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Molecular Brain Research 80 (2000) 244–251

MOLECULAR BRAIN RESEARCH

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Short communication

Differential RNA cleavage and polyadenylation of the glutamate transporter EAAT2 in the human brain

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Accepted 13 June 2000

Abstract

We cloned four novel transcripts of the excitatory amino acid transporter 2, named EAAT2/3UT1–4, resulting from differential cleavage and polyadenylation. Tandem poly (A) sites were found to be functional at 72, 654, 973 nucleotides and more than 2 kb downstream of the stop codon. A tissue-specific expression was identified for 3′-variants of the EAAT2 RNA, most prominently for EAAT2/3UT4 (hippocampus>cortex≫cerebellum>thalamus) as demonstrated by Northern blot analysis and quantitative PCR. We conclude, that alternative poly (A) selection may contribute to the reported differential EAAT2 protein expression under normal and diseased conditions.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Uptake and transporters

Keywords: Glutamate; Excitatory amino acid transporter; RNA; Cleavage; Polyadenylation; Neurodegeneration

The excitatory action of the neurotransmitter glutamate at postsynaptic receptors is followed by rapid removal from the synaptic cleft mediated by high-affinity glutamate transporters. This process may contribute to the termination of the glutamatergic neurotransmission. Furthermore, high-affinity glutamate uptake is important to prevent excitotoxic effects of glutamate on postsynaptic neurons [16]. Five members of the gene family of excitatory amino acid transporters have been cloned (EAAT1–5) [1,2,6], whereas the glial transporter EAAT2 provides more than 90% of total glutamate uptake [19]. The EAAT2 protein is preferentially expressed in the cerebral cortex, the hippocampus, the caudate nucleus, the nucleus basilaris of Meynert and the spinal ventral horn, but with lower levels of expression in the putamen, the thalamus, the cerebellum and other regions throughout the CNS [14]. A reduced EAAT2 protein expression has been shown in ischemia [20], in temporal lobe epilepsy [10], in Alzheimer's disease [8], amyotrophic lateral sclerosis (ALS) [18], and a transgenic model of familial ALS [4]. The mechanism of selective EAAT2 loss in neurological disease is not understood [9,13], yet little is known about EAAT2 gene regulation under normal conditions [7].

The EAAT2 gene consists of 10 protein coding exons spanning more than 50 kb genomic DNA on chromosome 11p13-12 [11]. It encodes an open reading frame of 1724 nt and more than 5 EAAT2 splice forms resulting from alternative splicing of coding and 5′-untranslated sequences [12,15]. In this study we report four novel EAAT2 transcripts that originate from alternative cleavage of 3′-untranslated sequences and show a differential expression in the human brain. Furthermore, we describe a sequence variation in the 3′-untranslated region of EAAT2 that may be subject of RNA editing.

For rapid amplification of cDNA ends (RACE) we used human brain cDNA ligated with a 3′-adaptor sequence

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0169-328X/00/$ – see front matter
PII: S0169-328X(00)00139-X

Table 1
Oligonucleotide primers used in PCR amplification [2]

Primer	Sequence	Position
A (sense)	5′-CGTCGGCGCGGCCAGTATC-3′	1341–1358
B (sense)	5′-CCCTTAAAATGTCTAGTAAAGCTTGC-3′	475–501
C (sense)	5′-CACCTGGTGTAGTAAAATC AAATGAG-3′	850–875
D (sense)	5′-GTCTGAGCTGGAT ACCATTGAC-3′	1527–1547
E (sense)	5′-GGATTGATTAAAAGCAAATACCTG-3′	506–529
F (sense)	5′-CCAAAAGATTATTTTTTGGTTCCTAAAG-3′	917–935
G (sense)	5′-CTGCAGTGTTGAGGAAGAACC-3′	1718–1738
H (anti-sense)	5′-CAACCATTGTTCTCACAGATTAC-3′	663–685
I (sense)	5′-CTCCCATCCACCCTGATGTGG-3′	236–256
J (anti-sense)	5′-AGGCTTGGACAAGGTTTTCAGGG-3′	561–583
K (anti-sense)	5′-TAAGCCTCGGCTAACAGATTAAG-3′	109–132

(Clontech). The 3′-ends of the EAAT2 RNA were PCR-amplified using an adaptor primer (AP1) and gene-specific primers (A, B and C (Table 1)). The cDNA was passed through a two-step program consisting of 5 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). The PCR product was the subject of a second amplification using a nested adapter primer (AP2) and gene-specific primers (D, E and F). The amplification product was subcloned (pCR2.1, TA cloning kit, Invitrogen) and insert-containing constructs were sequenced (ABI 377 DNA sequencer, Applied Biosystems). For the identification of region-specific 3′-variants of EAAT2 we performed RACE analysis as described above in cDNA-libraries derived from the human cortex, hippocampus, cerebellum and hypothalamus (marathon ready cDNA, Clontech). To study the quantitative expression of the EAAT2 mRNA we hybridized an expression array of multiple brain regions (Multiple Tissue Expression Array, Clontech) with the EAAT2/3UT1-probe. The mRNA amount per dot has been normalized based on the expression of various housekeeping genes and allowed the comparative analysis of regional gene expression. The mRNA expression of EAAT2/3UT1 and /3UT2 was studied by Northern blot analysis in different brain regions (Multiple Tissue Northern Blot, Clontech). Two RNA probes corresponding to the 3′-untranslated sequences (probe 1: nucleotides (nt) 1 to 74 and probe 2: nt 546 to 637 (Fig. 1)) were synthesized by in vitro-transcription (SP6/T7 Transcription Kit, Roche). Blots were hybridized at 60°C for 1 h, washed for 40 min in 2X SSC and 0.05% SDS at room temperature followed by 40 min at 50°C in 0.1X SSC and 0.1% SDS. Membranes were exposed to X-ray films with intensifying screens at −80°C. For quantitative PCR of EAAT2/3UT1 and EAAT2/3UT4 (primers G, H and I, J) cDNA from the cerebral cortex, hippocampus, thalamus and cerebellum (Invitrogen) was used as template. Semiquantitative PCR was performed as multiplex PCR with coamplification of 40S ribosomal protein S12 mRNA, an internal standard. The multiplex PCR of EAAT2/3UT1, EAAT2/3UT4 and the S12 mRNA was carried out for 44 cycles of denaturation at 94°C (15 s), annealing at 60°C (30 s) and extension at 72°C (45 s) (AmpliTaq, Perkin-Elmer). Five μl of PCR reaction products were removed after the cycles 26, 28, 30, 32, 34, 36, 38 and 40 separated on 2.5% agarose gel and ethidium–bromid stained. Images were captured by UV–translumination (GelDoc1000, BioRad) and quantified (MultiAnalyst Software, BioRad). Semiquantitative expression of EAAT2/3UT1 and EAAT2/3UT4 was determined during linear regression of amplification in relation to the internal standard S12 RNA. To determine the genomic structure of the 3′-end sequence, a DNA library of the bacterial artificial chromosome BAC 3640-1 (Research Genetics) was constructed (GATC GmbH). The library was screened with radiolabeled oligonucleotides (Amersham) complementary to 3′-untranslated sequences (probe1, Fig. 1). Positive subclones were identified using the dot blot technique (Biorad) and DNA sequenced. A polymorphic site at position 80 (A/G) of the EAAT2 cDNA was analyzed at the genomic DNA level. Genomic DNA of peripheral lymphocytes was isolated from 50 non-neurological individuals (DNA blood kit, Qiagen). The sequence of interest was amplified by PCR (primers G and K) at conditions as described above and DNA sequenced (ABI 377, Applied Biosystems).

RACE-analysis of a human whole brain cDNA library yielded 200 EAAT2 subclones containing the 3′-end of the transporter RNA. Four different novel isoforms of EAAT2 RNA were identified and named EAAT2/3UT1, /3UT2, /3UT3 and /3UT4. The EAAT2 RNA variants were characterized by differential cleavage at 72, 654, 973 nt and >2000 nt downstream of the stop codon (Fig. 1). In EAAT2/3UT1 and EAAT2/3UT3 the cleavage/polyadenylation site was preceded by the conserved polyadenylation signals AATAAA and ATTAAA at positions 21–26 and 626–631, respectively. The hexanucleotide AATAAA is the most predominant sequence directing cleavage and polyadenylation of pre-mRNA [3] and has been found in the 3′-untranslated region near the mature mRNA end of 80% of examined genes [5]. Other less conserved AT or A-rich sequences have been observed in the 3′-end of a smaller fraction of transcripts and have

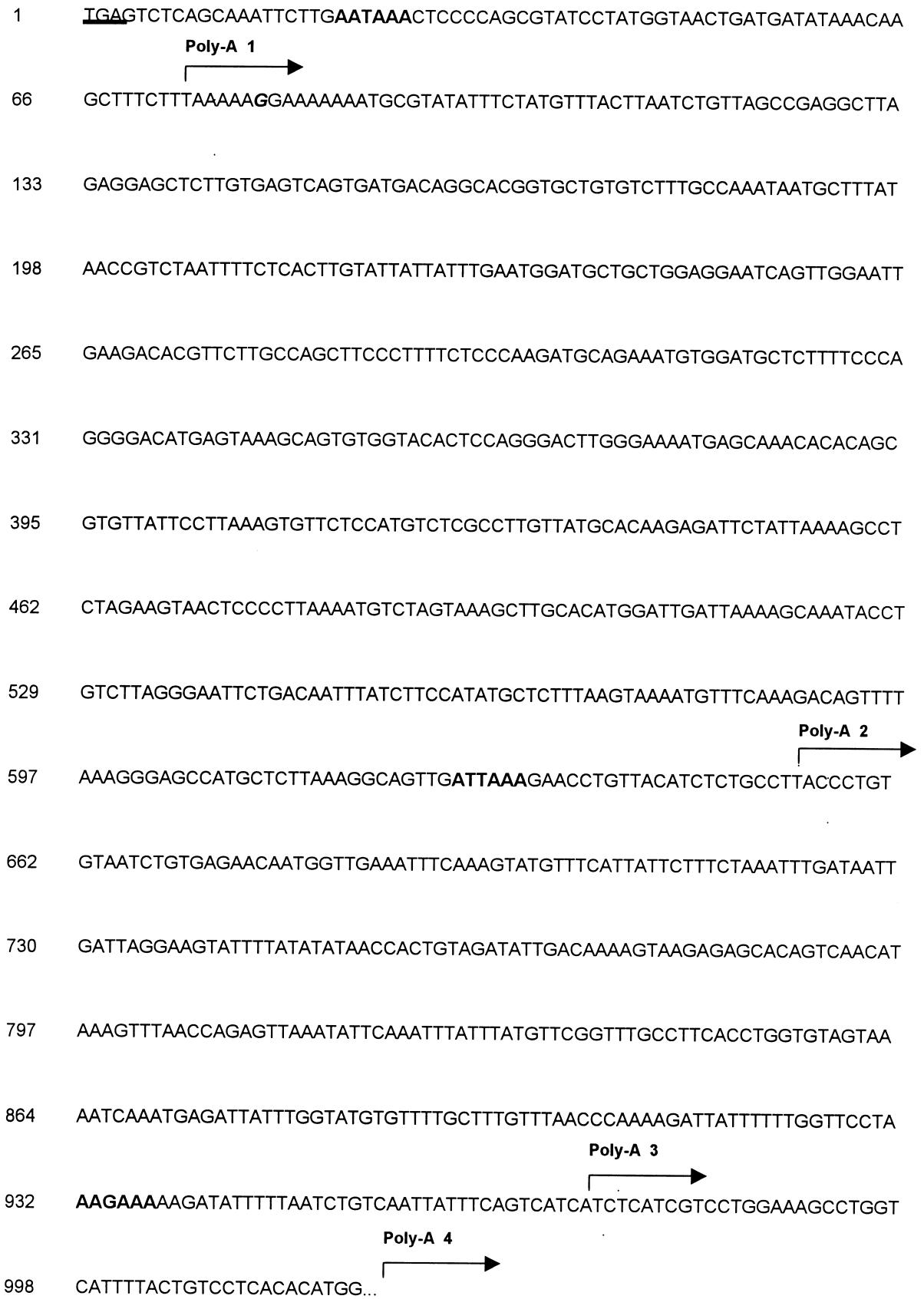


Fig. 1. Nucleotide sequence of the 3′-untranslated region of the human EAAT2 mRNA. The stop codon is underlined and the polyadenylation signals are shown in bold. Arrows indicate the position of the transcripts EAAT2/3UT1-4 (poly (A) 1-4). The rare nucleotide exchange G→A in EAAT2/3UT2 is indicated in bold italics.

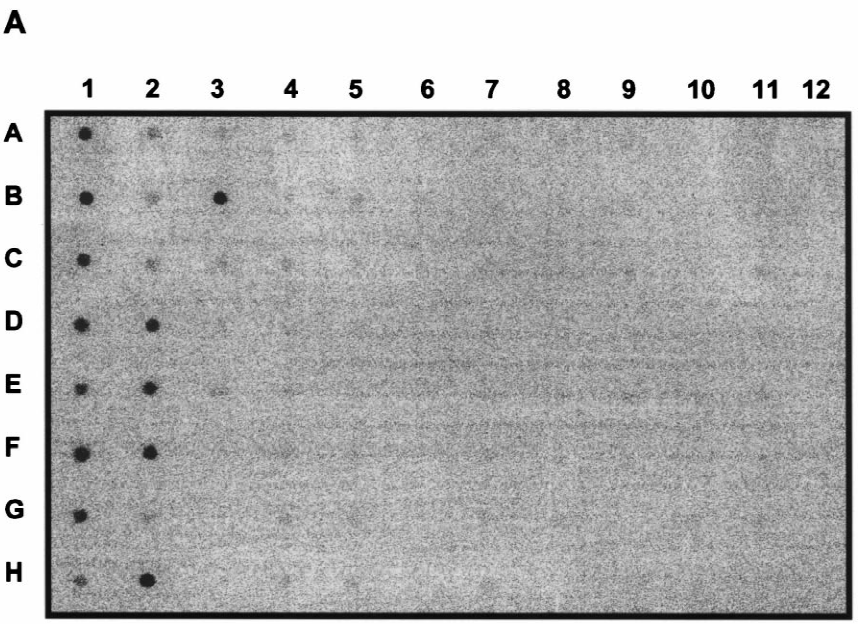
been found to function less effective in vitro than AATAAA. A rare cleavage/polyadenylation signal AAGAAA was identified in EAAT2/3UT3 42 nucleotides upstream of the poly (A) addition site at position 932–937. For the AAGAAA sequence it is known that it yields 3′-ends at very much reduced expression levels. Since the EAAT2/3UT4 has not been completely sequenced, the exact poly (A) selection site in this transcript was not determined (Fig. 3). The region-specific 3′-RACE analysis using cDNA libraries derived from the cortex, hippocampus, cerebellum, and hypothalamus did not show additional or tissue-specific 3′-variants (Table 2).

The regional expression pattern of the EAAT2 RNA was examined by multiple tissue expression array, Northern blot analysis and semiquantitative PCR. Hybridization of the expression array (probe 1, Fig. 3) showed high expression in the cortical areas followed by the putamen> nucleus accumbens>hippocampus>amygdala>caudatus nucleus (Fig. 2). A weak signal was found in the cerebellum, the thalamus and the substantia nigra. The RNA expression was in principle agreement with the immunohistochemical data on EAAT2 protein expression in the human CNS [14]. Only in the putamen a strong RNA expression was found in contradiction to a reportedly reduced EAAT2 protein expression in this region [14]. The expression array was based on the dot blot technique and did not allow to differentiate the expression of alternative EAAT2 transcripts. The hybridization of Northern blots (Clontech) of multiple human brain regions (Fig. 3) using a probe complementary to EAAT2/3UT1 revealed the presence of four EAAT2 transcripts of 1.8, 2.4, 7, and 12 kb in length. The 1.8- and 2.4-kb transcripts were likely to correspond with EAAT2/3UT1 and EAAT2/3UT2, respectively. For EAAT2/3UT3 no corresponding signal in the Northern blot has been found. This observation is in agreement with the known properties of the poly (A) signal AAGAAA in this RNA resulting in substantially diminished expression of AAGAAA containing transcripts. EAAT2/3UT4 may be represented by the 7- or 12-kb transcripts. Each brain region was characterized by a distinct expression of different EAAT2 transcripts (Fig. 3). In the cortex, the hippocampus and the amygdala the 11-kb transcript was predominantly expressed, whereas in the putamen, the cerebellum and the caudate nucleus the 12- and 7-kb transcripts were equally present. In the thalamus, the 12-kb transcript was not detected while the 1.8- and the 8-kb transcripts were strongly expressed. The 1.8-kb transcript was expressed in all brain regions with predominance of the thalamus and putamen. When hybridized with a probe complementary to EAAT2/3UT2 the 1.8-kb transcript was not detected proving the specificity of this transcript for EAAT2/3UT1 (probe 2, Fig. 3). Interestingly, brain regions with low EAAT2 protein such as the thalamus, the cerebellum and the putamen were found to express low levels of the full length 12-kb transcript. Most prominently, in the thalamus the weak immunohistochemical staining for EAAT2 [19] was associated with a strong total EAAT2 RNA signal in the expression array. However, the differentiation of 3′-variants of the EAAT2 RNA in the Northern analysis revealed a reduced signal for the 12-kb transcript. A strong expression of the 7- and 1.8-kb transcripts accounted for most of the total EAAT2 RNA signal in the expression array. We conclude, that a strong EAAT2 protein expression may be related to the presence of the 12-kb EAAT2 transcript (EAAT2/3UT4). The shorter EAAT2 transcripts may show functional properties such as reduced RNA stability or translation that preclude an efficient protein expression. The differential expression data obtained by the array and the Northern blot analysis were confirmed by quantitative PCR amplification of EAAT2/3UT1 and EAAT2/3UT4 on total RNA derived from the human cortex, hippocampus, cerebellum and thalamus. EAAT2/UT1 was abundantly expressed in the hippocampus and cerebral cortex and moderately expressed in the cerebellum and the thalamus. In contrast, EAAT2/UT4 showed a more differential expression pattern with high and moderate levels in the hippocampus and in the cerebral cortex, respectively, whereas in the thalamus and cerebellum this transcript was barely detected (Fig. 4).

Sequence analysis of two EAAT2/3UT2 clones derived from independent human whole brain cDNA libraries showed a sequence variation at position 80 (Fig. 1). The two clones revealed a G/A exchange at this position while the sequence in other 48 clones from whole brain libraries and in the 200 EAAT2 clones isolated from region-specific libraries was unaltered (Fig. 5). The cloning and DNA sequencing of a 1-kb genomic DNA fragment following the stop codon suggested that the 3′-untranslated region of the transcripts EAAT2/3UT1-3 are encoded by a single exon. The genomic structure of the full-length EAAT2/3UT4 has not been determined. The genomic DNA of 100 allels of control individuals did not show a G/A polymorphism at position 80. The molecular basis of the G/A sequence variation may be a rare polymorphism or a RNA sequence not encoded by genomic DNA. RNA editing of the EAAT2/3UT2 transcript in a CNS region present in

Table 2
Overview of EAAT2 subclones isolated from region-specific human brain cDNA libraries

Region of cloning	EAAT2/3UT1	EAAT2/3UT2	Total
Whole brain	19	25	44
Cortex	28	22	50
Hippocampus	42	8	50
Cerebellum	34	16	50
Hypothalamus	20	30	50
Total	143	101	244



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebellum, left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain	yeast total RNA
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney	*E. coli* rRNA
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver	*E. coli* DNA
E	occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen	Poly r(A)
F	temporal lobe	hippo-campus		ventricle, right	ilocecum		lymph node	testis	mammary gland	Burkitt's lymphoma, Daudi	fetal thymus	human $C_{0}t$ -1 DNA
G	cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone morrow	ovary		colorectal adeno-carcinoma, SW480	fetal lung	human DNA 100 ng
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549		human DNA 500 ng

Fig. 2. Multiple tissue expression array of mRNA from 76 human tissues and cell lines showing the quantitative expression of the EAAT2 RNA.

the whole brain library but not in the studied region-specific libraries has to be considered. A G/A transition resulting from RNA editing is a rare event but has been described in the mammalian gene regulation [17]. Editing of non-coding sequences has been found in 5′-regulatory sequences of a human brain transcript [21], but has not been reported for 3′-untranslated sequences before.

In summary, we cloned four novel EAAT2 RNA that were characterized by alternative 3′-untranslated sequences resulting from the differential usage of tandem poly (A) sites. We found tissue-specific expression of 3′-variants of the EAAT2 RNA, most prominently for EAAT2/3UT4. We propose that strong EAAT2 protein expression like in the cortex and the hippocampus is related to the EAAT2/3UT4 transcript. We conclude that the differential cleavage and polyadenylation of the EAAT2 RNA may be part of the tissue and cell-specific EAAT2 gene regulation. Transport of the mRNA from the nucleus to cytoplasm is dependent on polyadenylation and splicing. The poly (A) strength plays a role in the RNA stability, translatability,

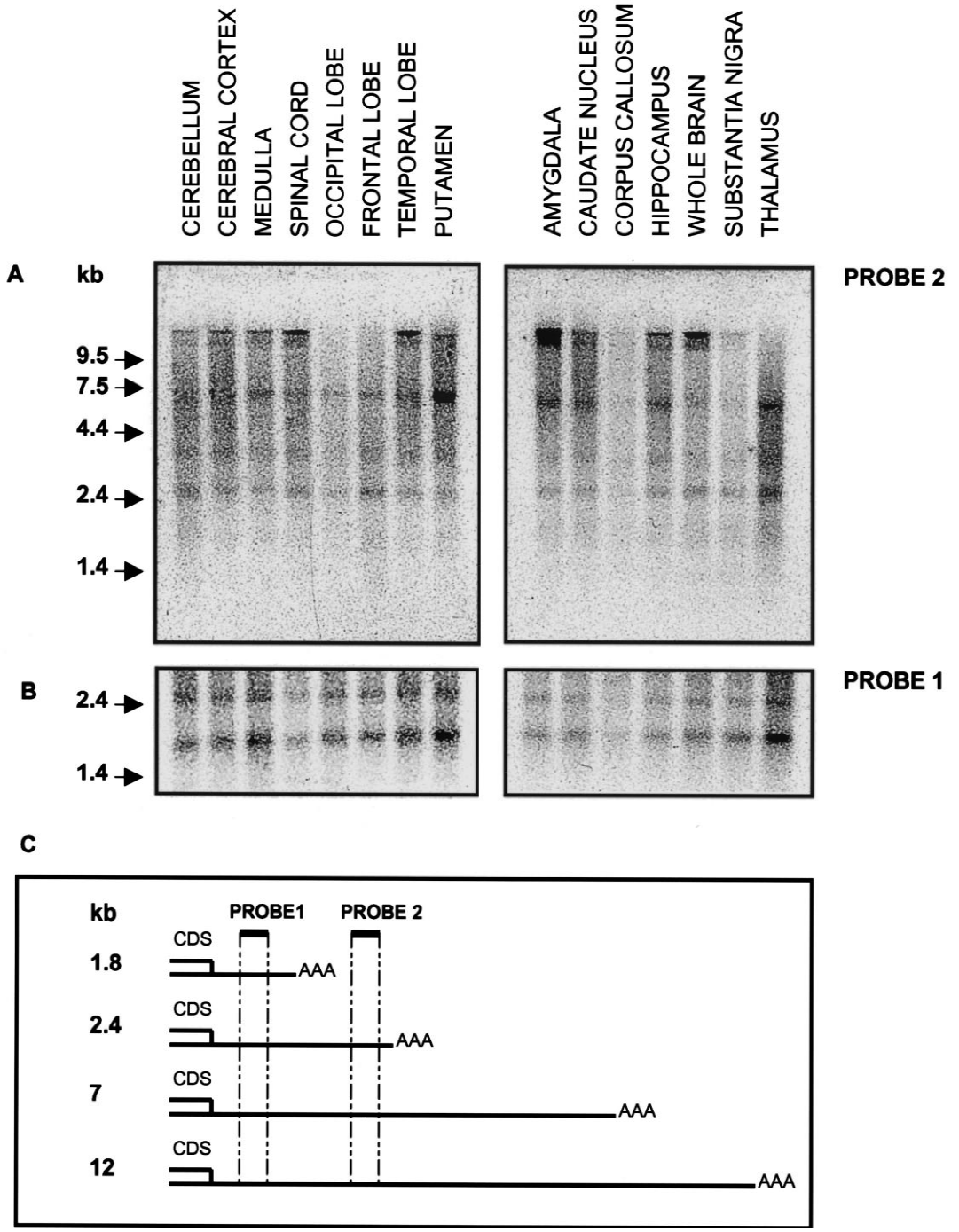


Fig. 3. Northern blot analysis of the EAAT2 mRNA in 14 CNS regions and the whole brain. (A): The blots were hybridized with probe 2 complementary to a distal sequence present in EAAT2/3UT2-4. (B): The expression of EAAT2/3UT1 was shown using probe 1. (C): Schematic presentation of the position of the probes used for Northern blot analysis.

and prevention of RNA degradation. It is known that poly (A) selection can directly influence the amount of cytoplasmatic RNA produced from the transcript [5]. Poly (A) site selection in EAAT2 can therefore serve as an important control point for gene expression in a tissue or developmental-stage specific manner under normal and diseased conditions. This aspect of EAAT2 RNA regulation is of great pathogenetic interest in several neurological disorders for which a disturbance of EAAT2 protein expression has been reported [4,8–10,19,20].

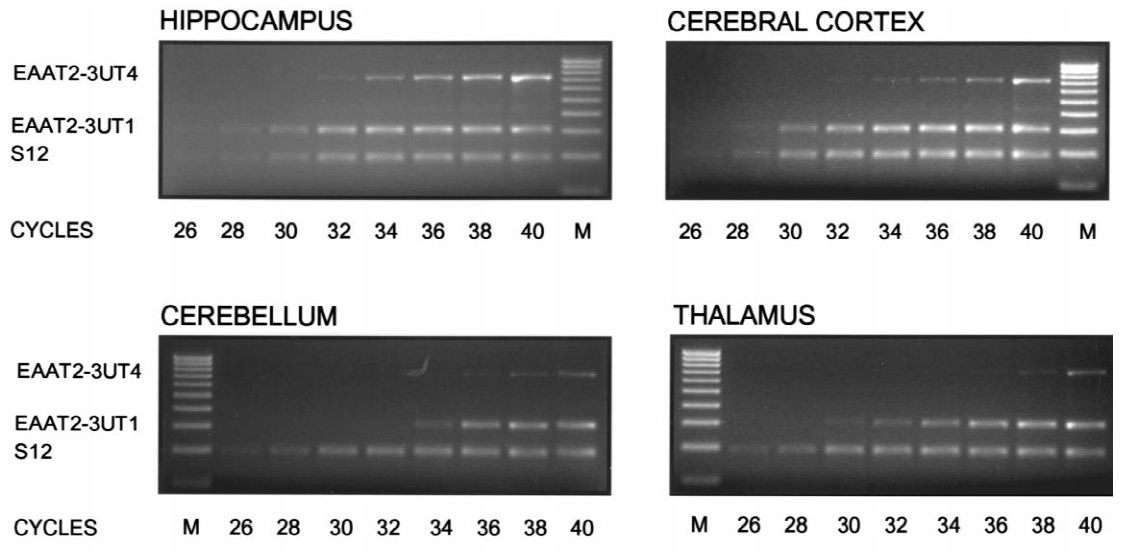


Fig. 4. Semiquantitative amplification of EAAT2/3UT4, EAAT2/3UT1 and S12 in the human hippocampus, cerebral cortex, cerebellum and thalamus. PCR products from cycle 26 to 40 were subjected to agarose gel electrophoresis and visualized by ethidium–bromid staining.

Fig. 5. G→A sequence variant at position 80 of the EAAT2/3UT2 complementary DNA as compared to the unaltered EAAT2/3UT2 transcript and the genomic DNA sequence.

Acknowledgements

This work was supported by a grant from the VERUM Foundation.

References

[1] J.L. Arriza, W.A. Fairman, J.I. Wadiche, G.H. Murdoch, M.P. Kavanaugh, S.G. Amara, Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex, J. Neurosci. 14 (1994) 5559–5569.

[2] J.L. Arriza, S. Eliasof, M.P. Kavanaugh, S.G. Amara, Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance, Proc. Natl. Acad. Sci. USA 94 (1997) 4155–4160.

[3] M.L. Birnstiel, M. Busslinger, K. Strub, Transcription termination and 3′ processing: The end is in site!, Cell 41 (1985) 349–359.

[4] L.I. Bruijn, M.W. Becher, M.K. Lee, K.L. Anderson, N.A. Jenkins, N.G. Copeland, S. S Sisodia, J.D. Rothstein, D.R. Borchelt, D.L. Price, D.W. Cleveland, ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions, Neuron 18 (1997) 327–338.

[5] G. Edwalds-Gilbert, K.L. Veraldi, C. Milcarek, Alternative poly(A) site selection in complex transcription units: Means to an end?, Nucleic Acids Res. 25 (1997) 2547–2561.

[6] W.A. Fairman, R.J. Vandenberg, J.L. Arriza, M.P. Kavanaugh, S.G. Amara, An excitatory amino-acid transporter with properties of a ligand-gated chloride channel, Nature 375 (1995) 599–603.

[7] G. Gegelashvili, A. Schousboe, High affinity glutamate transporters: Regulation of expression and activity, Mol. Pharmacol. 52 (1997) 6–15.

[8] S. Li, M. Mallory, M. Alford, S. Tanaka, E. Masliah, Glutamate transporter alterations in Alzheimer disease are possibly associated with abnormal APP expression, J. Neuropathol. Exp. Neurol. 56 (1997) 901–911.

[9] C.L. Lin, L.A. Bristol, L. Jin, M. Dykes-Hoberg, T. Crawford, L. Clawson, J.D. Rothstein, Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis, Neuron 20 (1998) 589–602.

[10] G.W. Mathern, D. Mendoza, A. Lozada, J.K. Pretorius, Y. Dehnes, N.C. Danbolt, N. Nelson, J.P. Leite, L. Chimelli, D.E. Born, A.C. Sakamoto, J.A. Assirati, I. Fried, W.J. Peacock, G.A. Ojemann, P.D. Adelson, Hippocampal GABA and glutamate transporter immunoreactivity in patients with temporal lobe epilepsy, Neurology 52 (1999) 453–472.

[11] T. Meyer, A.C. Ludolph, M. Morkel, C. Hagemeier, A. Speer,

Genomic organization of the human excitatory amino acid transporter gene GLT-1, NeuroReport 8 (1997) 775–777.
[12] T. Meyer, C. Münch, B. Knappenberger, S. Liebau, H. Völkel, A.C. Ludolph, Alternative splicing of the glutamate transporter EAAT2 (GLT-1), Neurosci. Lett. 241 (1998) 68–70.
[13] T. Meyer, A. Fromm, C. Münch, B. Schwalenstocker, A.E. Fray, P.G. Ince, S. Stamm, G. Grön, A.C. Ludolph, P.J. Shaw, The RNA of the glutamate transporter EAAT2 is variably spliced in amyotrophic lateral sclerosis and normal individuals, J. Neurol. Sci. 170 (1999) 45–50.
[14] I.D. Milton, S.J. Banner, P.G. Ince, N.H. Piggott, A.E. Fray, N. Thatcher, C.H. Horne, P.J. Shaw, Expression of the glial glutamate transporter EAAT2 in the human CNS: an immunohistochemical study, Brain Res. Mol. Brain Res. 52 (1997) 17–31.
[15] C. Münch, B. Schwalenstöcker, B. Knappenberger, S. Liebau, H. Völkel, A.C. Ludolph, T. Meyer, 5′-heterogeneity of the human excitatory amino acid transporter cDNA EAAT2 (GLT-1), NeuroReport 9 (1998) 1295–1297.
[16] D. Nicholls, D. Attwell, The release and uptake of excitatory amino acids, Trends Pharmacol. Sci. 11 (1990) 462–468.
[17] B. Rajput, J. Ma, I.K. Vijay, Structure and organization of mouse GlcNAc-1-phosphate transferase gene, J. Biol. Chem. 269 (1994) 9590–9597.
[18] J.D. Rothstein, M. Van Kammen, A.I. Levey, L.J. Martin, R.W. Kuncl, Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis, Ann. Neurol. 38 (1995) 73–84.
[19] K. Tanaka, K. Watase, T. Manabe, K. Yamada, M. Watanabe, K. Takahashi, H. Iwama, T. Nishikawa, N. Ichihara, T. Kikuchi, S. Okuyama, N. Kawashima, S. Hori, M. Takimoto, K. Wada, Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1, Science 276 (1997) 1699–1702.
[20] R. Torp, D. Lekieffre, L.M. Levy, F.M. Haug, N.C. Danbolt, B.S. Meldrum, O.P. Ottersen, Reduced postischemic expression of a glial glutamate transporter, GLT1, in the rat hippocampus, Exp. Brain Res. 103 (1995) 51–58.
[21] T. Yoshikawa, A.R. Sanders, L.E. Esterling, S. Detera-Wadleigh, Multiple transcriptional variants and RNA editing in C18orf1, a novel gene with LDLRA and transmembrane domains on 18p11.2, Genomics 47 (1998) 246–257.