Mechanisms of disease

Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy

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Summary

Background High extracellular glutamate concentrations have been identified as a likely trigger of epileptic seizures in mesial temporal lobe epilepsy (MTLE), but the underlying mechanism remains unclear. We investigated whether a deficiency in glutamine synthetase, a key enzyme in catabolism of extracellular glutamate in the brain, could explain the perturbed glutamate homoeostasis in MTLE.

Methods The anteromedial temporal lobe is the focus of the seizures in MTLE, and surgical resection of this structure, including the hippocampus, leads to resolution of seizures in many cases. By means of immunohistochemistry, western blotting, and functional enzyme assays, we assessed the distribution, quantity, and activity of glutamine synthetase in the MTLE hippocampus.

Findings In western blots, the expression of glutamine synthetase in the hippocampus was 40% lower in MTLE than in non-MTLE samples (median 44 [IQR 30–58] *vs* 69 [56–87]% of maximum concentration in standard curve; p=0·043; n=8 and n=6, respectively). The enzyme activity was lower by 38% in MTLE *vs* non-MTLE (mean 0·0060 [SD 0·0031] *vs* 0·0097 [0·0042] U/mg protein; p=0·045; n=6 and n=9, respectively). Loss of glutamine synthetase was particularly pronounced in areas of the MTLE hippocampus with astroglial proliferation, even though astrocytes normally have high content of the enzyme. Quantitative immunoblotting showed no significant change in the amount of EAAT2, the predominant glial glutamate transporter in the hippocampus.

Interpretation A deficiency in glutamine synthetase in astrocytes is a possible molecular basis for extracellular glutamate accumulation and seizure generation in MTLE. Further studies are needed to define the cause, but the loss of glutamine synthetase may provide a new focus for therapeutic interventions in MTLE.

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Introduction

Temporal lobe epilepsy is one of the commonest drug-resistant epilepsies.[1] In more than 40% of cases, this disorder cannot be controlled by medication, and anteromedial temporal lobectomy with hippocampectomy is needed for seizure control in some of these patients.[2] About 70% of the resected hippocampi are characterised by pronounced neuronal loss and astroglial proliferation, especially in areas CA1, CA3 (cornu ammonis subfields), and the dentate hilus (figure 1).[3] These neuropathological features are the hallmarks of hippocampal sclerosis, a distinctive characteristic of patients with mesial temporal lobe epilepsy (MTLE), noted first in 1880.[4] In MTLE, depth electrode recordings indicate that the seizures originate from the sclerotic hippocampus.[5] Moreover, surgical removal of the sclerotic hippocampus is associated with the best outcome, resulting in excellent seizure control in about 85% of patients.[6] Mathern and colleagues[7] have discussed more fully how these and other observations suggest that the sclerotic hippocampus has a key role in the generation of seizures in MTLE.

In-vivo BRAIN MICRODIALYSIS studies of patients with MTLE have shown that the extracellular concentration of glutamate rises in the sclerotic hippocampus just before a seizure and remains high for at least 15 min after the cessation of electrographic seizure activity.[8] Studies in animals show that administration to the hippocampus of glutamate or glutamate analogues triggers seizures, whereas coinjection of glutamate antagonists blocks the seizures.[9,10] Intracellular recordings from dentate granule cells in slice preparations of the hippocampus in human MTLE have shown hyperexcitability of these neurons that is glutamate dependent.[11] Furthermore, the glutamate-receptor subunits GluR1 and GluR2 are upregulated in the hippocampus in MTLE.[12] Taken together, these observations suggest that glutamate is crucial in the generation and maintenance of seizures in MTLE.

Glutamate released from synapses is normally taken up by astrocytes and rapidly converted to the non-excitotoxic aminoacid glutamine by glutamine synthetase.[13] This process is energy dependent and also requires metal ions for catalysis.[14] Glutamine is then transported from the astrocytes back to the neurons, where it may be converted to glutamate, mainly via phosphate-activated glutaminase.[13] This process is known as glutamine-glutamate cycling.[15] A study by magnetic resonance spectroscopy has shown possible disruption in the cycling of glutamate to glutamine in the hippocampus in MTLE.[16] The study showed that hippocampal glutamate content was increased and glutamine content decreased in MTLE compared with non-MTLE. Furthermore, the glutamate-glutamine cycling was slower in MTLE. Our hypothesis is that the accumulation of glutamate and

GLOSSARY

BRAIN MICRODIALYSIS
A technique to obtain real-time samples of extracellular solutes (such as glutamate) from the brain of living individuals. It is achieved by implantation of a dialysis probe in the brain followed by perfusion of a salt solution (dialysate) through the probe. Solutes in the extracellular space will equilibrate with the dialysate and their concentrations can be measured, generally by high-performance liquid chromatography or mass spectroscopy.

EXCITOTOXICITY
The concept that high extracellular concentrations of certain aminoacids (such as glutamate or kainic acid) can cause excitation and degeneration of neurons. The excitotoxic action is mediated by binding of the aminoacid to neuronal glutamate receptors. There are several types of glutamate receptors, each being assembled by unique receptor subunits.

slowing of glutamate-glutamine cycling in MTLE reflect downregulation of glutamine synthetase and, therefore, that a well-defined enzyme defect contributes to seizure generation. The aim of this study was to address this hypothesis by resolving whether glutamine synthetase is downregulated in the hippocampus in MTLE. Part of this work has been published in abstract form.[17]

Methods

Patients and tissue

Patients with medically intractable temporal lobe epilepsy underwent phased presurgical assessment at the Yale–New Haven Hospital. In those selected for surgery, the hippocampus was resected according to standard procedures.[2] Between July, 1996, and February, 2002, 86 hippocampi were resected. 29 were randomly selected for this study (table 1) and are representative of the population of patients who undergo surgery at this hospital. In seven patients who died from non-neurological causes, the hippocampus was removed at autopsy. Informed consent from each patient and institutional approval were obtained for the use of tissue in this project.

The mesial temporal lobe consists of the hippocampus, parahippocampal gyrus, entorhinal cortex, and amygdala. The hippocampus is commonly divided into the subiculum, Ammon's horn, and dentate gyrus.[18] Ammon's horn is further subdivided into the areas CA1, CA2, and CA3, and the dentate gyrus is subdivided into the hilus, granule-cell layer, and molecular layer.[18]

Tissue was classified separately by two investigators (TE and NCdeL), and the samples were encoded for further use. Thus, the researchers who did the western

Patient and classification	Sex	Age (years)	Time since first unprovoked seizure (years)	Antiepileptic drugs at surgery	MRI findings	Pathology
Non-MTLE						
1	M	39	16	Lamotrigine	Normal	Normal
2	F	22	10	Topiramate	Normal	Normal
3	F	46	28	Carbamazepine	Normal	Normal
4	M	17	1·5	Lithium	Cystic lesion, R parahippocampal gyrus	Focal fibrosis
5	M	38	10	Lamotrigine, phenytoin	L hippocampal atrophy, no signal changes	Normal
6	M	62	14	Carbamazepine, phenytoin	Hyperintense abnormality, R amygdala and parahippocampal gyrus; normal hippocampus	Oligodendroglioma
7	M	35	17	Lamotrigine, carbamazepine	Cavernous haemangioma, R amygdala, adjacent to hippocampal head; normal hippocampus	Cavernous haemangioma
8	F	41	6	Lamotrigine, carbamazepine	Bilateral hippocampal atrophy	Normal
9	M	46	9	Oxcarbazepine	R temporal-lobe lesion, adjacent to hippocampal head and amygdala	Normal
10	F	29	2	Phenytoin, valproate	Rounded hyperintense mass, L amygdala, extending to the hippocampal head	Normal
11	M	38	20	Valproate, gabapentin, phenytoin	R parietal-lobe abnormality; mild L hippocampal atrophy; no signal changes	Normal
12	M	27	1	Carbamazepine	L mass lesion in mesial temporal lobe, encroaching sub-basal ganglia, replacing and involving amygdala and anterior hippocampus	Oligodendroglioma
13	F	57	20	Lamotrigine, carbamazepine	Hyperintense abnormality of R amygdala and perihippocampal gyrus, extending into hippocampal head and inferior aspect of basal ganglia	Ganglioglioma
14	F	7	6	Valproate, carbamazepine	Tumour, L hippocampus	Ganglioglioma
15	M	12	0	Carbamazepine	Cystic lesion, R medial temporal lobe	Oligodendroglioma
MTLE						
16	M	47	22	Carbamazepine	R hippocampal atrophy	Hippocampal sclerosis
17	F	36	14	Carbamazepine	L hippocampal sclerosis	Hippocampal sclerosis
18	F	45	27	Carbamazepine, primidone, gabapentin	R hippocampal atrophy	Hippocampal sclerosis
19	M	40	39	Carbamazepine, phenytoin	Normal	Hippocampal sclerosis
20	M	27	26	Gabapentin, felbamate	R hippocampal atrophy	Hippocampal sclerosis
21	F	29	27	Carbamazepine	Atrophy of R hemisphere, including amygdala and hippocampus; increased T2 signal of R hippocampus	Hippocampal sclerosis
22	M	28	25	Valproate, lamotrigine	L temporal-lobe atrophy	Hippocampal sclerosis
23	F	29	25	Valproate, carbamazepine	L mesial temporal sclerosis	Hippocampal sclerosis
24	M	24	16	Topiramate, phenobarbital	R hippocampal atrophy	Hippocampal sclerosis
25	F	28	19	Carbamazepine	L hippocampal atrophy	Hippocampal sclerosis
26	F	24	24	Carbamazepine	L hemiatrophy involving amygdala, basal ganglia, and thalamus; L hippocampal atrophy with signal changes	Hippocampal sclerosis
27	M	48	43	Carbamazepine	L hemiatrophy due to MCA infarction; L hippocampal sclerosis	Hippocampal sclerosis
28	M	51	40	Carbamazepine, lamotrigine	R hippocampal sclerosis	Hippocampal sclerosis
29	F	43	35	Carbamazepine, valproate, tiagabine	R temporal-lobe atrophy	Hippocampal sclerosis

MCA=middle cerebral artery.

Table 1: **Characteristics of patients selected for the study**

blots (MJT and ER-P) and enzyme assays (JCKL and GVM) were unaware of the patients' categories until the experiments had been completed and the data were ready for statistical analysis.

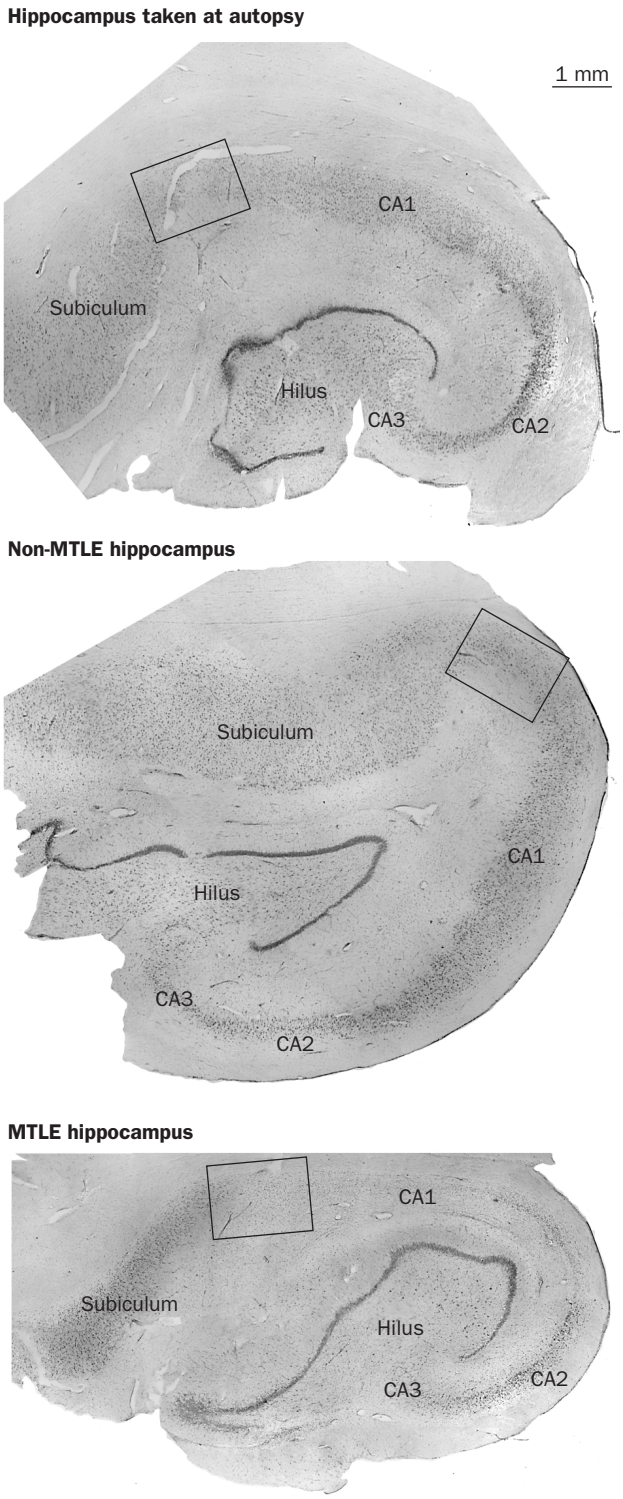


Figure 1: **Nissl-stained coronal sections of hippocampi from representative cases**

Density and distribution of neurons are similar in autopsy and non-MTLE hippocampi. By contrast, the MTLE hippocampus shows severe loss of neurons, particularly in areas CA1 and CA3 and the dentate hilus. Adjacent sections to those shown were processed for glutamine synthetase immunohistochemistry, and the boxed areas (ie, transition between the subiculum and CA1) are shown in figure 2. Magnification is the same for all sections.

The surgically resected hippocampi were classified into two groups: MTLE and non-MTLE (figure 1). Non-invasive studies and depth and subdural electrode recordings have suggested that in MTLE the seizure activity originates from the hippocampus.[5] The MTLE hippocampus is characterised by pronounced neuronal loss (>50%) and extensive astroglial proliferation in CA1, CA3, and the hilus.[19] Also, the MTLE hippocampus shows reorganisation of peptidergic (dynorphin, somatostatin, neuropeptide Y, substance P) neurons in the dentate gyrus.[3] The non-MTLE hippocampus is characterised by smaller (<25%) neuronal loss throughout all hippocampal subfields, little astroglial proliferation, and no reorganisation of peptidergic neurons in the dentate gyrus.[19] The histological pattern in the non-MTLE hippocampus is similar to that of autopsy controls.[19] In about 50% of non-MTLE hippocampi, the seizures are thought to originate from a mass lesion (a tumour in most cases) outside the hippocampus, but within the temporal-lobe territory.[19] In the remaining cases, no mass lesion is apparent and the hippocampi are selected for resection on the basis of intracranial recordings of seizure onset.[5]

Immediately after surgical resection or autopsy, two 5 mm slices were cut from the mid-portion of the hippocampus. One sample was immersed in a fixative containing 4% paraformaldehyde and 15% (by volume) saturated picric acid in 0·1 mol/L phosphate buffer, pH 7·4, for 1 h, then in 5% acrolein in phosphate buffer for 3 h. The tissue was then rinsed and stored in phosphate buffer at 4°C. 50 μm coronal sections were cut on a Vibratome and processed for pre-embedding immunohistochemistry.

The other sample was rapidly frozen on dry ice and cut coronally into 200 μm sections on a cryostat. These sections contained all hippocampal subfields, and some of the sections were microdissected further into three portions: dentate gyrus and CA3; CA1; and subiculum. All frozen sections were stored at –80°C for use later for western blots and enzyme assays. Owing to the small quantities of frozen tissue available for analysis, western blots and enzyme assays could not be done on tissue from the same patient. Instead, frozen tissue from each of the categories of patients was randomly but equally allocated to western blotting (total 14) or enzyme-activity studies (total 15).

We used a highly specific monoclonal antibody against sheep glutamine synthetase that cross-reacts with the human enzyme (G45020, Transduction Laboratories, Lexington, KY, USA) and a polyclonal antibody against human glial fibrillary acidic protein (GFAP), a marker for fibrous astrocytes (YMPS32, Accurate Chemical and Scientific Corporation, Westbury, NY, USA). The antibody against glutamate transporter EAAT2 (excitatory aminoacid transporter 2) was raised in rabbits against a synthetic peptide, KQVEVRMHDSHLSSE-amide, corresponding to aminoacid residues 12–26 of rat EAAT2 (GLT1) and affinity purified.[20] This antibody has been extensively tested and characterised and cross-reacts with human EAAT2.[21] Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

Immunohistochemistry

For single-labelling immunohistochemistry, Vibratome sections were incubated at 4°C, free-floating in a solution of glutamine synthetase antibody (1 in 32 000 dilution) for 72 h, then processed according to the avidin-biotin peroxidase method[22] with a commercial kit (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA). For double-labelling immunocytochemistry, Vibratome sections

were incubated at 4°C, free-floating in a mixture of glutamine synthetase antibody (1 in 10 000 dilution) and GFAP antibody (1 in 5000 dilution) for 72 h or glutamine synthetase antibody (1 in 10 000 dilution) and EAAT2 antibody (1 in 500 dilution). After washing, sections were incubated for 2 h in a mixture of Alexa Fluor 488 goat antibody to mouse immunoglobulin (1 in 400 dilution) and Alexa Fluor 594 goat antibody to rabbit immunoglobulin (1 in 1000 dilution; both from Molecular Probes Inc, Eugene, OR, USA). The single-labelled sections were examined by light microscopy and the double-labelled sections by confocal laser scanning microscopy (Zeiss LSM 510, Carl Zeiss Microimaging, Thornwood, NY, USA).

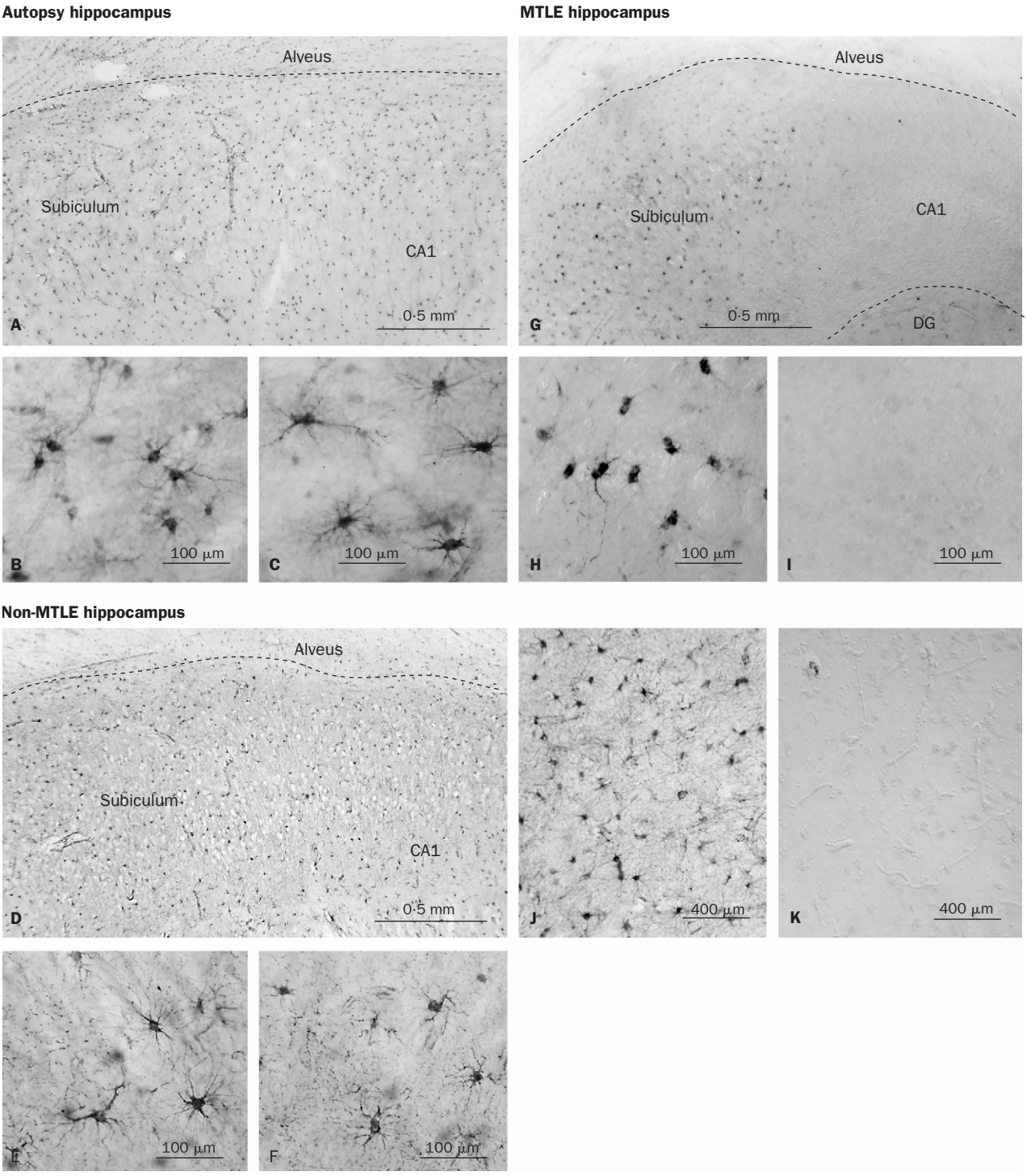


Figure 2: **Glutamine synthetase immunoreactivity of representative autopsy (A–C), non-MTLE (D–F), and MTLE (G–I) hippocampi**
Adjacent sections to those in figure 1. There is dense and even distribution of glutamine-synthetase-positive cells in subiculum and area CA1 of autopsy (A) and non-MTLE hippocampi (D). High-power fields of subiculum in autopsy (B) and non-MTLE (E) hippocampi show that staining is confined to astroglial cells. High-power fields of area CA1 in autopsy (C) and non-MTLE (F) hippocampi also show many positive astroglial cells. In MTLE hippocampus (G), there are many glutamine-synthetase-positive cells in the subiculum but area CA1 is severely deficient in staining for glutamine synthetase. High-power field of subiculum in G (H) confirms presence of staining in astroglial cells, which have somewhat fewer processes than positive astrocytes in the corresponding area of autopsy (B) and non-MTLE hippocampi (E). High-power field of area CA1 in G (I) confirms lack of staining in this region. Specificity controls with glutamine synthetase antiserum (J) and preimmune serum (K) on adjacent sections of the non-MTLE hippocampus shown in D–F reveal no staining in K. DG=dentate gyrus.

Western blots

Western-blot analysis was restricted to the surgical specimens to avoid problems with band identification and quantification due to post-mortem autolytic cleavage of proteins. Frozen non-MTLE and MTLE whole hippocampi and microdissected subregions were sonicated on ice in homogenisation buffer—50 mmol/L 3-(N-morpholino)propane-sulphonic acid/HCl and 2 mmol/L dithiothreitol, pH 7·6, 3 mmol/L ethylene glycol tetra-acetic acid, 0·5 mmol/L magnesium acetate, 0·1 mmol/L sodium orthovanadate, 0·1 mmol/L phenylmethylsulphonyl fluoride, 20 mg/L leupeptin, 10 mg/L pepstatin A, 5 mg/L aprotinin, and 0·32 mol/L sucrose. Protein concentrations were measured with a bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL, USA) and a microplate reader at 570 nm (MRX, BioLinx 2.20 software, Dynatech Laboratories, Alexandria, VA, USA). Protein homogenates were mixed (4 to 1) with sample loading buffer (50% sucrose, 7·5% sodium dodecyl sulphate, 62·5 mmol/L stacking gel buffer [which contains 0·5 mol/L Tris-HCl, 0·4% sodium dodecyl sulphate, pH 6·8], 2 mmol/L edetic acid, pH 7·5, 3·1% dithiothreitol, 0·01% bromophenol blue), and applied at concentrations of 10 μg or 20 μg protein per lane (for EAAT2 and glutamine synthetase, respectively). The proteins were separated by electrophoresis on 12% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were incubated overnight in primary antibody against glutamine synthetase (1 in 10 000) or EAAT2 (0·2 mg/L) diluted in 5% blocking agent (Enhanced Chemi-fluorescence kit, Amersham-Pharmacia, Piscataway, NJ, USA) in Tris-buffered saline (200 mmol/L Tris-HCl, 1·37 mol/L sodium chloride, pH 7·6) with 0·1% Tween 20. After washes in Tris-buffered saline with Tween 20, the membranes were incubated in fluorescein-conjugated antibodies to mouse immunoglobulin (glutamine synthetase) or rabbit immunoglobulin (EAAT2/GLT1) (Enhanced Chemi-fluorescence kit, diluted 1 in 600), washed again in Tris-buffered saline with Tween 20, and incubated in alkaline-phosphatase-conjugated anti-fluorescein (Enhanced Chemi-fluorescence kit, diluted 1 in 2500).

The chemifluorescent signal was detected with a CCD camera for glutamine synthetase (Fluor-S MultiImager, Bio-Rad) or a laser scanner for EAAT2 (Molecular Imager FX, Bio-Rad). For each blot, a global background subtraction was done, which removed the high-frequency, low-intensity pixels. After zooming in until individual pixels were visible, we traced each band manually using the remaining background pixel intensity immediately adjacent to the band as cutoff. A standard curve was made for each blot with increasing concentrations (within the linear range of the curve) of sodium-dodecyl-sulphate-homogenised human neocortex from a non-MTLE patient, with the highest concentration set at 100%. The volume values (sum of intensities of the pixels within the volume boundary multiplied by the pixel area) of the bands were calculated for glutamine synthetase and EAAT2, and the relative concentrations of glutamine synthetase and EAAT2 from the patients' samples were obtained by comparison with the standard curve.

The antibody to glutamine synthetase gave a single band at 45 kDa on western blots, consistent with the expected molecular mass of glutamine synthetase. The EAAT2 antibody also gave a strong band at the expected 70 kDa location. Substitution of the primary antibodies with normal serum or preimmune serum completely abolished the staining.

Enzyme assay

The enzyme activity assay was restricted to the surgical specimens because no systematic studies on the stability of glutamine synthetase in autopsy material are available. A homogenate of about 10% (weight/volume) of the hippocampal tissue was prepared in isolation medium (0·32 mol/L sucrose, 1 mmol/L edetic acid, 5 mmol/L HEPES, pH adjusted with Tris to 7·4). Activity of glutamine synthetase in human hippocampal homogenates was measured by the method of Dennis and colleagues[23] over a 30 min incubation at 25°C in a 1 mL reaction mixture containing (at final concentration) 44 mmol/L Tris-HCl, pH 7·2, 44 mmol/L magnesium chloride, 1 mmol/L dithiothreitol, 1 mmol/L ATP, 50 mmol/L sodium glutamate, 0·16% (by volume) Triton X-100, 5 mg/L oligomycin, 1 mmol/L ouabain (dissolved in 0·16% ethanol), freshly neutralised 0·1 mmol/L hydroxylamine, and an ADP trap consisting of 10 mmol/L phospho-enolpyruvate and 20 units of pyruvate kinase. The reaction mixture was then transferred to separate microfuge tubes containing 66 μL 37 mmol/L iron (III) chloride and 47 mmol/L trichloroacetic acid in 0·5 mol/L hydrochloric acid. The amount of the complex of L-γ-glutamyl-hydroxamate and iron (III) chloride formed was measured as the absorbance of the supernatant at 540 nm after the precipitated protein had been removed by centrifugation (15 000 *g* for 3 min). The extinction coefficient for this complex under these conditions was $7{\cdot}7\times10^5$ cm^2/mol. Protein concentrations in tissue homogenate were measured by the Pierce bicinchoninic acid assay method with bovine plasma albumin as the standard. The enzyme activities were the means of at least two separate measurements and were expressed as U/mg protein. The amount of protein used per assay was in the range 0·98–1·90 mg. Under our assay conditions, enzyme activities increased linearly with time and with homogenate protein concentration.

Statistical analysis

To assess the western blots and enzyme assays, Kruskal-Wallis one-way ANOVA was used to test whether there were any overall differences between groups. A two-tailed Mann-Whitney *U* test was then used to compare paired groups.

Role of the funding source

The sponsors of this study had no involvement in the study design, in collection, analysis, or interpretation of data, in writing of the report, or in the decision to submit the paper for publication.

Results

The immunohistochemical distribution of glutamine synthetase was similar in hippocampi removed at autopsy (n=7) and non-MTLE (n=15) hippocampi (figure 2); glutamine synthetase was strongly expressed in astroglial cells throughout all subfields. By contrast, in the MTLE hippocampi (n=14), pronounced loss of glutamine synthetase immunoreactivity was apparent, particularly in areas of peak neuronal loss and astroglial proliferation, such as CA1 (figure 2, G, I) and CA3. In areas of negligible neuronal loss and less astroglial proliferation, such as the subiculum and parts of the dentate gyrus (granular and molecular layer), glutamine synthetase was expressed in astroglial cells (figure 2, G, H). Typically, a sharp demarcation in staining was evident between the glutamine-synthetase-rich subiculum and depleted CA1 (figure 2, G). The staining pattern for glutamine synthetase was consistent within each of the autopsy, non-MTLE, and MTLE groups.

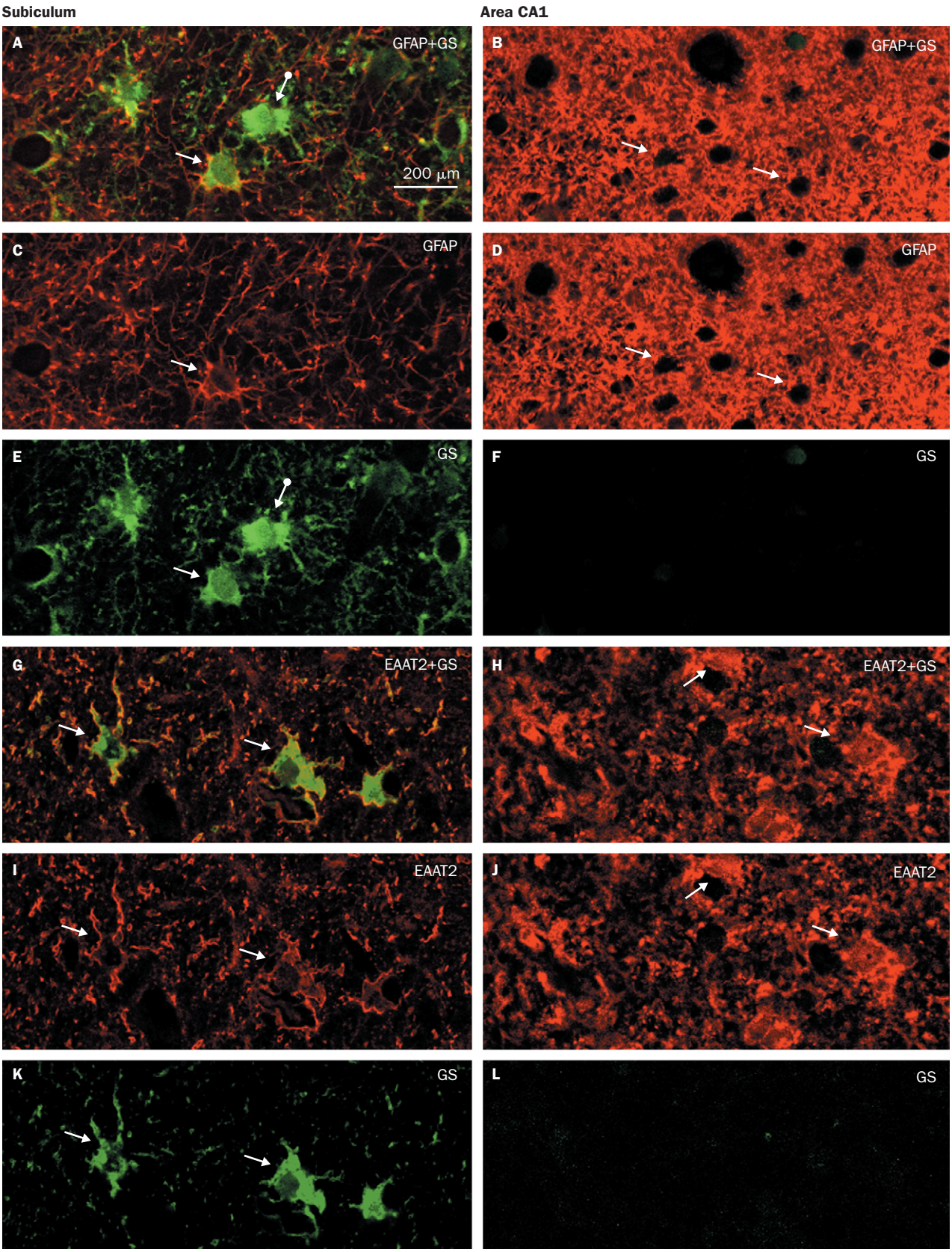
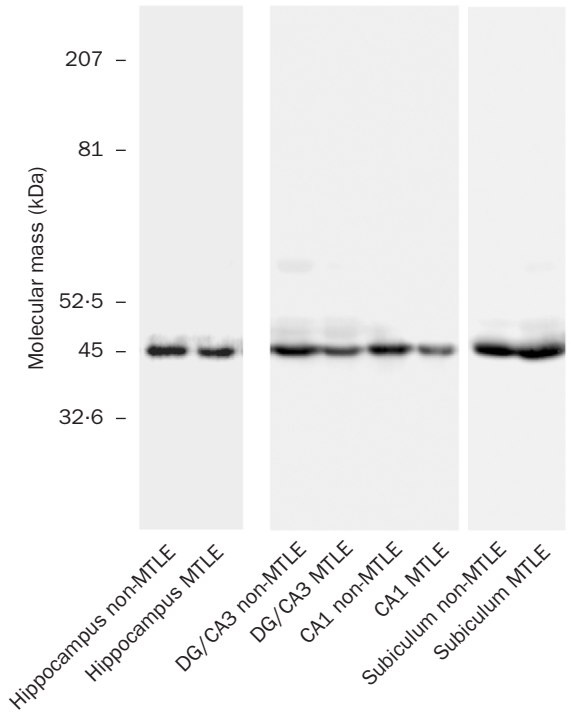


Figure 3: **Double-labelling immunohistochemistry of GFAP (red) and glutamine synthetase (GS; green) or EAAT2 (red) and GS (green) in MTLE subiculum (left) and MTLE area CA1 (right)**

Antibodies applied are indicated in the top right-hand corner of each image. Many GS-positive astroglial cells are present in MTLE subiculum (arrows with or without dots in A and E); some colocalise with GFAP (arrow in A, C, and E), but others do not (arrows with dot in A and E). In area CA1, a large number of GFAP-positive astroglial cells (arrows in B and D) are present along with a dense network of GFAP-positive processes, but there is virtually no staining for GS (F). Many GS-positive astroglial cells that also colocalise with EAAT2 are present in the MTLE subiculum (arrows in G). Virtually all GS-positive cells also localise with EAAT2 (G, I, K). In area CA1, a large number of EAAT2-positive glial cells (arrows in H and J) are present. None of these cells contains GS (L). For comparison, A–F and G–L, respectively, were taken from the same section and captured at the same laser intensity. Magnification is the same for all pictures.



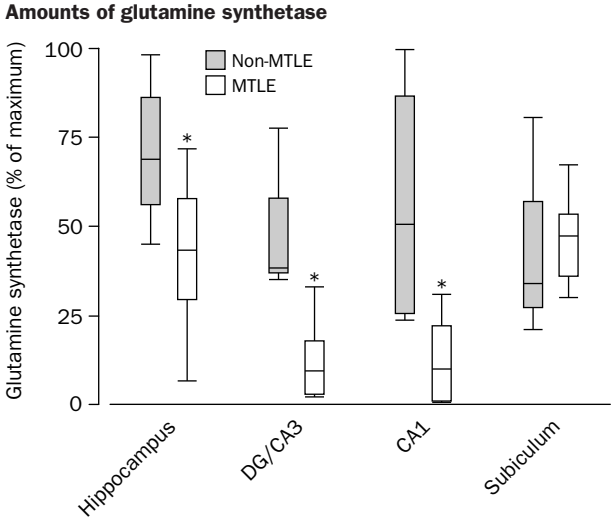


Figure 4: **Western blots of glutamine synthetase in non-MTLE and MTLE hippocampi and amounts of glutamine synthetase from western blots**

Blots show a single band at the predicted size of 45 kDa. DG=dentate gyrus. Amounts of glutamine synthetase are presented as percentage of highest concentration applied for the standard curve. Box=IQR; line across box=median; whiskers=maximum and minimum. *p<0·05. Number of patients (in order depicted): six, three, three, six for non-MTLE and eight, five, six, eight for MTLE.

Double-labelling studies showed that in areas of little neuronal loss such as the MTLE subiculum, glutamine synthetase was associated with the marker for fibrous astrocytes, GFAP (figure 3). In these areas, most of the GFAP-positive cells expressed glutamine synthetase; furthermore, glutamine synthetase was also present in a subpopulation of GFAP-negative cells that were similar in size and shape to the GFAP-positive cells. In the neuron-depleted areas of the MTLE hippocampi, such as CAI, strong staining for GFAP was noted in cell bodies and cellular processes. However, few if any of these cells expressed detectable amounts of glutamine synthetase. By contrast, in area CA1 of the non-MTLE hippocampus the majority of GFAP-containing cells also expressed glutamine synthetase (data not shown).

To estimate amounts of glutamine synthetase quantitatively, western blotting was done on whole hippocampal sections and microdissected subsections from non-MTLE and MTLE hippocampi (figure 4). The amount of glutamine synthetase in whole sections was 40% lower in the MTLE hippocampi than in non-MTLE hippocampi (p=0·043, Mann Whitney *U*, two-tailed). Within specific hippocampal subfields the reductions in glutamine synthetase in MTLE compared with non-MTLE were 74% in the dentate gyrus/CA3 (p=0·036) and 78% in area CA1 (p=0·038). No difference between MLTE and non-MLTE was found in the subiculum. Within the MTLE group, expression of glutamine synthetase was 73% lower in the dentate gyrus/CA3 than in the subiculum (p=0·0087) and 74% lower in area CA1 than in the subiculum (p=0·0043). No significant difference in glutamine synthetase was found between CA1 and dentate gyrus/CA3 in this group, nor between any of the subfields in the non-MTLE hippocampi. Thus, the data from western blotting were consistent with the observations from the immunohistochemical study.

To establish whether the reduced expression of glutamine synthetase was associated with reduced activity of the enzyme, functional assays were done (table 2). The mean activity was significantly (38%; p=0·045) lower in hippocampal homogenates derived from MTLE patients (0·0060 [SD 0·0031]; n=6) than in those from the non-MTLE group (0·0097 [0·0042]; n=9).

To assess whether the observed deficiency in glutamine synthetase was associated with reduced expression of transporters for glutamate, immunohistochemical and western-blot studies of the glutamate transporter EAAT2 were done. EAAT2 was chosen because it is the main glial glutamate transporter in the hippocampus.[24] Double-labelling immunohistochemical experiments showed that EAAT2 was present in virtually all glutamine-synthetase-positive astrocytes of non-MTLE and MTLE hippocampi (figure 3). EAAT2 was also found in the glutamine-synthetase-deficient astrocytes of MTLE hippocampi (figure 3). Western-blot analysis showed no significant difference in expression of EAAT2 between non-MTLE and MTLE hippocampi (figure 5).

Patient	Glutamine synthetase activity (U/mg protein)
Non-MTLE	
4	0·0180
5	0·0140
6	0·0053
8	0·0050
9	0·0076
10	0·0110
11	0·0091
13	0·0061
14	0·0120
Mean	0·0097 (SD 0·0042)
MTLE	
21	0·0047
22	0·0053
23	0·0053
26	0·0030
27	0·012*
28	0·0057
Mean	0·0060 (SD 0·0031)

*This patient had had a stroke of the middle cerebral artery several years before hippocampectomy.

Table 2: **Activity of glutamine synthetase**

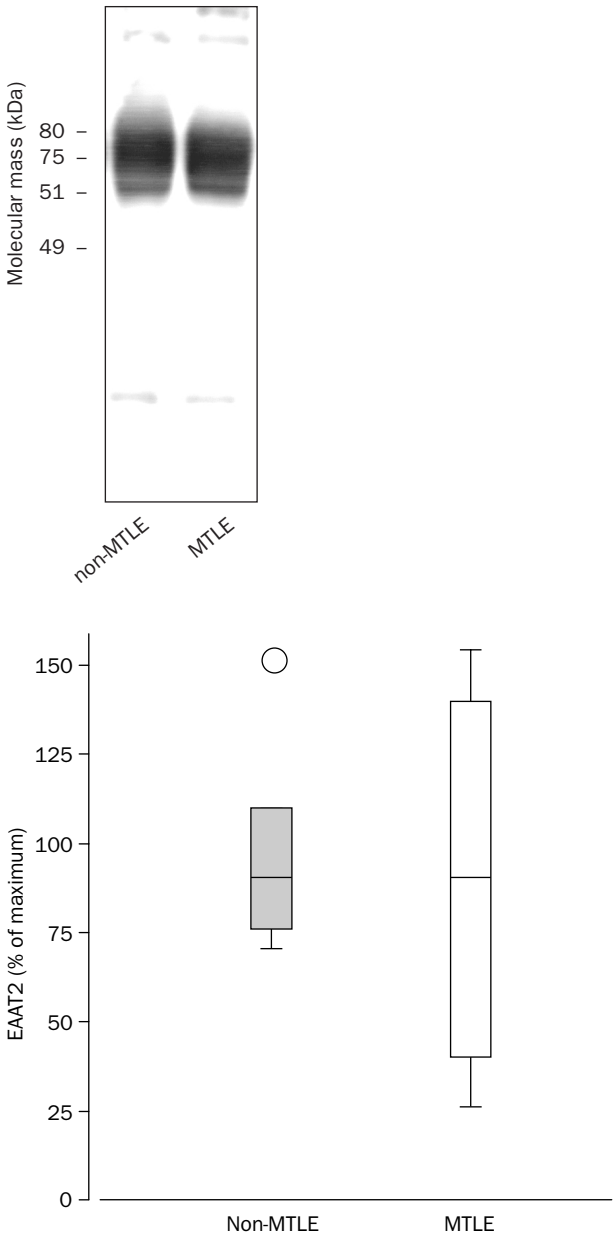


Figure 5: **Western blots of EAAT2 in non-MTLE (n=6) and MTLE (n=8) hippocampi and amounts of EAAT2**
Box plot as in figure 4; circle indicates outlier.

Discussion

Several lines of evidence suggest that the sclerotic MTLE hippocampus has a key role in the maintenance of temporal lobe seizures.[7] The hypothesis addressed by this study was that the accumulation of glutamate[8] and the slowing of glutamate-glutamine cycling,[16] features thought to be associated with triggering of seizures in the MTLE hippocampus, are caused by downregulation of glutamine synthetase.

We found that astrocytes are deficient in glutamine synthetase in surgically resected MTLE hippocampi, particularly in areas of peak neuronal loss and astroglial proliferation, such as the CA1, CA3, and dentate hilus. Because glutamine synthetase is necessary for the conversion of glutamate to glutamine, its deficiency would be expected to cause an increase in glutamate in the astrocytes negative for glutamine synthetase. This rise would slow the cycling of astrocytic glutamate to glutamine, and by mass action lead to accumulation of astrocytic and extracellular glutamate. The known stoichiometry of glutamate transport across the glial plasma membrane suggests that rapid metabolism of intracellular glutamate is a prerequisite for efficient glutamate clearance from the extracellular space.[25]

In-vitro experiments with organotypical hippocampal cultures have shown that inhibition of glutamine synthetase by the competitive antagonist methionine sulphoximine[26] causes depletion of glutamine and accumulation of glutamate in astrocytes.[27] It is also well known that methionine sulphoximine causes EXCITOTOXICITY.[28] Overexpression of glutamine synthetase in the neural retina is associated with an increased rate of glutamate uptake.[29] This observation accords with previous findings pointing to a neuroprotective effect of raised glutamine synthetase.[30]

The higher than normal amount of extracellular glutamate in the MTLE hippocampus could be due not to a deficiency in glutamine synthetase but to impaired clearance owing to loss of glutamate transporters. If glutamate cannot enter the astrocyte, the issue of whether the cell contains glutamine synthetase becomes irrelevant. To investigate this possibility, we assessed the presence of the most abundant astrocytic glutamate transporter in the hippocampus,[24] EAAT2, by immunohistochemistry and quantitative western blots. We found no difference in EAAT2 expression between non-MTLE and MTLE hippocampi; furthermore, the glutamine-synthetase-deficient astrocytes in the MTLE hippocampi expressed EAAT2. Thus, glutamate appears to have access to the cytosol of the glutamine-synthetase-deficient astrocytes, so glutamine synthetase downregulation seems the most likely factor for decreased glutamate clearance.

The importance of glutamine synthetase as a crucial factor for epileptic seizures has also been suggested by animal studies. Blocking of glutamine synthetase by systemic administration of methionine sulphoximine readily causes seizures in rats[31] and mice.[32] In a model of epilepsy, glutamine synthetase was deficient in the brain of seizure-prone gerbils compared with animals not prone to seizures.[33] As in MTLE, the loss of glutamine synthetase in seizure-prone gerbils was not associated with a lack of astrocytes.[33]

What is the mechanism behind the loss of glutamine synthetase in MTLE? The loss is not an unspecific effect of glial-cell proliferation per se, since no change in glutamine synthetase has been evident in two models of experimentally induced gliosis.[34,35] Nor is there a change in the activity or amount of glutamine synthetase in the wobbler mouse (although reactive astrocytes were negative for glutamine synthetase).[36] The wobbler mouse is characterised by progressive muscular atrophy, motoneuron degeneration, and astroglial proliferation.[36]

Glutamine synthetase could be downregulated through the loss of neurons and hence, reduced demand for glutamate detoxification. This possibility must be considered since the findings of Derouiche and colleagues[37] in hippocampal slice cultures can be interpreted to support the notion that neuronal glutamate release is required to induce glutamine synthetase in astrocytes. However, microdialysis studies show that the astrocytes in the MTLE hippocampus are exposed to unusually high extracellular glutamate concentrations,[8] indicating that downregulation of glutamine synthetase cannot be attributed to a loss of glutamatergic input.

RELEVANCE OF THIS PAPER TO PRACTICE

BACKGROUND

Glutamate excess in the hippocampus is known to trigger epileptic seizures. What is not clear is whether the excess is caused by changes in glutamate metabolism or abnormalities in glutamate transport. Elucidation of which of these pathogenetic mechanisms is involved is important for the development of rational therapies.

The study assessed the distribution of enzymes involved in glutamate metabolism and transport, protein concentrations, and enzyme activity. There was lower protein expression and activity of glutamate synthetase, which converts glutamate to glutamine, in specific areas of the hippocampus of individuals with temporal lobe epilepsy than in healthy individuals. Conversely, expression levels of the dominant glutamate transporter, EEAT2 (excitatory aminoacid transporter 2) were not altered.

IMPLICATIONS

These findings suggest that the main cause of glutamate build up is decreased enzymatic catabolism, rather than altered transport, although they did not establish why this decreased catabolism occurs. The findings support previous work in animals, and if further work establishes a direct pathogenetic role for the enzyme deficiency in temporal lobe epilepsy, then it might be possible to design a therapy based on direct manipulation of glutamate synthetase.

Several reports indicate that the expression of glutamine synthetase is regulated by cAMP analogues and by glucocorticoids.[38–40] The inductive effect of glucocorticoids is depressed by c-Jun,[40] which is upregulated after epileptic seizures.[41] Thus, interference with a glucocorticoid-dependent stimulatory pathway, possibly through c-Jun or cytokines,[42] might underlie the loss of glutamine synthetase in MTLE. c-Jun mediates the decrease of glutamine synthetase induced by basic fibroblast growth factor,[43] which is known to have a seizure-inducing effect in rats.[44]

This study therefore suggests that glutamate accumulation and epileptic seizures could be coupled to a highly localised enzyme defect. Animal studies are required to resolve the pathogenetic role of the loss of glutamine synthetase and the mechanisms behind it. If there is a causal relation, manipulations of glutamine synthetase activity might constitute a novel principle for curtailing seizures in patients with MTLE.

Contributors

T Eid took part in the immunohistochemical experiments and experimental design. M J Thomas and E Rundén-Pran participated in the western-blot analysis and statistical analysis. D D Spencer took part in selection of patients and surgery. J C K Lai and G V Malthankar did the assays of glutamine synthetase activity. J H Kim was responsible for experimental design and retrieval of autopsy tissue. N C Danbolt was responsible for production of the EAAT2 antiserum. O P Ottersen was involved in experimental design and statistical analysis. N C de Lanerolle conceived the studies and was involved in experimental design. The report was prepared by T Eid, M J Thomas, E Rundén-Pran, N C Danbolt, O P Ottersen, and N C de Lanerolle.

Conflict of interest statement

None declared.

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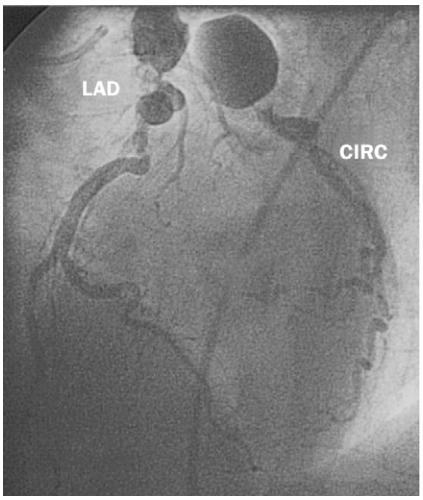
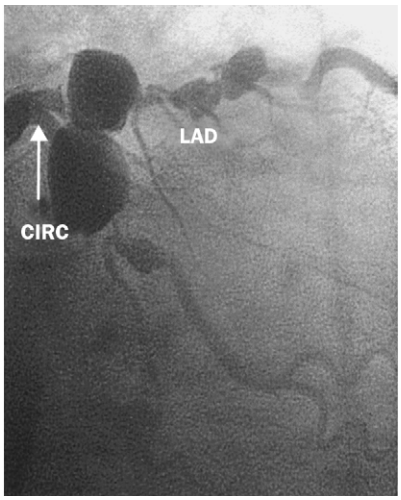
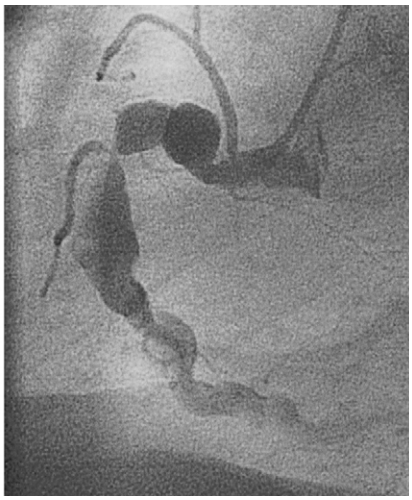
Clinical picture

Giant coronary aneurysms

D A Sarkar, E E J Smith, S J Brecker







A 74-year-old professional trumpet player had a 3-month history of exertional angina and presented with recent onset of pain at rest. An electrocardiogram on admission showed anterior T-wave inversion. Cardiac enzymes, including troponin T, were within normal limits. His risk factors included hypercholesterolaemia, hypertension, and previous cigarette smoking. Coronary angiography showed a large aneurysm of the proximal circumflex artery (CIRC), 16·3 mm by quantitative coronary angiography, with a tight post-aneurysmal stenosis. It also showed an 11·7 mm proximal aneurysm of the left anterior descending artery (LAD), with two smaller areas of aneurysm dilatation (figure, left: left anterior oblique [LAO] projection and figure, centre: right anterior oblique caudal projection). The right coronary artery was generally ectatic with pronounced dilatation of the proximal segment (figure, right: LAO projection). The aneurysms were calcified. Surgical revascularisation was successful. The patient was unable to recall any childhood illness consistent with Kawasaki disease though this remains a possible cause of his multiple coronary aneurysms.

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