Up-regulation of hippocampal metabotropic glutamate receptor 5 in temporal lobe epilepsy patients

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Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors involved in the regulation of glutamatergic transmission. Recent studies indicate that excitatory group I mGluRs (mGluR1 and mGluR5) contribute to neurotoxicity and hyperexcitability during epileptogenesis. In this study, we examined the distribution of mGluR1α and mGluR5 immunoreactivity (IR) in hippocampal resection tissue from pharmaco-resistant temporal lobe epilepsy (TLE) patients. IR was detected with panels of receptor subtype specific antisera in hippocampi from TLE patients without (non-HS group) and with hippocampal sclerosis (HS group) and was compared with that of non-epileptic autopsy controls (control group). By immunohistochemistry and immunoblot analysis, we found a marked increase of mGluR5 IR in hippocampi from the non-HS group. High mGluR5 IR was most prominent in the cell bodies and apical dendrites of hippocampal principal neurons and in the dentate gyrus molecular layer. In the HS group, this increase in neuronal mGluR5 IR was even more pronounced, but owing to neuronal loss the number of mGluR5-immunoreactive neurons was reduced compared with the non-HS group. IR for mGluR1α was found in the cell bodies of principal neurons in all hippocampal subfields and in stratum oriens and hilar interneurons. No difference in mGluR1α IR was observed between neurons in both TLE groups and the control group. However, owing to neuronal loss, the number of mGluR1α-positive neurons was markedly reduced in the HS group. The up-regulation of mGluR5 in surviving neurons is probably a consequence rather than a cause of the epileptic seizures and may contribute to the hyperexcitability of the hippocampus in pharmaco-resistant TLE patients. Thus, our data point to a prominent role of mGluR5 in human TLE and indicate mGluR5 signalling as potential target for new anti-epileptic drugs.

Keywords: hippocampus; human; immunocytochemistry; metabotropic glutamate receptors; temporal lobe epilepsy

Abbreviations: HS = hippocampal sclerosis; IR = immunoreactivity; mGluR = metabotropic glutamate receptor; TLE = temporal lobe epilepsy


Introduction

Mesial temporal lobe epilepsy (TLE) is the most common form of epilepsy in adult humans and usually associated with hippocampal sclerosis (HS), a characteristic pattern of hippocampal cell loss (Margerison and Corsellis, 1966; Houser, 1999), but can also be lesional or cryptogenic (Engel, 1996). Approximately 30% of the patients suffering from TLE are pharmaco-resistant, which means that seizures cannot be controlled by tolerable doses of anti-epileptic drugs. Surgical resection of the epileptogenic tissue, in particular the hippocampus, most often results in seizure control,
implicating the hippocampus in the generation and/or propagation of seizures in these patients (Mathern et al., 1995b; Zentner et al., 1995).

The molecular mechanisms underlying human TLE are still largely unknown (reviewed, Dalby and Mody, 2001). Excessive synaptic excitation mediated by glutamate interacting with ionotropic and metabotropic glutamate receptors (mGlurS) has been recognized as an important process in the pathophysiology underlying TLE. In hippocampal resection tissue from TLE patients, alterations in AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate), kainate and NMDA (N-methyl-D-aspartate) receptor subunit number or distribution have been reported (reviewed, Meldrum et al., 1999; see also Mathern et al., 1998, 1999). Only limited information is available on the expression of mGlurS in human TLE (Blümcke et al., 2000; Lie et al., 2000; Tang and Lee, 2001; Tang et al., 2001).

The mGlurS are G protein-coupled receptors, which modulate neuronal excitability and synaptic transmission by regulating the activity of various membrane ion channels and intracellular second-messenger systems (for a review see Cartmell and Schoepf, 2000). Eight mammalian mGlurS subtypes (mGlur1–mGlur8) have been cloned and assigned to three groups on the basis of their sequence homology, second messenger coupling and pharmacology. Group I consists of mGlur1 and mGlur5, which couple primarily to the activation of phospholipase C/phosphoinositide hydrolysis in heterologous expression systems. Group II and III include all other subtypes and couple negatively to adenylyl cyclase/cyclic AMP formation but differ in their agonist selectivity. In general, group I mGlurS are considered to be excitatory, whereas group II and III receptors have predominantly inhibitory properties (Cartmell and Schoepf, 2000).

In vitro and in vivo studies have shown a pivotal role of hippocampal group I mGlurS in epileptogenesis (reviewed, Bordi and Ugolini, 1999; see also Merlin, 1999, 2002). Further diversity in group I mGlurS is generated by alternative splicing at the C-terminal cytoplasmic domain. Three splice variants have been described for the human mGlur1 receptor (α or a, β or b, and d) (Desai et al., 1995; Laurie et al., 1996; Stephan et al., 1996; Lin et al., 1997) and three for human mGlur5 (a, b and d) (Minakami et al., 1994; Daggett et al., 1995; Malherbe et al., 2002).

In situ hybridization studies have indicated a widespread, but discrete, distribution of group I mGlur subtypes in the human brain (Daggett et al., 1995; Lin et al., 1997; Malherbe et al., 2002). In the human hippocampus, mGlur1 mRNAs are expressed with the highest density in neurons of the CA3 region and dentate gyrus, whereas mGlur5 mRNAs are most predominant in the CA1 region. A similar distribution pattern has been reported for both receptor subtypes by immunohistochemistry (Blümcke et al., 1996).

To date only two studies have been published on the expression of group I mGlurS in human TLE. The first one studied mGlur1α and mGlur5 in the hippocampus of non-HS and HS TLE patients (Blümcke et al., 2000). These authors report up-regulation of mGlur1α immunoreactivity (IR) and similar or slightly reduced mGlur5 expression in the dentate gyrus molecular layer of TLE patients as compared with a limited number of peritumoral control specimens. Yet the second immunocytochemical study, using only biopsies of HS patients, describes high mGlur1α and mGlur5 IR in the dentate gyrus molecular layer and of mGlur5 in hippocampal pyramidal neurons (Tang et al., 2001).

In view of the importance of group I mGlurS as potential targets for pharmacological treatment of medically intractable chronic epilepsies (see Spooren et al., 2001; Gasparini et al., 2002) a detailed description of their expression in human TLE is essential. We, therefore, analysed mGlur1α and mGlur5 IR in the hippocampus of pharmaco-resistant TLE patients with and without HS and compared it with non-epileptic autopsy controls.

Materials and methods

Patient evaluation and tissue collection

Hippocampal tissue of pharmaco-resistant TLE patients with complex partial seizures was obtained at surgery. Patients were selected for epilepsy surgery according to the criteria of the Dutch Epilepsy Surgery Program (Debets et al., 1991). Surgical removal of the hippocampus was necessary in all patients to achieve seizure control and was performed under general or local anaesthesia. The excision was based on clinical evaluations, interictal and ictal EEG studies (video EEG monitoring), MRI, and intraoperative electrocorticography. Informed and written consent was obtained from the patients for all procedures. After en bloc resection, the hippocampus was cut into three slices perpendicular to its long (rostrocaudal) axis and the middle portion was used for analysis. Tissue samples for immunohistochemistry were immediately immersed-fixed in 4% formaldehyde (pH 7.4) overnight at room temperature and embedded in paraffin. Samples for immunoblotting were freshly frozen on powdered dry ice and stored at −80°C until further use. Hippocampal control tissue was obtained at autopsy and processed as described for surgical resection samples.

To determine post-mortem stability of mGlur1α and mGlur5 epitopes human neocortical tissue was subjected to autopsy-like conditions. In brief, neocortical tissue resected from HS TLE patients during selective amygdalohippocampectomy was cut into 10 mm thick slices and was immersion-fixed in 4% formaldehyde and the middle portion was used for analysis. Tissue samples for immunohistochemistry were immediately immersed-fixed in 4% formaldehyde (pH 7.4) overnight at room temperature and embedded in paraffin. Samples for immunoblotting were freshly frozen on powdered dry ice and stored at −80°C until further use. Hippocampal control tissue was obtained at autopsy and processed as described for surgical resection samples.

To determine post-mortem stability of mGlur1α and mGlur5 epitopes human neocortical tissue was subjected to autopsy-like conditions. In brief, neocortical tissue resected from HS TLE patients during selective amygdalohippocampectomy was cut into 10 mm thick slices and was immersion-fixed in 4% formaldehyde immediately or after 12 or 24 h storage at ambient temperature in PIPES (1,4-piperazinediethanesulfonic acid) buffer (in mM: 120 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 20 Pipes, 25 D-glucose; pH 7.0) until further processing as described for hippocampal tissue. mGlur5 IR was found to be stable for at least 24 h post-mortem and mGlur1α IR for at least 12 h. At 24 h post-mortem overall mGlur1α IR and the number of mGlur1α-immunoreactive neurons was slightly decreased. Therefore, in this study we have used autopsy controls with post-mortem delays <17 h. In this autopsy control group no difference could be detected in hippocampal mGlur1α or mGlur5 between patients with various post-mortem delays (ranging from 5 to 17 h).

For neuropathological evaluation representative paraffin sections (7 μm) were stained with cresyl violet (Nissl stain). HS was diagnosed and classified according to Wyler (Wyler et al., 1992). Only tissue samples from non-HS (Wyler grade 0; n = 12) and severe
HS (Wyler grade 4; n = 12) cases were used. In the non-HS group, pathological diagnoses included oligodendroglia (n = 1), astrocytoma and related low-grade astrocytic tumours (n = 2) or glioneuronal neoplasms (n = 4), cavernous haemangioma (n = 1), neocortical gliosis (n = 2) or dysplasia (n = 1). None of these focal lesions extended into the hippocampus proper. In one patient, neither hippocampal nor extrahippocampal pathology could be demonstrated by MRI and histopathological examination. The HS group included patients without focal lesions, who generally had a clinical history of an initial precipitating injury and in whom the hippocampus was characterized by extensive neuronal loss and gliosis in all CA segments (Fig. 2C and F; compare with Fig. 2A and B, and 2D and E, respectively; see also Proper et al., 2000). Initial precipitating injuries (Mathern et al., 1995a) included febrile convulsions (n = 4), infantile meningitis with febrile seizures (n = 2) or significant head trauma (n = 1). Three patients had no known initial insult and for two patients these data had not been recorded. One HS patient had dual pathology with a low-grade ganglioglioma in the neocortex. At the time of surgery, all TLE patients received anti-epileptic medication either as monotherapy or polytherapy. Two non-HS and one HS TLE patient were treated with carbamazepine monotherapy. Two non-HS patients were on monotherapy with oxcarbazepine and one patient with clobazam. Seven non-HS and eleven HS TLE patients received various combinations of the following anti-epileptic drugs: carbamazepine, clobazam, clonazepam, lamotrigine, oxcarbazepine, phenytoin, topiramate and valproic acid. In the autopsy control group (n = 12), cardiovascular failure (n = 3), cerebral (n = 2) or cerebellar (n = 1) haemorrhage, cerebral metastases (n = 1), cerebral trauma (n = 1), herniation due to acute vascular accident (n = 1), ileus (n = 1) or septic shock (n = 2) was recorded as cause of death. None of the autopsy controls had a history of neurological or psychiatric disorders and all hippocampal specimens were normal as confirmed by neuropathological examination.

Relevant clinical data for autopsy controls and TLE patients included in this study are summarized in Table 1. Multiple group comparison or \( \chi^2 \) tests between the autopsy control, non-HS and HS group revealed only one significant difference in all the clinical parameters. The mean age at tissue collection in the control group was higher than in the non-HS group (\( P = 0.02, \) post hoc Bonferroni test) but did not differ between the control and HS group (\( post \ hoc \ P = 0.14 \)) or the non-HS and HS group (\( post \ hoc \ P = 1.00 \)).

### Antibodies

Panels of affinity purified rabbit antisera against mGluR1\( \alpha \) or mGluR5 were employed. The mGluR1\( \alpha \) antiserum (#AB1595; Chemicon, Temecula, CA) used in this study is directed against amino acids 1116–1130 of the rat mGluR1\( \alpha \) splice variant (Houamed et al., 1991; Masu et al., 1991). This amino acid sequence differs only at one residue from the human mGluR1\( \alpha \) 1108–1124 (Desai et al., 1995; Stephan et al., 1996) and exhibits no similarity to other known mGluRs based on alignment of reported sequences. The mGluR5 antiserum (#06–451; Upstate, Lake Placid, NY) was raised against a synthetic peptide containing the C-terminal 20 amino acids of the receptor. This amino acid sequence is common to rat mGluR5a (Abe et al., 1992) and mGluR5b (Minakami et al., 1993), and to the human a, b and d splice variants (Minakami et al., 1994; Daggett et al., 1995; Malherbe et al., 2002). Additional group I mGluR antisera (listed in Table 2) included three mGluR1\( \alpha \) and two mGluR5 affinity purified rabbit anti-peptide antibodies. Specificity characteristics of these antisera have been described elsewhere (Hampson et al., 1994; Ferraguti et al., 1998; Alvarez et al., 2000). Glial fibrillary acidic

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**Table 1** Comparison of clinical data in the three patient categories

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Autopsy</th>
<th>Non-HS</th>
<th>HS</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45.6 ± 5.5 (21–74)</td>
<td>27.0 ± 3.1 (13–41)</td>
<td>32.3 ± 4.6 (11–55)</td>
<td>0.02</td>
</tr>
<tr>
<td>Epilepsy onset (years)</td>
<td>NA</td>
<td>15.1 ± 3.3</td>
<td>10.9 ± 3.2</td>
<td>0.37</td>
</tr>
<tr>
<td>Epilepsy duration (years)</td>
<td>NA</td>
<td>11.9 ± 2.4</td>
<td>21.5 ± 4.5</td>
<td>0.08</td>
</tr>
<tr>
<td>Seizure frequency (no./month)</td>
<td>NA</td>
<td>17.1 ± 5.5</td>
<td>12.8 ± 5.1</td>
<td>0.57</td>
</tr>
<tr>
<td>Male/female</td>
<td>6/6</td>
<td>4/8</td>
<td>4/8</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM values (n = 12). P-values were computed using one-way ANOVA (age), independent samples t-test (epilepsy onset, epilepsy duration and seizure frequency), or Fisher’s exact test (male/female). P-values < 0.05 were considered significant. Epilepsy duration was calculated as the time between epilepsy onset (onset of habitual seizures) and surgery. Seizure frequency refers to the number of complex partial seizures in the month before surgery. HS = hippocampal sclerosis; NA = not applicable.

**Table 2** mGluR antibodies used

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Source</th>
<th>Immunizing peptide</th>
<th>Reactivity</th>
<th>References</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>#AB1595</td>
<td>Chemicon, Temecula, CA</td>
<td>EFYVYERGNTTEDEEL</td>
<td>Rat mGluR1( \alpha )</td>
<td>1 : 25</td>
<td></td>
</tr>
<tr>
<td>#m1a</td>
<td>This laboratory (Hampson)</td>
<td>EFYVYERGNTTEDEEL</td>
<td>Rat mGluR1( \alpha )</td>
<td>1 : 100</td>
<td></td>
</tr>
<tr>
<td>#24426</td>
<td>Diasorin, Stillwater, MN</td>
<td>EFYVYERGNTTEDEEL</td>
<td>Rat mGluR1( \alpha )</td>
<td>1 : 50</td>
<td></td>
</tr>
<tr>
<td>#AB1551</td>
<td>Chemicon</td>
<td>PNYTTASYLIRLYQSSSTL</td>
<td>Rat mGluR1( \alpha ), 5( ^{\alpha} )</td>
<td>Alvarez et al. (2000); Ferraguti et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>#06–451</td>
<td>Upstate, Lake Placid, NY</td>
<td>SSPKYDQLRLDRFNNSSL</td>
<td>Rat mGluR5( ^{\alpha} )</td>
<td>Alvarez et al. (2000)</td>
<td></td>
</tr>
<tr>
<td>#M3884–79</td>
<td>US Biological, Swampscott, MA</td>
<td>SSPKYDQLRLDRFNNSSL</td>
<td>Rat mGluR5( ^{\alpha} )</td>
<td>1 : 200</td>
<td></td>
</tr>
<tr>
<td>#AB5232</td>
<td>Chemicon</td>
<td>LIRIDYQSSSSL</td>
<td>Rat mGluR5( ^{\alpha} )</td>
<td>1 : 100</td>
<td></td>
</tr>
</tbody>
</table>

References provide specificity characteristics of the antisera. Dilution applies to immunohistochemistry on human tissue; NA = not applicable; *Cross reacts with human; †No reliable reactivity under the conditions tested.
protein (GFAP) was detected with a mouse monoclonal antibody (clone G-A-5; Boehringer, Mannheim, Germany; diluted 1 : 50).

**Immunoblotting**

Homogenates from human hippocampus were prepared as reported earlier (van der Hel et al., 2005). The subiculum was removed from all hippocampal specimens before homogenization. Synaptosomal plasma membranes were isolated from hippocampal homogenates as described by Kristjansson et al. (1982). Rat brain microsomal proteins were from Upstate. Proteins were heated for 30 min at 37°C, separated on 7% SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) gels, and transferred to nitrocellulose membranes by semi-dry blotting. Equal protein loading was checked by Ponceau-S staining. Blots were stained with antibodies according to the manufacturer’s instructions (Upstate). IR was detected with peroxidase-conjugated goat anti-rabbit immunoglobulin (diluted 1 : 15,000–30,000; Sigma Chemical Co., St Louis, MO). The blots were developed with enhanced chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate; Pierce, Rockford, IL) and exposed to high performance chemiluminescence film (Hyperfilm ECL; Amersham, Buckinghamshire, UK). Prestained Precision Protein standards were from Bio-Rad (Hercules, CA). In negative controls, the primary antiserum was omitted.

On quantitative immunoblots, chemiluminescent signals were captured with a Fluor-S multispectral imaging (Bio-Rad) and analysed using Bio-Rad’s Quantity One software. Band intensities were measured in duplicate for each homogenate under conditions of signal linearity. Relative optical densities corrected for background were averaged per patient and the mean was used for statistical analysis.

**Immunohistochemistry**

Immunostaining was performed on dewaxed, 7 μm thick serial sections by the indirect unlabelled antibody peroxidase–antiperoxidase method (Sternberger et al., 1970). Per primary antiserum, tissue sections from all patients were stained simultaneously to minimize variation in the immunohistochemical reactions. Endogenous peroxidase activity was blocked with 3% H2O2 in phosphate-buffered saline (PBS). Tissue sections to be stained for mGluR1α or with the mGluR5 US Biological (Swampscott, MA; #M3884–79) antiserum were immersed in 10 mM sodium citrate (pH 6.0) for 20 min and microwaved in the same solution for 7 min at 750 W to expose immunoreactive sites (Shi et al., 1991). Non-specific binding of immunoglobulins was reduced with 0.5% non-fat dry milk powder in 10 mM Tris–HCl, 5 mM EDTA (ethylenediaminetetraacetic acid), 150 mM NaCl, 0.25% gelatin and 0.05% Tween-20 (pH 8.0). Primary antiserum (diluted in PBS) were applied overnight at room temperature. Optimal working concentrations (Table 2) were determined by serial dilutions on rat brain and human autopsy tissue. Goat anti-rabbit immunoglobulin G (H + L) serum adsorbed with human, mouse and rat serum proteins (Jackson Immunoresearch, West Grove, PA) or rabbit anti-mouse immunoglobulin G (H + L) serum (diluted 1 : 250) and the appropriate peroxidase–antiperoxidase immunocomplexes (diluted 1 : 750) (Nordic, Tilburg, The Netherlands) were applied in PBS for 1.5 h each. For pre-adsorption controls, the primary antiserum was incubated overnight at 4°C with 50 (mGluR1α) or 100 (mGluR5) μg/ml of blocking peptide and then applied to the sections. Antiserum samples without blocking peptide were run in parallel. After each incubation, the sections were thoroughly rinsed in PBS. IR was visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in 25 mM imidazole, 1 mM EDTA (pH 7.0) containing 0.01% H2O2. Colour was developed for 15 min. Controls without the primary antiserum did not reveal staining (data not shown).

**Specificity of the mGluR antisera**

Adult male Wistar (U:WU; GDL, Utrecht, The Netherlands; 250–300 g; n = 3) or Sprague–Dawley (Hsd:SD; Harlan Winkelmann GmbH, Börchen, Germany; 250–300 g; n = 3) rats were used in testing the specificity of the mGluR antiserum. Rat experiments conformed to institutional guidelines. Rats were either killed by decapitation or by transcardial perfusion (5–10 min) with 4% formaldehyde under deep pentobarbital (200 mg/kg body weight, i.p.) anaesthesia. Their brains were rapidly removed from the skull and then processed and immunostained as described for human tissue. No differences in the staining pattern of the different mGluR antibodies were observed between the two rat strains or fixation methods.

The specificity of the mGluR antisera was evaluated in several ways. First, on immunoblots loaded with rat brain microsomal proteins, the mGluR1α and mGluR5 antisera labelled a single protein band with an estimated molecular weight of 150 kDa and 145 kDa, respectively (Fig. 1A in the Supplementary material), consistent with previous reports (cf. Baude et al., 1993; Shigemoto et al., 1993; Hampson et al., 1994; Romano et al., 1995). Immunoreactive bands on immunoblots of synaptosomal plasma membrane (Fig. 1A) or homogenate (e.g. Fig. 6A) proteins from human hippocampus showed similar molecular weights as in rat. Secondly, in immunohistochemistry on whole rat brain sections, both antisera produced distinct and highly reproducible IR patterns in various brain regions (Supplementary Fig. IB, C, D, H, J and N). The specificity of the mGluR5 antiserum was further confirmed in regions known to express high levels of mGluR1α IR (cerebellar cortex, thalamus) but virtually no or only low mGluR5 (cf. Martin et al., 1992; Baude et al., 1993; Shigemoto et al., 1993; Hampson et al., 1994; Romano et al., 1995). In addition, the labelling pattern observed with the mGluR1α and mGluR5 antiserum (in rat hippocampus and cerebellum) was identical to the immunostaining obtained, respectively, with the mGluR1α #m1a (Supplementary Fig. IE and K) or Diasonin (Stillwater, MN; #24426) (Supplementary Fig. IF and L) and the mGluR5 US Biological antisera (data not shown). These antisera have been characterized in detail previously (Hampson et al., 1994; Ferraguti et al., 1998; Alvarez et al., 2000). Finally, in immunohistochemistry on human and rat brain tissues, pre-adsorption of the antisera with appropriate peptide sequences from rat mGluR1α (amino acids 1116–1130; Diasonin) or mGluR5 (amino acids 1159–1171; US Biological) resulted in a loss of signal (e.g. Fig. 1B; see also Supplementary Fig. IG, I, M and O).

The other mGluR antisera tested on rat and human hippocampal sections produced no reliable (Chemicon’s mGluR5, #AB5322) or an overlapping IR pattern (Chemicon’s mGluR1α, #AB1551) with the mGluR5 antiserum used in this study (data not shown). This latter antiserum (#AB1551; directed against the C-terminal 20 amino acids) should, therefore, probably be considered a specific antiserum (cf. Ferraguti et al., 1998; Alvarez et al., 2000) and, therefore, was not further used in this study.

**Statistical analysis**

Densitometric measurements were analysed for significant group differences using one-way ANOVA, combined with post hoc Bonferroni test as a multiple comparison method. P < 0.05 was considered significant.
IR was obtained with the mGluR1α #m1a and Diasorin antisera (data not shown).

Non-HS and HS TLE hippocampi showed a similar mGluR1α IR pattern as the controls (Fig. 2H and I; compare with Fig. 2G). However, owing to severe neuronal loss as revealed by Nissl staining, fewer mGluR1α-immunopositive neurons were present in the HS hippocampus (Fig. 2C and I; compare with Fig. 2A and B, and 2G and H, respectively). No consistent difference in the staining intensity for mGluR1α IR was found between neurons in the control and non-HS or HS group (Figs 3A–C, 4A–C and 5A–C). In both patient groups, glial cells were negative for mGluR1α IR.

**mGluR5 immunoreactivity**

The mGluR5 antiserum yielded an immunoreactive staining pattern in control tissue similar to that described previously for the human hippocampus (Blümcke et al., 1996). A dense and homogeneously distributed neuropil staining decorated the pyramidal cell layer and stratum lacunosum-moleculare of CA1–3 (Fig. 2J). Staining of these layers was strongest in CA1 and less intense in CA3. IR for mGluR5 in CA4 was extremely weak (Figs 2J and 4D). In the dentate gyrus (Figs 2I and 4D), mGluR5 IR consistently labelled the outer molecular layer. In contrast, IR in the inner molecular layer was variable. The polymorphic layer of the dentate hilus exhibited virtually no detectable staining. Neuronal cell bodies in the control hippocampus were almost devoid of IR, except in CA1 where pyramidal neurons showed a dense, cytoplasmic staining of the perikarya (Fig. 3D; compare with e.g. Fig. 4D). The cytoplasm within dendritic shafts of CA1 pyramidal cells exhibited no or only weak IR. In the control tissue, no staining for mGluR5 was seen in individual glial cells.

The most striking finding in both TLE patient groups was an increased intracellular staining of principal neurons. In the HS group, the perikarya and apical dendrites of virtually all surviving pyramidal neurons displayed this strong, cytoplasmic staining (Figs 3F and 4F). In the dentate gyrus, granule cell bodies and basal dendrites often were also intensely stained (Fig. 5F). This staining pattern was also apparent in the non-HS group, but fewer strongly mGluR5-immunoreactive neurons were found and in these neurons strong staining was more confined to pyramidal shafts (Figs 1B and 3E) or dendritic processes (Fig. 4E).

In the non-HS group, mGluR5 IR of the granule cells (cell bodies and basal dendrites) varied between patients. In some patients granule cells were almost devoid of staining, whereas in others about one-third of the cell population was moderately stained (Fig. 5E).

A further interesting finding was the strong neuropil staining in the non-HS and HS group compared with control tissue. However, in the HS group in regions with marked sclerosis, such as CA1 and CA4, neuropil IR was less dense (Fig. 2L; compare with Fig. 2C, asterisks). In both the non-HS and HS group, prominent mGluR5 IR was apparent in the...
The staining for mGluR5 IR in this sublayer was consistently stronger in the HS group (Fig. 5F) than in the non-HS group (Fig. 5E). Except for some strongly immunoreactive dendritic profiles in the supragranular zone, IR for mGluR5 in the inner molecular layer of the HS hippocampus did not differ much from that in the control hippocampus (Fig. 5F; compare with Fig. 5D). In contrast, in the non-HS hippocampus the inner molecular layer showed a stronger neuropil staining compared with control and sclerotic tissue (Fig. 5E; compare with Fig. 5D and F). Moreover, in the non-HS group, a discrete, punctate staining decorated the granule cell apical dendrites in the supragranular zone.

In the HS group, mGluR5 IR was also observed in a subset of strongly GFAP-immunoreactive glial cells (data not shown); most notably in those hippocampal segments with substantial neuronal loss and gliosis. Although the nature of these cells was not further investigated, they resembled...
reactive astrocytes by their morphological appearance. In the non-HS hippocampus, mGluR5-immunoreactive glial cells were only observed in regions of poor neuropil staining such as the alveus or close to the hippocampal fissure.

All mGluR5 IR was specific, because immunostaining (i.e. including that in non-HS or HS neuronal cell bodies and apical dendrites) could be abolished by pre-adsorption with the mGluR5 blocking peptide (Fig. 1B). Moreover,
similar IR patterns were obtained with the US Biological antibody (data not shown).

Immunoblot analysis of mGluR5 in control, non-HS and HS hippocampi revealed a single immunoreactive band of ~145 kDa (Fig. 6A) in all samples. Consistent with our immunohistochemical data, mGluR5 IR was significantly increased in hippocampal homogenates from the non-HS compared with the control and HS group (~15-fold and 7.5-fold, respectively) (Fig. 6B; \( P < 0.001 \)), but mGluR5 IR did not differ significantly between the control and the HS group (Fig. 6B; post hoc \( P = 1.00 \)).

**Discussion**

The main conclusion that emerges from this study is that mGluR5 IR is significantly up-regulated in the epileptogenic hippocampus from TLE patients as compared with non-epileptic controls. The most drastic increase in mGluR5 IR was observed in hippocampal principal neurons (i.e. pyramidal and granule cells) and in the dentate gyrus molecular layer. This increase in mGluR5 IR was found in the hippocampus of both TLE patient groups. No differences in mGluR1α IR were observed between hippocampi from TLE patients with or without HS, and non-epileptic controls.

IR for mGluR1α in the hippocampus from autopsy controls and in the rat was localized to the somata and proximal dendrites of interneurons, particularly in the stratum oriens and the hilus, and was also detectable in principal neurons. This distribution pattern was observed with all three mGluR1α antibodies (against a unique proline/glutamine-rich region of the mGluR1α molecule; Houamed et al., 1991; Masu et al., 1991) and could be completely abolished by immuno-adsorption with a corresponding synthetic mGluR1α peptide. The pattern correlates well with that found by in situ hybridization for mRNAs coding for all mGluR1 (Masu et al., 1991; Shigemoto et al., 1992; Lin et al., 1997) and by immunohistochemistry for the human \( \beta \) splice variant (Blümcke et al., 1996). In situ hybridization studies aimed to identify the expression of individual mGluR1 splice variants, however, have failed to detect mGluR1α transcripts in hippocampal principal neurons (Berthele et al., 1998). In view of the relatively low abundance of mGluR1α protein in principal neurons it is not surprising that others had difficulties in detecting the mGluR1α transcript. Previous immunohistochemical studies on rat brain also do not consistently report mGluR1α IR in hippocampal principal neurons (Martin et al., 1992; Baude et al., 1993; Hampson et al., 1994; Ferraguti et al., 1998, 2004; Alvarez et al., 2004).
morphology were visible in the neuronal mGluR1α. As expected, sclerosis-related alterations in hippocampal HS and the HS group and autopsy controls were similar. In CA3 to light IR in CA1. The presence of mGluR1α staining in all principal neurons varying from strong IR then in CA3, and finally in interneurons. Thus, we found antibodies staining was first lost in principal neurons in CA1, then in CA3, and finally in interneurons. Thus, we found staining in all principal neurons varying from strong IR in CA3 to light IR in CA1. The presence of mGluR1α in hippocampal principal neurons is consistent with electrophysiological data showing a role for mGluR1 in regulating pyramidal cell function (Mannaioni et al., 1991). In fact, using serial dilutions of the mGluR1α antibodies staining was first lost in principal neurons in CA1, then in CA3, and finally in interneurons. Thus, we found staining in all principal neurons varying from strong IR in CA3 to light IR in CA1. The presence of mGluR1α in hippocampal principal neurons is consistent with electrophysiological data showing a role for mGluR1 in regulating pyramidal cell function (Mannaioni et al., 2001).

The mGluR1α IR patterns in the hippocampus of the non-HS and the HS group and autopsy controls were similar. As expected, sclerosis-related alterations in hippocampal morphology were visible in the neuronal mGluR1α-staining pattern in the HS group. HS is characterized by severe neuronal cell loss and dispersion of the granule cell layer (Margerison and Corsellis, 1966; Houser, 1990, 1999; Proper et al., 2000). Associated with these pathological alterations, we detected a decrease in the number of mGluR1α-immunoreactive neurons (most pronounced in CA1 and CA4) and a broader band of mGluR1α-immunoreactive granule cells in the HS hippocampus. We did not observe an increased mGluR1α IR in the dentate gyrus molecular layer in TLE patients, as reported by Tang et al. (2001) and Blümcke et al. (2000), but found such an increase for mGluR5 IR. This apparent discrepancy may not be so surprising, because in control experiments on rat brain tissue (see Material and methods) we found that the mGluR1α antiserum used in the study of Blümcke shows cross-reactivity with mGluR5. Other studies using this antibody, including studies in mice lacking mGluR1, confirm cross-reactivity with mGluR5 (Ferraguti et al., 1998; Alvarez et al., 2000). It is, therefore, likely that the increased labelling in the dentate gyrus molecular layer in the aforementioned human studies represents mGluR5 rather than mGluR1α IR. The mGluR1α antiserum that we used in this study was raised against unique epitopes of the rat mGluR1α splice variant and showed a consistent neuronal somatodendritic distribution in rat and human hippocampus.

In the control group, mGluR5 IR was most pronounced in the dendritic compartments (cf. Blümcke et al., 1996), but in TLE patients mGluR5 IR was also detected in hippocampal principal neurons and glial cells. The most drastic increase in mGluR5 IR in TLE patients (compared with autopsy controls) was observed in the cell bodies and main dendritic processes of pyramidal neurons and granule cells. IR for mGluR5 in neuronal cell bodies has also been described in other CNS regions (Romano et al., 1995; Alvarez et al., 2000). Thus, whereas neuronal mGluR5 IR was weak in autopsy controls, it was markedly increased in the non-HS group and very prominent in the HS group. Immunoblot experiments confirmed the increase in mGluR5 IR in the non-HS hippocampus, but the increase in the HS hippocampus did not reach significance. Conceivably, the severe neuron loss in the HS hippocampus masks the large increase of mGluR5 in individual neurons.

The increase in mGluR5 IR in hippocampal neurons in TLE patients is not due to a reduced IR in the autopsy control group (e.g. as a result of post-mortem protein degradation and/or modification) as control experiments with freshly resected neocortical biopsies kept under post-mortem conditions showed mGluR5 IR to be stable for at least 24 h. Moreover, the distribution of mGluR5 IR in our autopsy tissue closely resembled the pattern described for surgically removed (i.e. freshly excised) peritumoral hippocampal tissue from non-epileptic patients (Blümcke et al., 1996). Of course, the observed increase in neuronal mGluR5 IR in the HS compared with the non-HS group cannot be attributed to post-mortem effects. Thus, our results show that mGluR5 IR is up-regulated in hippocampal neurons of patients with pharmaco-resistant TLE.
A further interesting finding was the increase in mGluR5 IR in the outer molecular layer of the dentate gyrus in the HS and the non-HS group. In the dentate inner molecular layer mGluR5 IR is decreased in the HS compared with the non-HS group. Because patients in both groups have a long history of seizures, this decrease in mGluR5 IR is likely to be pathology-related rather than seizure-related. In HS, extensive synaptic reorganization occurs in the inner molecular layer concomitant with mossy fibre sprouting (reviewed, Houser, 1999; see also Proper et al., 2000). Possibly decreased expression of mGluR5 in this hippocampal sublayer is part of a compensatory mechanism in response to increased, recurrent excitation caused by mossy fibre sprouting. The increase in mGluR5 IR in hippocampal neurons and the dentate molecular layer is in agreement with a study by Tang et al. (2001). These authors studied mGluR5 localization in TLE patients by light and electron microscopy and found high mGluR5 IR in both post-synaptic and pre-synaptic elements in the molecular layer of the dentate gyrus and hippocampal CA1 region, but this study did not include a non-HS nor an autopsy control group. Blümcke et al. (2000) have described a slight decrease in mGluR5 IR in the dentate gyrus molecular layer of TLE patients with HS, as compared with peritumoral hippocampal specimens from non-epileptic controls, but did not illustrate this.

In the HS group, mGluR5 IR was also found in glia-like cells. Since in these patients massive gliosis has been described (Margerison and Corsellis, 1966; Wyler et al., 1992; Proper et al., 2000), these cells may represent reactive astrocytes. Up-regulation of hippocampal mGluR5 expression in reactive astrocytes has been documented in kainic acid-lesioned rats (Ulase et al., 2000) and mice (Ferraguti et al., 2001), and in rats after electrically-induced status epilepticus (Aronica et al., 2000). Expression of mGluR5 in glia is consistently found in glial human brain tissue from epileptic (Aronica et al., 2001; Tang et al., 2001) as well as non-epileptic (Blümcke et al., 1996; Condorelli et al., 1997; Geurts et al., 2003) patients. Whether the mGluR5-immunoreactive cells in the HS hippocampus are reactive astrocytes needs to be investigated.

It is important to realize that studies on human epileptic tissue suffer from a number of limitations. For instance, only a selected group of TLE patients is treated for their epilepsy by surgical removal of the epileptogenic tissue. These pharmaco-resistant TLE patients suffer from a highly progressed stage of epilepsy and have had seizures for many years. Thus, it is not easy to determine whether the up-regulation of mGluR5 IR in hippocampal neurons is an underlying cause or merely a consequence of the repeated seizures (for a discussion see Meldrum, 1997). Conceivably, the up-regulation of mGluR5 receptors is a consequence rather than a cause of the epileptic seizures. It is unlikely that the increase in mGluR5 expression is drug-induced, because anti-epileptic drug treatment did not differ between TLE groups. Also the broad spectrum of anti-epileptic drugs prescribed makes a general effect on mGluR5 expression unlikely. This up-regulation of mGluR5 in surviving neurons may contribute to the hyperexcitability of the hippocampus. Alternatively, it may be part of a mechanism to protect against overexcitation and neurotoxicity (Nicoletti et al., 1999). Although activation of mGluR5 receptors normally seems to enhance glutamatergic transmission, their function may switch to inhibiting glutamatergic transmission when mGluR5 receptors become desensitized upon exposure to glutamate (Herrero et al., 1998; Rodriguez-Moreno et al., 1998; reviewed, De Blasi et al., 2001). Further physiological and pharmacological studies must clarify whether high mGluR5 expression in TLE corresponds to increased functional receptor activity. If high mGluR5 expression indeed contributes to hyperexcitability of the hippocampal network, mGluR5 signalling might be a potential target for new anti-epileptic drugs.

Supplementary material
Supplementary data are available at Brain Online.

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