstrating moderate (mEAAT2/5UT2) and weak expression signals (mEAAT2/5UT3–5). *In situ* hybridization of mEAAT2/5UT1–3 in the spinal cord presented a prominent and rather homogenous expression in the grey matter, whereas in the white matter a weak hybridization signal was found. In contrast, the transcripts mEAAT2/5UT4 and mEAAT2/5UT5 showed a lower signal intensity in the grey matter revealing a differential expression of alternative EAAT2 splice variants in the spinal cord (Fig. 5).

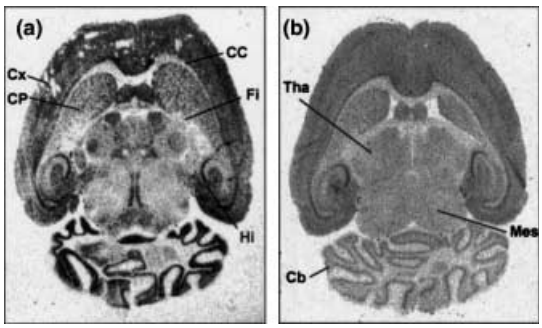

Fig. 3 *In situ* hybridization analysis of mEAAT2/5UT1 (a) and mEAAT2/5UT5 (b) in the normal mouse brain. Cx, cortex; CC, corpus callosum; CP, caudate putamen; Fi, fimbria; Hi, hippocampus; Tha, thalamus; Cb, cerebellum; Mes, mesencephalon.

Competitive RT–PCR

The expression analysis using competitive RT–PCR of mEAAT2/5UT1–5 demonstrated its differential expression in the mouse frontal cortex, hippocampus, cerebellum and spinal cord (Fig. 6). mEAAT2/5UT1–3 showed a strong expression in the neocortex, hippocampus, cerebellum and spinal cord. In contrast, the splice variants mEAAT2/5UT4–5 presented with an overall low expression level and a minimum of expression in the cerebellum. The internal standard of the 40S ribosomal protein S12 RNA and the EAAT2 coding sequence were invariably amplified in the series of brain and spinal cord tissues when equal amounts of total RNA were used as template for reverse transcription and subsequent competitive PCR.

Dot blotting

Dot blotting of multiple mouse tissues using probes complementary to the splice variants mEAAT2/5UT1–5 revealed the presence of EAAT2 RNA over a wide range of tissues (Fig. 7). mEAAT2/5UT1 RNA was abundantly

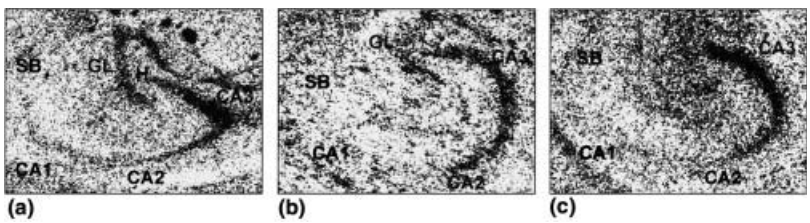

Fig. 4 *In situ* hybridization analysis of hippocampal expression in the normal mouse. A differential expression of mEAAT2/5UT1 (a), mEAAT2/5UT3 (b) and mEAAT2/5UT5 (c) was found in the dentate granular cell layer (GL) and the CA2 and CA1 pyramidal cell layers. The Ammon's horn (CA3) showed an invariably abundant expression of all EAAT2 transcripts. SB, subiculum; h, hilus.

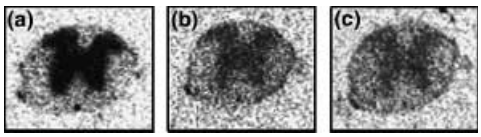

Fig. 5 *In situ* hybridization analysis of mEAAT2/5UT1 (a), mEAAT2/5UT4 (b) and mEAAT2/5UT5 (c) in the normal mouse spinal cord.

expressed in the CNS but also in extraneuronal tissues. There was a most prominent expression in the brain, skeletal muscle, heart, pancreas, testis, eye and a lesser presence in the lung, liver and smooth muscles. In contrast, mEAAT2/5UT2–5 showed a more differential expression outside the CNS. mEAAT2/5UT2–3 demonstrated a high expression in the epididymis > submaxillary gland > skeletal muscle and a much lower expression in other tissues. In mEAAT2/5UT4–5 a low expression level signal was found in the brain but a prominent signal intensity in the epididymis and the submaxillary gland. The multiple tissue array supports the involvement of mEAAT2/5UT2–5 in the regional expression of the mouse EAAT2 gene.

Regulation in SOD1 mutants

The phenotype of the SOD1G93A mice became apparent at around day 170–180 consisting of the previously described symptoms of tremors, hind limb paresis and impairment in motor behaviour (Gurney *et al*. 1994). Motor dysfunction progressed rapidly and, at around day 190–200, all mutants showed marked paralysis and muscular atrophy of the hind limbs associated with difficulty in the performance of motor tasks to reach death by day 203 ± 7 (SD). The relative expression of the splice forms mEAAT2/5UT1–5 was studied in pre-symptomatic mutants and in non-transgenic controls at age of 30 and 120 days. Transgenic animals killed at day 30 revealed no significant changes in the expression of mEAAT2/5UT1–5 in the spinal cord and cerebral cortex as compared to age-matched controls (Table 2). In the spinal cord, the quantitative expression of mEAAT2/5UT1–3 was unaltered at day 30, 120 and 180 (Figs 8 and 9). In contrast, at day 120 and 180 the expression of mEAAT2/5UT4 RNA was significantly increased ($p < 0.01$), whereas mEAAT2/5UT5 showed a significant down-regulation ($p < 0.05$). In the cortex of the diseased and normal animals the relative expression of all EAAT2 transcripts was found to be unchanged.

In relation to the phenotype of the studied SOD1G93A mouse line we showed a pre-symptomatic RNA regulation of two novel glutamate transporter splice variants. The internal standard 40S ribosomal protein S12 RNA was invariably amplified in the series of brain and spinal cord tissues when equal amounts of total RNA were used as template for reverse transcription and subsequent competitive PCR.

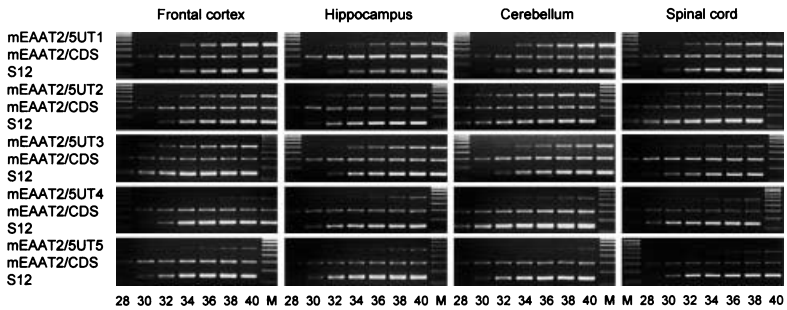

Fig. 6 Multiplex PCR analysis of splice variant sequences (mEAAT2/5UT1–5), the EAAT2 coding sequence (mEAAT2/CDS) and the internal standard RNA (ribosomal protein S12). Expression was studied in normal mouse frontal cortex, hippocampus, cerebellum and spinal cord. PCR products obtained from cycle 28–40 were subjected to agarose gel electrophoresis and visualized by ethidium bromide-staining. M, molecular DNA marker.

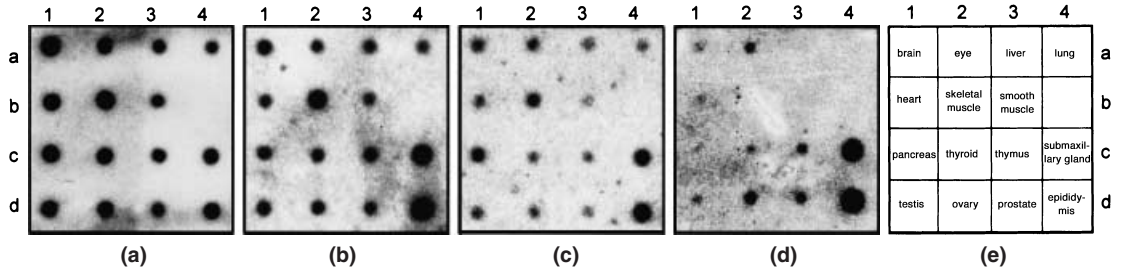

Fig. 7 RNA expression array of multiple normal mouse tissue specimens. Signal intensities represent the regional expression pattern of mEAAT2/5UT1 (a), mEAAT2/5UT2 (b), mEAAT2/5UT4 (c) and mEAAT2/5UT5 (d). (e) The legend of the multiple tissue array.

Table 2 Analysis (mean ± SD ratio mEAAT2/S12) of the mEAAT2/5UT1–5 RNA in the spinal cord of SOD1G93A mice and age-matched controls

	Day 30					Day 120					Day 180				
	Control ($n = 6$)		SOD1G93A ($n = 6$)			Control ($n = 6$)		SOD1G93A ($n = 6$)			Control ($n = 6$)		SOD1G93A ($n = 6$)		
	Mean	SD	Mean	SD	*p* to control	Mean	SD	Mean	SD	*p* to control	Mean	SD	Mean	SD	*p* to control
mEAAT2/5UT1	1.04	0.10	1.07	0.10	n.s.	1.00	0.15	0.98	0.15	n.s.	1.02	0.10	0.96	0.10	n.s.
mEAAT2/5UT2	1.00	0.10	0.96	0.10	n.s.	1.00	0.10	0.96	0.12	n.s.	1.05	0.10	0.95	0.15	n.s.
mEAAT2/5UT3	0.86	0.15	0.83	0.15	n.s.	0.85	0.15	0.84	0.20	n.s.	0.83	0.17	0.80	0.10	n.s.
mEAAT2/5UT4	0.50	0.10	0.50	0.10	n.s.	0.50	0.10	1.15	0.20	0.01	0.50	0.10	1.15	0.20	0.01
mEAAT2/5UT5	0.50	0.10	0.50	0.10	n.s.	0.50	0.10	0.11	0.10	0.05	0.50	0.10	0.11	0.10	0.05

The optical density values of PCR signals were quantified using an image analysis system. The values were expressed as a ratio of mEAAT2/5UT1–5 and S12. n.s., not significant.

Discussion

A regional protein expression of the glutamate transporter EAAT2 has been described in the normal brain. Furthermore, a diminished glutamate uptake resulting from a reduced EAAT2 protein has been reported in acute and chronic neurodegeneration. Yet little is known about the mechanisms of EAAT2 regulation in the normal and diseased brain (Gegelashvili and Schousboe 1997). Previously, in the human brain we identified a complex post-transcriptional regulation of EAAT2, including alternative splicing of 5′-untranslated and coding sequences and differential cleavage/polyadenylation (Meyer *et al*. 1998; Münch *et al*. 2000).

In this study we characterized several novel murine EAAT2 transcripts resulting from alternative splicing of 5′-sequences as cloned from the normal mouse brain. The 5′-heterogeneity was exerted at the level of 5′-untranslated sequences, N-terminal amino acid sequences and 5′-regulatory elements such as uORF. The conserved principles of 5′-heterogeneity in the human and murine CNS support the role of the mouse as an appropriate animal model to study alternative splicing mechanisms of EAAT2.

The structural diversity of the murine EAAT2 protein N-terminus is encoded by two alternative translation start codons. The functional importance of variable N-ends has been related to distinct biological properties such as protein signalling, cell sorting and protein half-life as summarized in the N-end rule (Bachmair *et al*. 1986). Previous studies have disclosed the fundamental role of the 5′-non-coding sequences for the regulation of translational control (Geballe and Morris 1994). One of the structural features within the 5′-sequences are uORF that can strongly influence the translational efficacy (Schluter *et al*. 2000). In three of the newly cloned EAAT2 transcripts we identified upstream translation initiation sites (uAUG). From other mRNA it is known that uAUG followed by uORF usually inhibit the translation from the major ORF (Morrish and Rumsby 2001). Functional studies in these RNA have revealed that the number of uAUG inversely relate to translational efficacy (Arrick *et al*. 1991). The different numbers of null (mEAAT2/5UT1), one (mEAAT2/5UT3) and two (mEAAT2/5UT2) uAUG and the presence of overlapping uORF in mEAAT2/5UT5 suggest different properties of translational control in distinct splice variants of the EAAT2 RNA. Furthermore, the exons D, E and G insert a premature stop codon that is more than 50 nucleotides upstream of the exon/exon junction and therefore fulfil the criteria of nonsense-mediated decay for mEAAT2/5UT2-3 and mEAAT2/5UT5 (Nagy and Maquat 1998; Frischmeyer and Dietz 1999).

Alternative splicing is extensively used to create the structural and functional proteomic diversity of the mammalian brain (Graveley 2001; Smith and Valcarcel 2000). To investigate the role of splicing for the differential EAAT2 gene expression we performed an expression analysis using

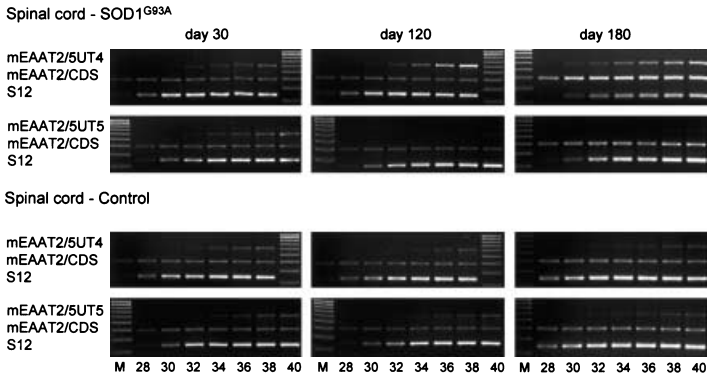

Fig. 8 Competitive PCR analysis of splice variant sequences (mEAAT2/5UT4, mEAAT2/5UT5), coding sequence of EAAT2 (mEAAT2/CDS) and the standard RNA (ribosomal protein S12). Expression in the spinal cord of SOD1G93A and control mice was analysed at age of 30, 120 and 180 days. The mEAAT2/5UT4 expression is up-regulated and mEAAT2/5UT5 decreased in the SOD1G93A mouse at day 120 and 180. PCR products obtained at cycle 28–40 were subjected to agarose gel electrophoresis and visualized by ethidium bromide-staining. M, molecular DNA marker.

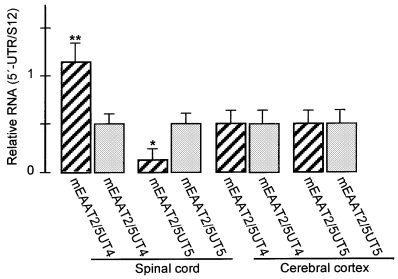

Fig. 9 Competitive PCR analysis of mEAAT2/5UT4 and mEAAT2/5UT5 in the cerebral cortex and spinal cord of SOD1G93A mutants compared to age-matched controls at age of 120 days. The values represent relative expression levels of mEAAT2/5UT4 and mEAAT2/5UT5 as compared to the expression of the standard RNA (S12). The results show the means (± SD) of six animals. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from the non-transgenic control group.

in situ hybridization, blotting and PCR techniques. The results demonstrated a regional pattern of splice variant expression in the mouse brain. Our findings suggest that alternative splicing of the EAAT2 RNA may contribute to the region-specific regulation of transporter properties mediated by distinct EAAT2 splice forms. It is conceivable that the topical expression of regulatory elements such as uAUG (mEAAT2/5UT2,3,5) and truncated N-ends (mEAAT2/5UT2–5) may result in a tissue- and cell-specific EAAT2 protein expression which has been reported before (Milton *et al*. 1997).

Disease-related alterations of alternative splicing have been increasingly identified involving the abundance, location or timing of normally expressed RNA isoforms (Daoud *et al*. 1999). In ALS patients, altered RNA splicing of the EAAT2 transcript has been found but its pathogenetic significance remains controversial (Lin *et al*. 1998). In the primary investigation several previously unknown EAAT2 splice forms have been cloned from post-mortem tissue specimens of ALS patients, whereas two of them have been proposed to be ALS-specific. Our own studies and the work of others suggested that the presence of these and other splice variants is not disease-specific (Meyer *et al*. 1999; Honig *et al*. 2000). However, it is yet unknown whether an altered regulation of normally expressed splice variants of EAAT2 may be part of the ALS pathogenesis and a molecular mechanism for the described EAAT2 protein loss in ALS patients and the transgenic model of this disease.

In this study we investigated disease-related splicing events of normal EAAT2 splice variants in the mouse model of familial ALS. To achieve this aim we undertook an expression analysis of all known murine EAAT2 splice forms in the transgenic disease model of ALS (Rosen *et al*. 1993; Gurney *et al*. 1994). The use of the SOD1 mouse model seems to be justified as a functional deficit of high-affinity glutamate uptake and a loss of the EAAT2 protein have been shown in this disease model before (Bruijn *et al*. 1997; Alexander *et al*. 2000; Bendotti *et al*. 2001). Furthermore, the coexpression of a specific SOD1 mutant gene *in vitro* resulted in an impairment of glutamate transport as mediated by an unknown toxic function of the mutated SOD1 protein (Trotti *et al*. 1999). Interestingly, in this study, the quantitative expression analysis of transporter splice variants in the SOD1G93A model revealed a disease-associated regulation of two of the five known EAAT2 transcripts. The alteration of EAAT2 splicing events occurred in a region-specific manner and was observed already in the early course of motor neurone degeneration. However, changes in the expression of the constitutively spliced and main EAAT2 transcript were not identified. This observation is consistent with a recently reported expression study showing a reduced transporter protein expression but preserved overall EAAT2 RNA expression in the SOD1G93A-model (Bendotti *et al*. 2001).

Our findings of the presence and the differential expression of EAAT2 splice variants support the hypothesis that alternative RNA splicing events may pose a potential mechanism how the reduced protein expression of EAAT2 is mediated.

We found that changes of quantitative expression of normally occurring EAAT2 splice forms accompany disease progression in the studied ALS model. The exact mechanism how EAAT2 splicing may affect transporter function is poorly understood. The altered RNA expression of EAAT2 variants encoding different 5′-regulatory sequences may contribute to the down-regulation of EAAT2 protein expression in the SOD1G93A model. This hypothesis is exemplified by the presence of three overlapping uORF in the down-regulated transcript mEAAT2/5UT5. In contrast, the overexpressed transcript mEAAT2/5UT4 proved to be free of uAUG. From other genes it is known that uAUG followed by small uORF may function as strong inhibitory elements of translational control (Schluter *et al*. 2000; Morrish and Rumsby 2001). In EAAT2, the functional properties of the identified 5′-regulatory sequences are not known and remain to be delineated by detailed *in vitro* expression studies. Moreover, in order to determine its principle pathogenic involvement, EAAT2 splicing events have to be analysed in other murine models for which a glutamate-mediated neurotoxicity has been described.

We conclude that at least five murine EAAT2 transcripts are generated by alternative splicing of 5′-sequences. Expression studies showed a regional presence of the novel EAAT2 splice variants in the mouse brain. Our observations support the role of RNA splicing for the structural diversity of EAAT2 and suggest that the mouse is a suitable model for studying EAAT2 splicing in the normal and diseased brain. The investigation of murine models is of substantial value to elucidate the pathogenic relevance of alternative EAAT2 splicing events in the SOD1G93A model for familial ALS and in other experimental models of neurodegeneration.

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