Distribution of glutamate transporters in the hippocampus of patients with pharmaco-resistant temporal lobe epilepsy


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Summary

In patients suffering from temporal lobe epilepsy (TLE), increased extracellular glutamate levels in the epileptogenic hippocampus both during and after clinical seizures have been reported. These increased glutamate levels could be the result of malfunctioning and/or down-regulation of glutamate transporters (also known as EAATs; excitatory amino acid transporters). In this study, the distribution of protein and mRNA of EAAT subtypes was examined in the hippocampus of TLE patients with hippocampal sclerosis (HS group) and without hippocampal sclerosis (non-HS group), and in autopsy controls without neurological disorders. EAAT protein localization was studied by immunohistochemistry on paraffin sections using specific poly- and monoclonal antibodies against the glial glutamate transporters EAAT1 and EAAT2 and the neuronal glutamate transporter EAAT3. Antibody specificity was shown by immunoblotting. In the HS group, a small decrease in EAAT1-immunoreactivity (IR) was observed in CA4 and in the polymorphic and supragranular layer of the dentate gyrus, compared with the control group. The strongest changes were found for EAAT2 levels. In the non-HS group, increased EAAT2-IR was detected in the CA1 and CA2 field, compared with non-epileptic controls. EAAT2-IR was decreased in the HS compared with the non-HS group. Fewer EAAT3-positive cells were found in the HS group than in the non-HS and control group. In both TLE groups, increased EAAT3 levels were observed in individual neurones. In the HS group, the percentage of EAAT3-IR neurones was increased in CA2 and in the granule cell layer of the dentate gyrus. Radioactive in situ hybridization for EAAT1-3 confirmed our immunohistochemical results. Non-radioactive in situ hybridization showed that not only astrocytes, but also neurones express EAAT2 mRNA. Taken together, differences in both mRNA and protein levels of glutamate transporter subtypes were found in specific regions in the TLE hippocampus, with most severe changes found for EAAT2 and EAAT3 levels. The results indicate an upregulation of EAAT2 protein expression in CA1 and CA2 in neurones in the non-HS group. This is in line with decreased EAAT2 protein levels in the HS group, since these hippocampi are characterized by severe neuronal cell loss. The functional consequences (glutamate transport capacity) of the reported changes in EAAT2 and EAAT3 remain to be determined.

Keywords: temporal lobe epilepsy; hippocampal sclerosis; excitatory amino acid transporter; glutamate

Abbreviations: DAB = 3,3’-diaminobenzidine tetrahydrochloride; DIG = digoxigenin; EAAT = excitatory amino acid transporter; GCL = granule cell layer of the dentate gyrus; HS = hippocampal sclerosis; IR = immunoreactivity; PML = polymorphic layer of the dentate gyrus; SGL = supragranular layer of the dentate gyrus; TBS = Tris-buffered saline; TLE = temporal lobe epilepsy

Introduction

Glutamate is the predominant excitatory neurotransmitter in the mammalian CNS. Glutamatergic transmission is terminated ultimately by binding of glutamate to its transporters and subsequent uptake into astrocytes and neurones (Kanai, 1997; Robinson and Dowd, 1997). Glutamate transport is driven by sodium, potassium and possibly by hydroxide ion gradients.
Thus by the coupling of glutamate transport to the ionic gradient, glutamate transporters [called excitatory amino acid transporters (EAATs)] are able to transport glutamate against a concentration gradient of >1000-fold (Attwell and Mobbs, 1994). EAATs thus can modulate neurotransmission by maintaining low concentrations of extracellular glutamate and preventing toxic levels being reached. Conversely, when the ion gradient or membrane potential drops, for instance during ischaemia or epileptic activity, glutamate transporters may reverse and release glutamate into the extracellular space in a calcium-independent manner (Rossi et al., 2000).

Five mammalian EAAT isoforms have been cloned and characterized electrophysiologically and pharmacologically: EAAT1 (GLAST) (Arriza et al., 1994; Kawakami et al., 1994), EAAT2 (GLT-1) (Arriza et al., 1994; Shashidharan et al., 1994), EAAT3 (EAAC1) (Kanai and Hediger, 1992; Arriza et al., 1994), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). EAAT family members display ~50–55% amino acid sequence identity and an almost identical hydrophobicity pattern, suggesting that each transporter exhibits its functional properties on the basis of similar characteristics. They each contain 10 hydrophobic domains, with both the N- and C-termini in the cytoplasmic side (Wahle and Stoffel, 1996).

The EAAT subtypes differ in regional, cellular and developmental distribution (Robinson and Dowd, 1997). EAAT1-immunoreactivity (IR) is highly abundant in cerebellar Bergmann glia, and EAAT2-IR is concentrated in astrocytes in the hippocampus and cerebral cortex (Rothstein et al., 1994; Lehre et al., 1995). EAAT3 is highly expressed in the cortex, hippocampus and caudate–putamen, and is neurone specific, most probably post-synaptic (Rothstein et al., 1994). EAAT4 is confined to cerebellar Purkinje cells, with little expression in other brain regions (Yamada et al., 1996). EAAT5 is expressed predominantly in the retina (Arriza et al., 1997). Although both neurons and glia contain glutamate transporters, it is generally accepted that the uptake capacity of astrocytes is much higher than that of neurones (Rothstein et al., 1996).

A disturbance in glutamate transport has been implicated in several neurodegenerative disorders such as amyotrophic lateral sclerosis (Rothstein et al., 1995), Alzheimer’s disease (Masliah et al., 1996), Parkinson’s disease (Blandini et al., 1996) and epilepsy (Meldrum, 1994). In epilepsy, glutamate is thought to play an important role in the initiation, spread and maintenance of seizure activity (e.g. Meldrum, 1994). In a microdialysis study, comparing epileptogenic hippocampi with non-epileptogenic hippocampi of patients with temporal lobe epilepsy (TLE), increased extracellular glutamate was found in the epileptogenic hippocampus just before and during clinical seizures (During and Spencer, 1993). Interestingly, increased glutamate levels were sustained after the seizure, specifically in the epileptogenic hippocampus. These results indicate impaired glutamate uptake capacity and thus possible malfunctioning and/or downregulation of glutamate transporters (for a review, see Meldrum et al., 1999). A causal relationship between EAAT dysfunction and epilepsy was established by Tanaka and colleagues, who demonstrated increased extracellular glutamate levels and spontaneous lethal epileptic seizures in EAAT2 knock-out mice (Tanaka et al., 1997).

So far, only two studies have been reported on glutamate transporters in the human epileptic brain. In cortical tissue from TLE patients, no changes in EAAT1 and EAAT2 mRNA levels were found compared with autopsy controls (Tessler et al., 1999). However, this study did not provide data on hippocampal EAAT expression levels. In a detailed study from Mathern and colleagues, no changes in EAAT1-IR were observed in hippocampi from TLE patients with hippocampal sclerosis (HS) compared with TLE patients without HS, while reduced EAAT2-IR was associated with neurone loss, and increased EAAT3-IR was detected in areas where neurones were spared and decreased in affected regions (Mathern et al., 1999).

The aim of this study was to investigate the distribution of mRNA and protein of the major glutamate transporter subtypes in the hippocampus of TLE patients with (HS group) and without signs of HS (non-HS) and in autopsy controls. Three glutamate transporters were studied: the glial glutamate transporters EAAT1 and EAAT2 and the neuronal glutamate transporter EAAT3. By means of immunohistochemical analysis, EAAT1, EAAT2 and EAAT3-IR were determined and quantified. To study the cellular localization of EAAT2 mRNA, we also performed non-radioactive in situ hybridization experiments.

Material and methods

Patients

Surgical tissue was obtained from patients suffering from pharmaco-resistant TLE. Using non-invasive and invasive techniques, it was determined that the epileptogenic focus was localized in the temporal lobe. In all cases, informed consent was given by the patients for using any data and tissue for research studies. Surgical procedures were performed under general or local anaesthesia. The excision was based on clinical evaluations, interictal and ictal EEG studies (video EEG monitoring), MRI and intra-operative corticography. During surgery, the anterior part of the temporal lobe and the hippocampal complex was resected, and the hippocampus was then cut into slices (0.5 cm thickness) perpendicular to the longitudinal hippocampal axis. Hippocampal specimens were obtained immediately after resection and fixed in 4% paraformaldehyde for 24–48 h (for immunohistochemistry and in situ hybridization) or freshly frozen in dry ice and stored at −80°C until used for immunoblotting. Control tissue was obtained from six post-mortem cases with no signs of hippocampal aberrations. Post-mortem delay ranged from 11 to 28 h and the duration of paraformaldehyde fixation was 24–48 h. Hippocampal specimens were divided into three groups: a non-epileptic autopsy
control group \((n = 6)\), a group of TLE patients without signs of HS (non-HS group; \(n = 6\)) and a group of TLE patients with severe HS (HS group; \(n = 8\)). Clinical information on the composition of the patient groups is provided in Table 1.

### Immunoblotting

In order to assess the specificity of the antibodies used for immunohistochemistry, mono- and polyclonal antibodies against EAAT1, -2 and -3 were tested by immunoblotting. Monoclonal antibodies used in this study included monoclonal mouse anti-EAAT1, EAAT2 and EAAT3 antibodies (EAAT1-M, EAAT2-M and EAAT3-M, respectively), purchased from Novocastra Labs, UK (EAAT1-M and EAAT2-M) and from Chemicon International, USA (EAAT3-M). Polyclonal antibodies included polyclonal guinea pig anti-EAAT1 or anti-EAAT2 (EAAT1-P, EAAT2-P, respectively), purchased from Chemicon International, USA; and polyclonal rabbit anti-EAAT3 antibodies (EAAT3-P) purchased from Biotrend Chemicals, Germany.

Human and rat cortical and hippocampal homogenates were prepared by homogenization (700 r.p.m.) in a Potter-Elvehjem tube in 10 volumes of lysis buffer [containing 100 mM Tris–HCl, 1 mM EGTA, 1 mM EDTA, 1 µg/ml leupeptin, 250 µM PMSF (phenylmethylsulfonyl fluoride), 1 µg/ml aprotinin and 50 µg/ml soybean trypsin inhibitor]. Protein was determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin as the standard. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, which was performed on 10% gels. Separated proteins were transferred to a polyvinylidene difluoride membrane (1 h, 400 mA). Non-specific binding was blocked with Tris-buffered saline (TBS; 100 mM Tris, 150 mM NaCl, pH 7.5) + 0.1% Tween (adding 2.5% skim milk for the EAAT1-M and -2-M, or 5% normal goat serum and 5% skim milk for EAAT3-M and EAAT1-3-P) for 1 h at room temperature. Blots were subsequently incubated with primary antibodies: EAAT1-M and EAAT2-M were used 1 : 200 in TBS/0.1% Tween, EAAT1-P (1 : 5000) and EAAT2-P (1 : 20 000) in TBS/0.1% Tween/5% normal goat serum, and EAAT3-M (1 : 500) and EAAT3-P (1 : 5000) in TBS/0.1% Tween/10% normal goat serum. All antibodies were incubated overnight at 4°C, except for EAAT2-P (1 h, room temperature). Next, membranes were washed and incubated with alkaline phosphatase-conjugated secondary antibody [goat anti-guinea pig (Jackson Labs Inc., USA) 1 : 2000; goat anti-mouse (Jackson Labs Inc., USA) 1 : 5000; or goat anti-rabbit (Jackson Labs Inc., USA) 1 : 7500]. Finally, membranes were washed, developed with ECF (enhanced chemifluorescence) substrate (Amersham Pharmacia Biotech, UK) and visualized by a fluor S multi-imager (Bio-Rad Labs, USA).

Results of the immunoblot experiments are shown in Fig. 1. In cortical and hippocampal homogenates, both EAAT1-M and EAAT1-P antibodies showed a single immunoreactive band with an apparent molecular weight of 67 kDa. EAAT2 and EAAT3 mono- and polyclonal antibodies labelled single bands at the apparent molecular weight of 73 and 70 kDa, respectively. No immunoreactivity was

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### Table 1 Comparison of clinical variables in the three patient groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Non-HS</th>
<th>HS</th>
</tr>
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<tbody>
<tr>
<td>Age (years) ± SEM</td>
<td>55.6 ± 5.7</td>
<td>30.6 ± 4.6</td>
<td>35.1 ± 2.0</td>
</tr>
<tr>
<td>Male : female</td>
<td>3 : 4</td>
<td>2 : 7</td>
<td>3 : 1</td>
</tr>
<tr>
<td>Epilepsy onset (years)</td>
<td>NA</td>
<td>11.1 ± 2.8</td>
<td>6.8 ± 2.0</td>
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<tr>
<td>Duration (years)</td>
<td>NA</td>
<td>19.4 ± 3.1</td>
<td>28 ± 2.6</td>
</tr>
<tr>
<td>pmi (h) ± SEM</td>
<td>17.8 ± 2.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hippocampal pathology</td>
<td>NA</td>
<td>No HS</td>
<td>Severe HS</td>
</tr>
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For more details, see also Proper et al. (2000). NA = not applicable; pmi = post-mortem interval; SEM = standard error of the mean.
detected on any of the blots after omission of the primary antibody (lanes 1 and 2). These apparent molecular weights for the three transporter proteins are consistent with previously published data (e.g. Rothstein et al., 1994).

**Immunohistochemistry**

To test the effect of differences in post-mortem interval and fixation time on IR patterns, immunohistochemical stainings were first performed on two different autopsy control groups. An age-matched control group (‘archive’ controls), with a long post-mortem interval and fixation time (i.e. >48 h), and a control group, with a relatively short post-mortem time and fixation time, but with an average age that was relatively high compared with the TLE groups (‘fresh’ control group). Comparison of these two autopsy control groups showed that the archive controls displayed more variation in EAAT1±3-IR than the fresh controls and that the signal to noise ratio in EAAT3 staining was slightly better in fresh controls. Thus, in all our immunohistochemical experiments, we used the fresh autopsy control group (short post-mortem interval and fixation time) to compare with both TLE groups. Within this group, no correlation was found between EAAT1-, EAAT2- or EAAT3-IR and the post-mortem delay. To assess further the stability of EAAT1-, EAAT2- and EAAT3-IR after different post-mortem intervals, we compared sections cut from brains of Wistar rat (300 g) freshly dissected and fixed after 6 and 12 h at room temperature. The EAAT1-, EAAT2- and EAAT3-IRs in the hippocampus were not affected by a post-mortem delay of 6 or 12 h.

After fixation, sections were dehydrated and embedded in paraffin. Sections (7 μm) were cut, mounted on slides, coated with 3-aminopropyltriethoxysilane and used for immunohistochemistry. In each experiment, control and epileptic tissues were stained simultaneously. Immunohistochemical staining was performed using the avidin–biotin complex method using EAAT1-M, EAAT2-M and EAAT3-P. For EAAT1, 2 and 3, similar IR patterns were observed using the other antibodies specified under immunoblotting. Prior to incubation with the primary antibodies, sections were submitted to microwave treatment (7 min 650 W and 5 min 350 W) in 0.01 M sodium citrate buffer (pH 6) followed by quenching of endogenous peroxidase activity by immersion in 0.3% H2O2 for 30 min. EAAT1-M and EAAT2-M were diluted 1 : 150 (~0.2 μg/ml), and EAAT3-P was diluted 1 : 500 (~0.2 μg/ml) and incubation was carried out overnight at 4°C. Sections were developed using 3,3’-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. For EAAT1 and 2, the reaction was intensified using 0.1% ammonium nickel sulphate. For each antibody, control experiments were performed in which the primary antibody was omitted. Immunoreactivity was not detected in any of the patients (data not shown).

For the analysis of immunohistochemical staining of EAAT1, -2 and -3, we randomly selected two sections per patient. Since EAAT1 and -2 displayed a rather diffuse staining pattern, we chose to determine average grey values (optical density measurements). An image-based analysis system was used, with a ×10 objective and 1.25 intermediate lens, which yielded a resolution of 1.6 pixels/μm. The different subareas in the hippocampus were defined according to the nomenclature of Amaral and Insausti (Amaral and Insausti, 1990). First, all hippocampal subareas were delineated and average pixel density was measured per selected area. Selected hippocampal subareas for analysis were: CA1, CA2, CA3, CA4, and the polymorphic layer (PML) and supragranular layer (SGL) of the dentate gyrus. Since EAAT3-IR displayed a clear staining of neuronal cell bodies, we used another quantification method for EAAT3-IR. From each patient, two sections were stained using the EAAT3 antibody and two sections were counterstained using a Nissl staining after the DAB staining. Since the counterstaining did not alter DAB staining intensity, we continued using the counterstained sections. In these sections, we expressed the number of EAAT3-IR cells as a percentage of the number of Nissl-positive cells (= total number of neurones). Cells were counted by using a grid.

**In situ hybridization**

Full-length human EAAT1 and EAAT2 cDNA (courtesy of Dr H. Kawakami and Dr D. Trotti, respectively) was used. From EAAT1, nucleotides 3597–3810 (Kawakami et al., 1994; forward primer 5’-ACTGAAGTGCATGTGT-ATGCA-3’ and reverse primer 5’-GTCGAGGCTCTAA-TTTGG-3’), and from EAAT2 nucleotides 1569–1699 (Shashidharan et al., 1994; forward primer 5’-CAACTCTAATAATGTGT-3’ and reverse primer 5’-CTATT-TCTCAGGGTCCAG-3’) were subcloned. Partial cDNAs were expressed in a pBluescript SK(--) (for EAAT1) or SK(+) vector (for EAAT2) and verified by sequence analysis. For the preparation of the EAAT3 probe, we used an image clone (Research Genetics, USA; clone no. 334225). Sequence analysis of this clone showed that it contained nucleotides 1217–1763 when compared with mouse EAAT3 (GenBank accession no. U73521.1). After linearization, riboprobes were prepared using T7 and T3 RNA polymerase by in vitro transcription and labelled with [α-33P]UTP (ICN Biomedicals, USA) or with DIG (digoxigenin) RNA labelling mix (Roche Molecular Biochemicals, Germany).

**In situ hybridization** was carried out on 7 μm paraffin sections. In brief, sections were deparaffinized and deproteinated (30 min 10 μg/ml proteinase K treatment at 37°C). After post-fixation (4% paraformaldehyde in 0.01 M PBS (phosphate-buffered saline) for 5 min; only in the DIG in situ hybridization protocol), sections were washed (PBS; 2 × 1 min), and subsequently permeabilized with 0.1% Triton X-100 (10 min). After washing steps (PBS; 2 × 5 min), sections were pre-hybridized with hybridization buffer containing 2× SSC (standard saline citrate), 50% deionized formamide, 10% dextran sulfate, 1× Denhardt’s solution, 5 mM EDTA, 10 mM phosphate buffer and 0.5 mg/ml tRNA. After removal of the pre-hybridization solution, the sections
were covered with the hybridization solution containing $^{33}$P-labelled or DIG-labelled RNA probe (400 ng/ml), overnight at 55°C. Post-hybridization washes were done with 2× SSC for 30 min at room temperature, 2× SSC for 60 min at 65°C and 0.1× SSC for 60 min at 65°C. In the $^{33}$P in situ hybridization experiments, RNase A treatment was included in the protocol (20 μg/ml; 15 min at 37°C). For the detection of $^{33}$P-labelled RNA, sections were dehydrated, air dried and exposed to X-ray film (Kodak Bio-Max MR) for 7–13 days.

Two sections from each patient were used for quantitative analysis. Film autoradiographs were analysed by video-based computerized densitometry, using a MicroComputer Imaging Device image analyser (Imaging Research, Ontario, Canada). Having image capture, CA1–4 and the granule cell layer of the dentate gyrus (GCL) were selected by free-hand drawing. Subsequently, relative optical density was measured in the different hippocampal subregions.

For detection of the DIG-labelled RNA probe, sections were rinsed in TBS for 5 min and subsequently incubated with TBS containing 5% heat-inactivated foetal calf serum and 0.3% Triton X-100 for 1 h at room temperature. Then, overnight incubation at room temperature started with the primary antibody was carried out [TBS containing 1% foetal calf serum and anti-DIG-alkaline phosphatase conjugate (Roche Molecular Biochemicals, Germany), diluted 1 : 5000]. Sections were washed in TBS for 3 × 5 min and transferred to a 0.1 M Tris buffer containing 100 mM NaCl and 50 mM MgCl$_2$ (pH 9.5). Alkaline phosphatase visualization was performed in a solution containing levamisole and NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; Roche Molecular Biochemicals, Germany) overnight at room temperature. The reaction was stopped, and the sections were dehydrated and covered with enthanal (Merck, Germany). As negative controls, EAAT sense probes were tested in both in situ hybridization experiments and did not show any signal (data not shown).

**Statistical analysis**

One-way ANOVA (analysis of variance), combined with a Student–Newman–Keuls test as a multiple comparison method, was used to test differences between the three groups (control, non-HS and HS group). In cases of non-normal distribution or unequal variance (which was found in the CA3 in the EAAT2 optical density measurements), the Kruskal–Wallis ANOVA on ranks with Dunn’s test as multiple comparison (pairwise) was performed. $P < 0.05$ was considered significant.
Results

**EAAT1, -2 and -3 immunohistochemistry**

Immunohistochemical staining of the glial glutamate transporter EAAT1 showed a rather homogeneous staining pattern in the control hippocampus (Figs 2A and 3A). No EAAT1-IR was detected in neuronal cell bodies (pyramidal and granule cells), and a rather diffuse EAAT1-IR pattern was found in neuropil areas (Fig. 2A). In the hilus, EAAT1-IR was relatively low, whereas the SGL displayed high EAAT1-IR. The non-sclerotic hippocampus displayed the same staining pattern as the control hippocampus (Figs 2B and 3B). In the sclerotic hippocampus, however, a small decrease in EAAT1-IR was observed in almost all sublayers compared with the non-sclerotic or the control hippocampus (Figs 2C and 3C). Quantification of EAAT1-IR revealed no statistically significant differences between the control and non-HS group (except for the PML), and decreased EAAT1-IR in the HS group compared with the control group, which was statistically significant in CA4, PML and SGL (see Fig. 4A).

EAAT2, which is the predominant subtype in the hippocampus, showed a ‘patchy’ IR pattern, as described in other studies (Figs 2D and 3D; e.g. Milton et al., 1997). In the control hippocampus, neuronal cell bodies were negative and neuropil areas displayed high EAAT2-IR. In some areas, such as the subiculum and the SGL, individual astrocyte-like cells were clearly visible, whereas other areas, such as CA2–4 showed a more diffuse staining pattern, mainly surrounding pyramidal and granule cells. The non-sclerotic hippocampus displayed a similar EAAT2-IR distribution, but with a more diffuse IR pattern in all sublayers, with no clearly detectable individual ‘patches’ (Figs 2E and 3E). Visual inspection revealed higher staining intensity in the pyramidal cell layers CA1–3 of the non-sclerotic hippocampus compared with the control hippocampus. In the sclerotic hippocampus, however, an overall loss of EAAT2-IR was observed in almost all hippocampal subareas when compared with the non-sclerotic hippocampus (Figs 2F and 3F). In the hilus (CA4 and PML), the reduction in overall EAAT2-IR again allowed identification of individual EAAT2-IR astrocytes (Fig. 3F). Quantification of EAAT2-IR revealed a significant increase in EAAT2-IR in CA1 and CA2 in the non-HS group compared with the control group, and a strong decrease in the HS group compared with the non-HS group in almost all subareas (Fig. 4B).

EAAT3-IR in the hippocampus displayed a typical neuronal localization (Figs 2G and 3G). In the control hippocampus, EAAT3-IR was localized mainly in neuronal cell bodies and dendrites, consistent with its presumed postsynaptic localization (Rothstein et al., 1994). In the control hippocampus, not all pyramidal and granule cells were...
EAAT3-IR, which was also detected in the non-sclerotic hippocampus (Figs 2H and 3H). The staining intensity, however, was different between these two groups. In the non-sclerotic hippocampus, EAAT3-IR neurones were stained more intensely compared with EAAT3-IR neurones in the control hippocampus, and this is clearly shown in the GCL (see Fig. 3I). Moreover, the apical dendrites of the granule cells, located in the SGL, were heavily stained for EAAT3-IR, whereas in the control hippocampus only a diffuse staining was found in the SGL, with a less distinct dendritic staining. In the sclerotic hippocampus, an overall loss of EAAT3-IR was detected, which coincided with the severe loss of neurones (Figs 2I and 3I). In addition, it was found that the remaining neurones, mostly in the relatively resistant areas, such as CA2 and the GCL, displayed high EAAT3-IR, which was comparable with the EAAT3 levels in the non-sclerotic hippocampus. Quantification of the EAAT3-IR revealed an increased percentage of EAAT3-IR neurones in the non-HS group in CA1, CA4 and the GCL, which was statistically significant in the GCL. In the CA2 and GCL of the HS group, a further significant increase in the percentage of EAAT3-IR neurones was observed (Fig. 4C). In other words, the overall EAAT3-IR is lost concomitant with total neuronal degeneration, but the percentage of EAAT3-IR cells was increased in the CA2 and GCL, the two relatively resistant subareas in the HS group compared with the non-HS and control group.

EAAT1, -2 and -3 in situ hybridization
In order to examine whether the EAAT1-3 mRNA levels showed similar changes to those found for the protein levels, radioactive in situ hybridization experiments were performed (Fig. 5). To test the specificity of the probes used, human cerebellar tissue was included. In the cerebellum, highly abundant EAAT1 mRNA was found (Fig. 5D), whereas EAAT2 and EAAT3 mRNA displayed only weak labelling (Fig. 5H and L), which is in agreement with the literature (Arriza et al., 1994; Rothstein et al., 1994).

Quantiﬁcation of EAAT1-3 mRNA expression levels, measured in different hippocampal subregions, is shown in Fig. 6. In the control and non-sclerotic hippocampus, the EAAT1 antisense probe displayed a weak, diffuse signal in the pyramidal cell layers of CA1–4 (Fig. 5A and B). In the sclerotic hippocampus, a small decrease in EAAT1 mRNA was found in all hippocampal subareas compared with the non-sclerotic hippocampus, which was significant in CA3 (Fig. 6B). In the sclerotic hippocampus, on the other hand, all hippocampal subareas showed a reduction of EAAT2 mRNA as compared with the non-sclerotic hippocampus, which was significant in CA3, 4 and the PML. The CA2 region and the

![Fig. 4](https://example.com/fig4.png)

**Fig. 4** Optical density (OD) measurements of EAAT1 (A) and EAAT2-IR (B) and the percentage of EAAT3-IR positive cells (C) in the control, non-HS and HS group. Data are expressed as averages ± standard error of the mean. *P < 0.05.
dentate gyrus displayed relatively high EAAT2 mRNA levels. EAAT3 mRNA expression was found in the principal cell layers of the hippocampus (Fig. 5I), in line with a neuronal localization. In the non-sclerotic hippocampus, the distribution pattern resembled the control hippocampus (see Fig. 5J). In the sclerotic hippocampus, however, a strong overall reduction in EAAT3 mRNA expression was detected, but relatively high EAAT3 mRNA levels remained in the resistant subareas such as the GCL and the CA2 region (Fig. 5K). Quantification of EAAT3 mRNA levels revealed hardly any changes between the three patient groups, except for a small significant decrease in the HS group compared with the control group in CA3 (Fig. 6C).

To investigate the cellular localization of EAAT2 mRNA, we performed non-radioactive in situ hybridization experiments, which are shown in Fig. 7A–C. In the non-epileptic control hippocampus, EAAT2 mRNA signal was detected in neurones and in astrocyte-like cells (see Fig. 7A), but their labelling intensity was different. Neurones were heavily stained, whereas astrocytes displayed a less pronounced cellular labelling. In some control hippocampi, reduced EAAT2 glial mRNA signals were detected, most probably...
due to the post-mortem delay, resulting in a weakening or disappearance of EAAT2 mRNA staining in astrocytes. Non-radioactive EAAT2 in situ hybridization on rat hippocampus also demonstrated weak glial and strong neuronal staining of EAAT2 mRNA (data not shown).

In the non-sclerotic and sclerotic hippocampus, the cellular localization of EAAT2 mRNA was comparable with the control hippocampus (Fig. 7B and C). However, in the non-sclerotic hippocampus, a high level of EAAT2 mRNA staining was found in all principal neurones. Corresponding to severe neuronal cell loss and astrogliosis, relatively few neurones and numerous astrocytes contained EAAT2 mRNA in the sclerotic hippocampus. Although the sclerotic hippocampus is characterized by astrogliosis, individual astrocytes do not contain high amounts of EAAT2 mRNA.

Discussion
Several lines of evidence indicate that alterations in glutamate transporter levels are associated with epilepsy. For instance, microdialysis studies in the human epileptogenic hippocampus have shown that, after epileptic seizures, prolonged elevated extracellular glutamate levels exist, supporting the hypothesis that glutamate transporters are dysfunctional or absent in the epileptogenic hippocampus (During and Spencer, 1993). Recently, studies on animal models of epilepsy reported on glutamate transporter levels, but the results of these studies were contradictory (e.g. Akbar et al., 1997; Nonaka et al., 1998; Simantov et al., 1999). Therefore, it is important to determine glutamate transporter expression levels in the human epileptogenic hippocampus.

As in all human studies, limitations in experimental design and methodology must be considered when interpreting the results. In our study, we used non-epileptic control tissue, with a post-mortem interval <28 h. In a previous study by Milton et al. (1997), no changes were found in EAAT2-IR pattern up to 48 h post-mortem. TLE patients were divided into a non-HS group (no signs of hippocampal sclerosis) and a HS group (severe hippocampal sclerosis), and have been characterized in detail previously (Proper et al., 2000). The use of these three different groups allowed us to examine both seizure-related (control versus non-HS) and sclerosis-related (HS versus non-HS) morphological changes.

In the sclerotic hippocampus, a moderate general decrease in EAAT1-IR was observed when compared with the non-sclerotic or control hippocampus, which was most pronounced in the CA4, PML and SGL. This decrease in EAAT1 protein levels was accompanied by a decrease in EAAT1 mRNA levels. The results of this study are different from those obtained by Mathern et al. (1999), who found no differences in EAAT1 protein levels between the three groups. These authors used slightly different methodology, including free-floating sections and a different source of antibody. In addition, we found a 35% loss of neurones in the non-HS group (see Proper et al., 2000), whereas Mathern and colleagues did not find differences in neuronal density.
between the control and non-HS group. Comparison between our data and those from different rat models of epilepsy is difficult. In the kainic acid model, a transient increase was reported in EAAT1 mRNA (Nonaka et al., 1998), and no changes were found in hippocampal EAAT1 levels in fully kindled rats (Miller et al., 1997; Ghijsen et al., 1999). Differences between animal models might be caused by differences in mode of seizure induction and the confounding effects of the neurotoxin. Moreover, the morphological changes in the rat models vary and only partially resemble those found in humans.

In this study, the most dramatic differences were found in the levels of EAAT2, which is the predominant glial glutamate transporter in the hippocampus (Arriza et al., 1994; Rothstein et al., 1994). When compared with the control group, increased EAAT2 mRNA and protein levels were found in CA1 and CA2 of the non-sclerotic hippocampus. The increased EAAT2 levels found in the non-sclerotic hippocampus are not due to decreased IR of autopsy control tissue, as the increase in EAAT2 protein was restricted to CA1 and CA2, and was accompanied by an increase in EAAT2 mRNA. This increase was only observed for EAAT2 and not for EAAT1 and EAAT3 levels. In the sclerotic hippocampus, an overall decrease in EAAT2 mRNA and protein was observed, which was the highest in the most vulnerable regions, such as the CA1, CA3 and the hilus. This is in line with the results of Mathern et al. (1999) who found a decrease in EAAT2 protein in the HS group, which was associated with neuronal cell death. These authors did not find increased EAAT2 expression in their non-HS group. In the kainic acid model, a modest increase in EAAT2 protein was found (Simantov et al., 1999), whereas in fully kindled rats no changes in EAAT2 protein levels were reported (Akbar et al. 1997; Miller et al., 1997; Ghijsen et al., 1999).

Since our immunohistochemical studies did not allow conclusions on the cellular localization of EAAT2 protein, we performed non-radioactive in situ hybridization experiments to examine the cellular localization of EAAT2 mRNA. To our knowledge, this is the first study reporting on the cellular localization of EAAT2 mRNA in the human (epileptogenic) hippocampus. We demonstrated that EAAT2 mRNA is present in astrocytes as well as in neurones, which is in agreement with its localization in rat hippocampal tissue (Sutherland et al., 1996; Torp et al., 1997). The observation that both astrocytes and neurones contain EAAT2 mRNA explains the relatively ‘diffuse’ EAAT2 mRNA distribution compared with the clear neuronal EAAT3 mRNA expression pattern from the radioactive in situ hybridization experiments. The EAAT2 mRNA expression pattern is comparable in the control and the non-sclerotic epileptic hippocampus. Corresponding to neuronal degeneration and astrogliosis, relatively few neurones and numerous astrocytes express EAAT2 mRNA in the sclerotic hippocampus. The presence of EAAT2 protein in neurones has already been reported during rat and sheep development, where EAAT2 is localized in astrocytes and transiently expressed in neurones which subsequently lose EAAT2 as development proceeds (Furuta et al., 1997; Northington et al., 1998, 1999), but still maintain low EAAT2 mRNA transcripts (Torp et al., 1997). Several rat studies have reported that in the normal adult brain, EAAT2 protein is found specifically in astrocytes (Rothstein et al., 1994; Northington et al., 1998). In addition, under certain pathological conditions, such as after hypoxic–ischaemic insults, both EAAT2 mRNA and protein expression can be found in neurones (Martin et al., 1997). Thus, it is likely that neuronal EAAT2 protein expression contributes to the increased EAAT2-IR levels found in the non-HS group and may represent a compensatory mechanism of seizures.

In the sclerotic hippocampus, decreased EAAT2 protein levels were confirmed by the reduction of EAAT2 mRNA and were associated with neuronal degeneration. Considering that EAAT2 is found in a subset of glial cells and high amounts of (reactive) astrocytes in the sclerotic hippocampus, relative EAAT2 levels would be decreased even further in this group. Interestingly, the remaining astrocytes in the hilus of the sclerotic hippocampus displayed a normal EAAT2-IR. The neuronal localization of EAAT2 also gives an explanation as to why massive neuronal cell loss results in a reduction of EAAT2 levels in the HS group. In addition, if neuronal factors regulate the expression of EAAT2 in astrocytes (Swanson et al., 1997; Perego et al., 2000), neuronal degeneration would also result in decreased EAAT2 levels in astroglia in the HS group.

Fig. 7 Photomicrographs of EAAT2 mRNA localization in the dentate gyrus of a control (A), a non-sclerotic (B) and a severe sclerotic TLE hippocampus (C). Scale bar = 80 μm.
In both TLE groups, an increase in EAAT3 levels in individual neurones was found when compared with the control group. In the sclerotic hippocampus, an overall decrease in the total amount of EAAT3-positive neurones was detected, which is not surprising considering the amount of neuronal cell loss. It was striking, however, that surviving neurones in the sclerotic hippocampus not only contained high EAAT3 levels, but the percentage of EAAT3-IR neurones was also increased in the relatively resistant regions, such as CA2 and GCL. The study from Mathern et al. (1999) on EAAT3-IR did not report large differences between the three groups but, in the HS group, increased EAAT3-IR was also found in remaining neurones. A difference from our study is that Mathern and colleagues performed optical density measurements to quantify EAAT3-IR, whereas we determined the percentage of EAAT3-positive neurones. In the kainic acid model, an overall decrease in EAAT3 was found (Simantov et al., 1999), whereas fully kindled rats displayed increased hippocampal EAAT3 levels (Miller et al., 1997; Ghijsen et al., 1999).

The increase in neuronal EAAT3 levels in the non-sclerotic hippocampus is likely to be triggered by the elevation of glutamate during seizures and could reflect a compensatory mechanism. The increase in the number of EAAT3-IR neurones in the resistant areas in the HS group suggests that either neurones lacking EAAT3 expression will die or that EAAT3 protein expression is upregulated in all neurones as a result of increased extracellular glutamate levels. It is interesting that EAAT3 is expressed not only in glutamatergic, but also in GABAergic neurones (Rothstein et al., 1994). Since glutamate is a precursor for GABA synthesis, an increase in EAAT3-mediated glutamate transport could also reflect an increase in GABA synthesis and neurotransmission (see also Rothstein et al., 1996; Robinson and Dowd, 1997).

Taken together, the results of this study have shown differences in both mRNA and protein levels of glutamate transporter subtypes in the human epileptogenic hippocampus, which are most pronounced for EAAT2 and EAAT3. The experimental design of this study does not allow conclusions to be drawn about whether the changes in glutamate transporter levels precede or result from neuronal cell death. The possibility that a deficit in glutamate transport capacity leads to neuronal death was confirmed by different knock-out studies (Rothstein et al., 1996; Tanaka et al., 1997). On the other hand, in vitro studies indicate that neurones participate in the regulation of glial glutamate transporter expression (e.g. Swanson et al., 1997). In this case, a severe loss of neurones in the sclerotic hippocampus could lead to a decrease in astroglial glutamate transporter levels. Moreover, it is not clear what the mechanism is behind the postulated neuronal EAAT2 protein production. It most probably reflects a protective mechanism against high extracellular glutamate levels caused by epileptic seizures. It should also be noted, however, that EAAT levels do not necessarily correspond to functional transporter activity. In fact, decreased EAAT levels may even be protective in epileptic tissue, since EAATs are known to reverse during depolarization and may contribute to high extracellular glutamate levels. Therefore, it will be important to investigate functional properties of glutamate transporters in pathological tissues to elucidate the relationship between transporter levels and transport activity.

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References
Glutamate transporters in temporal lobe epilepsy


