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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 compared with the control 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

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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.*, 1995*b*; 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 Schoepp, 2000). Eight mammalian mGluR 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 Schoepp, 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 immersion-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-piperazinediethanesulphonic acid) buffer (in mM: 120 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 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 oligodendroglioma ($n = 1$), 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.*, 1995*a*) 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 χ^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* $P = 1.00$).

Table 1 Comparison of clinical data in the three patient categories

Clinical variable	Autopsy	Non-HS	HS	*P*-value
Age (years)	45.6 ± 5.5	27.0 ± 3.1	32.3 ± 4.6	0.02
(range)	(21–74)	(13–41)	(11–55)	
Epilepsy onset (years)	NA	15.1 ± 3.3	10.9 ± 3.2	0.37
(range)		(2–38)	(0.5–35)	
Epilepsy duration (years)	NA	11.9 ± 2.4	21.5 ± 4.5	0.08
(range)		(3–28)	(3–52.5)	
Seizure frequency (no./month)	NA	17.1 ± 5.5	12.8 ± 5.1	0.57
(range)		(2–60)	(2–64)	
Male/female	6/6	4/8	4/8	0.76

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.

Antibodies

Panels of affinity purified rabbit antisera against mGluR1α or mGluR5 were employed. The mGluR1α antiserum (#AB1595; Chemicon, Temecula, CA) used in this study is directed against amino acids 1116–1130 of the rat mGluR1α splice variant (Houamed *et al.*, 1991; Masu *et al.*, 1991). This amino acid sequence differs only at one residue from the human mGluR1α 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 mGluR1α 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

Table 2 mGluR antibodies used

Antiserum	Source	Immunizing peptide	Reactivity	References	Dilution
#AB1595	Chemicon, Temecula, CA	EFVYEREGNTEEDEL	Rat mGluR1α*		1 : 25
#m1a	This laboratory (Hampson)	EFVYEREGNTEEDEL	Rat mGluR1α*	Hampson *et al.* (1994)	1 : 100
#24426	Diasorin, Stillwater, MN	EFVYEREGNTEEDEL	Rat mGluR1α*	Alvarez *et al.* (2000)	1 : 50
#AB1551	Chemicon	PNVTYASVILRDYKQSSSTL	Rat mGluR1α, 5*	Alvarez *et al.* (2000); Ferraguti *et al.* (1998)	1 : 100
#06–451	Upstate, Lake Placid, NY	SSPKYDTLIIRDYTNSSSSL	Rat mGluR5*	Alvarez *et al.* (2000)	1 : 200
#M3884–79	US Biological, Swampscott, MA	SSPKYDTLIIRDYTNSSSSL	Rat mGluR5*		1 : 100
#AB5232	Chemicon	LIIRDYTQSSSSL	Rat mGluR5†	Alvarez *et al.* (2000)	NA

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 immunoglobulins (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 apposed 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® Multi-Imager (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% H_2O_2 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 (ethylenediaminetetraacetate), 150 mM NaCl, 0.25% gelatin and 0.05% Tween-20 (pH 8.0). Primary antisera (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 antisera were 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% H_2O_2. Colour was developed for 15 min. Controls without the primary antisera 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, Borchen, Germany; 250–300 g; $n = 3$) rats were used in testing the specificity of the mGluR antisera. 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. IA 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 antisera (in rat hippocampus and cerebellum) was identical to the immunostaining obtained, respectively, with the mGluR1α #m1a (Supplementary Fig. IE and K) or Diasorin (Stillwater, MN; #24426) (Supplementary Fig. IF and L) and the mGluR5 US Biological antiserum (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; Diasorin) 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, #AB5232) 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 group I rather than an mGluR1α 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.

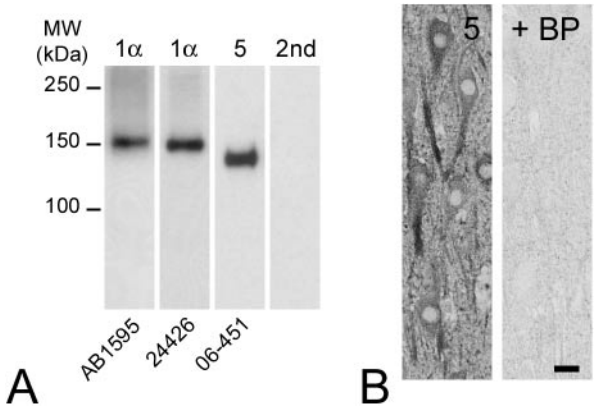

Fig. 1 Characterization of mGluR1α and mGluR5 antisera on human hippocampal tissue. (**A**) Immunoblots of synaptosomal plasma membranes (20 μg, lanes 1 and 2; 5 μg, lanes 3 and 4) from human (HS TLE) hippocampus were stained with mGluR1α (lane 1, Chemicon's #AB1595; diluted 1 : 125; lane 2, Diasorin's #24426; diluted 1 : 250) or mGluR5 (lane 3, Upstate's #06–451; diluted 1 : 40 000) antiserum. The mGluR1α and mGluR5 antisera detected a single immunoreactive band of ~150 and ~145 kDa, respectively. Blots without the primary antisera (i.e. second antibody controls) (lane 4) were blank. Positions of molecular weight (MW) markers in kDa are indicated on the left. (**B**) Immuno-adsorption of Upstate's mGluR5 antiserum on tissue sections from human (non-HS TLE) hippocampus (CA1 field). The strong mGluR5 signal in pyramidal cell bodies and apical dendrites (left panel) is eliminated by pre-adsorption with a C-terminal, 13-amino acids peptide from mGluR5 (right panel). 1α = mGluR1α; 5 = mGluR5; 2nd = second antibody control; +BP = with blocking peptide. Bar in **B** = 25 μm. Further details on the specificity of the mGluR antisera can be found in Fig. 1 of the Supplementary material.

Nomenclature

The hippocampal nomenclature used in this study is essentially as described by Amaral and Insausti (1990), except that the proximal part of the CA3 pyramidal layer (also termed 'end blade' or 'endfolium;' Margerison and Corsellis, 1966) that inserts into the dentate gyrus is referred to as CA4 as in our previous studies (Proper *et al.*, 2000).

Results

mGluR1α immunoreactivity

The mGluR1α antiserum labelled the perikarya and proximal apical dendrites of principal neurons and interneurons in the control hippocampus (Figs 2G, 3A, 4A and 5A). The strongest IR was observed within the hippocampus proper in pyramidal neurons of CA2 and CA3 (Fig. 2G). Labelling of CA1 and CA4 pyramidal neurons was less intense. Immunoreactive neuronal cell bodies were also observed in CA1–3 stratum oriens, consistent with a distribution of a subpopulation of somatostatin-containing interneurons (cf. Chan-Palay, 1987). In the dentate gyrus, granule cells and polymorphic hilar neurons were consistently immunoreactive (Figs 2G and 5A). Glial cells as identified by GFAP IR in adjacent sections were negative for mGluR1α IR. A similar pattern of mGluR1α 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 2J and 5D), 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

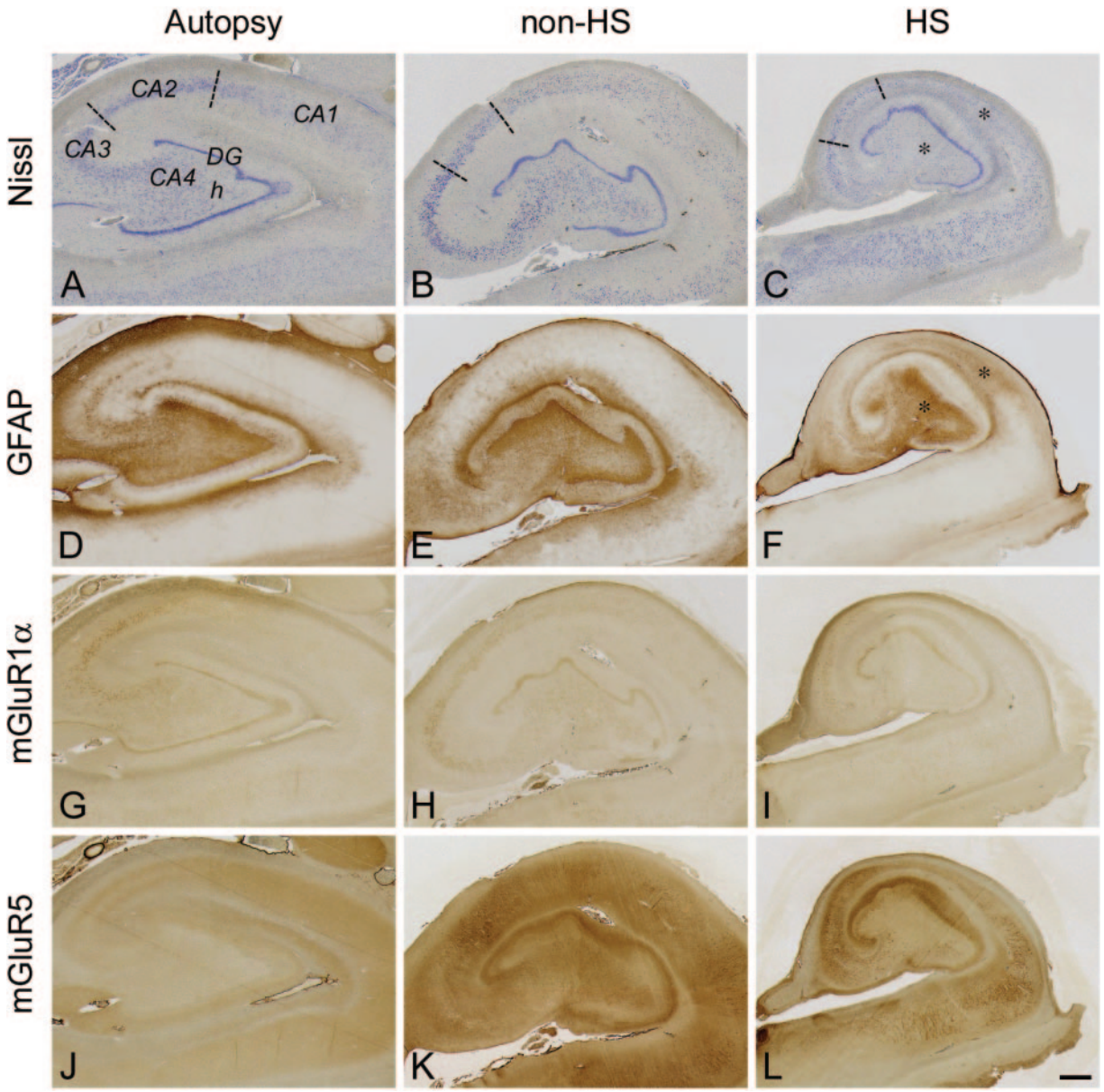

Fig. 2 Cytoarchitecture (**A–F**) and regional distribution of mGluR1α (**G–I**) and mGluR5 (**J–L**) IR in serial sections of a non-epileptic autopsy control (**A**, **D**, **G**, **J**), a non-HS (**B**, **E**, **H**, **K**) and an HS (**C**, **F**, **I**, **L**) hippocampus. Sections in **A–C** were stained with cresyl violet (Nissl stain) and in **D–F** for GFAP IR. Dashed lines in **A–C** delineate the CA2 region. Asterisks in **C** and **F** indicate areas with massive pyramidal cell loss (**C**) and gliosis (**F**) in CA1 and CA4 of the HS hippocampus. Note a similar neuronal distribution of mGluR1α IR (**G–I**) in the control (**G**), non-HS (**H**) and HS (**I**) hippocampus and the overall increase of mGluR5 IR (**J–L**) in the non-HS (**K**) and HS (**L**) hippocampus as compared with the control (**J**). Lack of mGluR1α IR (**I**; compare with **C**, asterisks) and reduced immunoreactive neuropil staining for mGluR5 (**L**; compare with **C**, asterisks) in the HS hippocampus is associated with segmental neuron loss. CA = CA field(s) of the hippocampus; DG = dentate gyrus; h = hilus. Bar = 1 mm.

neuropil of the dentate gyrus outer molecular layer (Fig. 2K and L). 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

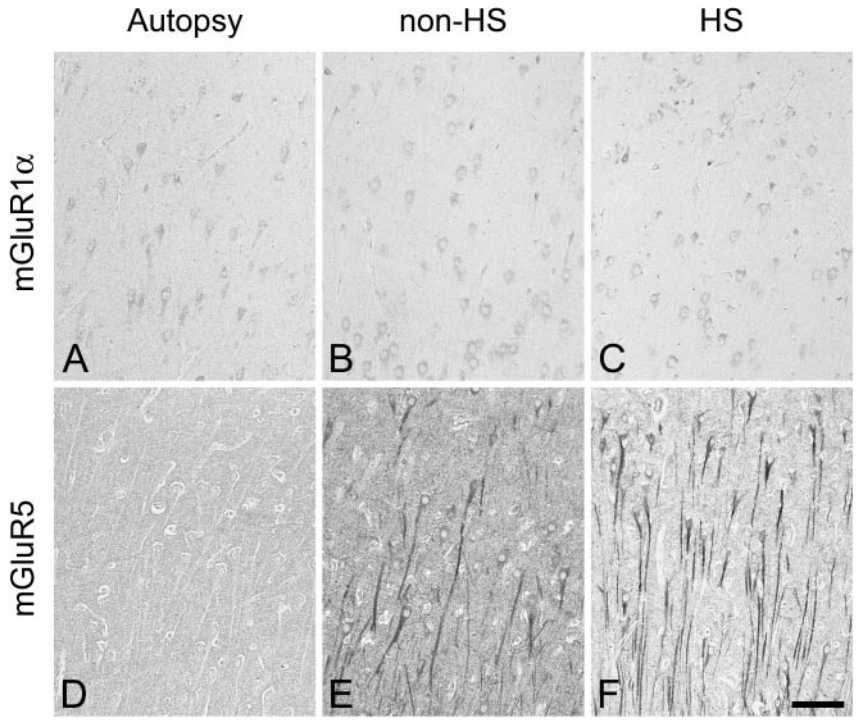

Fig. 3 Distribution of mGluR1α (**A–C**) and mGluR5 (**D–F**) IR in the CA1 pyramidal cell layer of a non-epileptic autopsy control (**A** and **D**), a non-HS (**B** and **E**) and an HS (**C** and **F**) hippocampus. The panels show a region of CA1 with relative sparing of pyramidal neurons in the HS hippocampus. (**A–C**) Pyramidal neurons show a weak somatodendritic labelling for mGluR1α IR with no difference in staining intensity between neurons in the control (**A**), non-HS (**B**) and HS (**C**) hippocampus. (**D–F**) Compared with the control (**D**), IR for mGluR5 is markedly increased in pyramidal neurons of the non-HS (**E**) and HS (**F**) hippocampus. Note that strong pyramidal staining for mGluR5 in the non-HS hippocampus (**E**) is almost exclusively restricted to apical dendrites. Note also the increased staining of the neuropil in the non-HS hippocampus (**E**) and the reduced immunoreactive neuropil staining in the HS hippocampus (**F**). Bar = 124 μm.

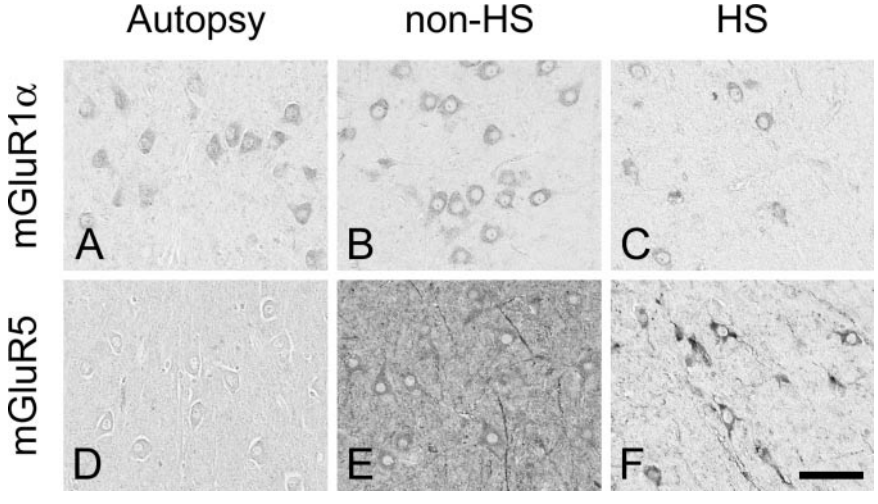

Fig. 4 Distribution of mGluR1α (**A–C**) and mGluR5 (**D–F**) IR in the CA4 pyramidal cell layer of a non-epileptic autopsy control (**A** and **D**), a non-HS (**B** and **E**) and an HS (**C** and **F**) hippocampus. Note for mGluR1α IR (**A–C**) a similar somatodendritic labelling of pyramidal neurons in the control (**A**), non-HS (**B**) and HS (**C**) hippocampus, and for mGluR5 IR (**D–F**) the abundant immunoreactive neuropil staining in the non-HS hippocampus (**E**) and the strong pyramidal staining in the HS hippocampus (**F**). The lower number of immunopositive neurons in the HS hippocampus (**C** and **F**) is associated with pyramidal loss. Bar = 100 μm.

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,

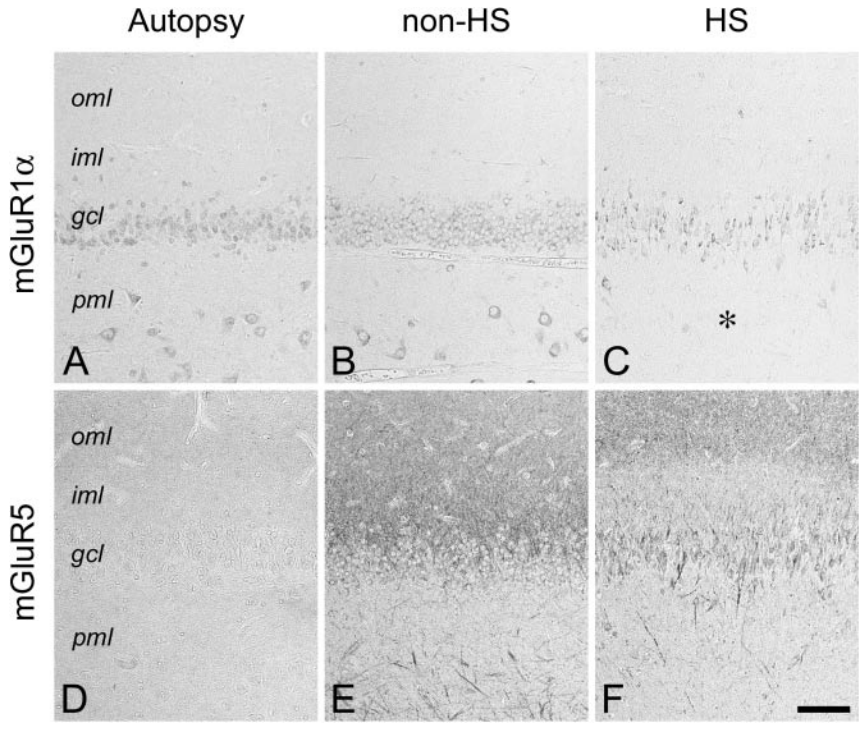

Fig. 5 Distribution of mGluR1α (**A–C**) and mGluR5 (**D–F**) IR in the dentate gyrus of a non-epileptic autopsy control (**A** and **D**), a non-HS (**B** and **E**) and an HS (**C** and **F**) hippocampus. (**A–C**) Granule cells and polymorphic neurons show a somatodendritic labelling for mGluR1α IR with no difference in staining intensity between neurons in the control (**A**), non-HS (**B**) and HS (**C**) hippocampus. Lack of mGluR1α IR in the polymorphic layer of the HS hippocampus (**C**, asterisk) is associated with hilar neuron loss. (**D–F**) Compared with the control (**D**), IR for mGluR5 is markedly increased in the dentate gyrus of the non-HS (**E**) and HS (**F**) hippocampus. Note the prominent mGluR5 IR in the inner and outer molecular layer of the non-HS hippocampus (**E**) and the strong staining of the outer molecular layer in the HS hippocampus (**F**). Note also the dense, supragranular staining immediately above the granule cells in the non-HS hippocampus (**E**) and the numerous mGluR5-immunoreactive granule cells (cell bodies and dendrites) in the HS hippocampus (**F**).
gcl = granule cell layer; iml = inner molecular layer; oml = outer molecular layer; pml = polymorphic layer. Bar = 124 μm.

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 β 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

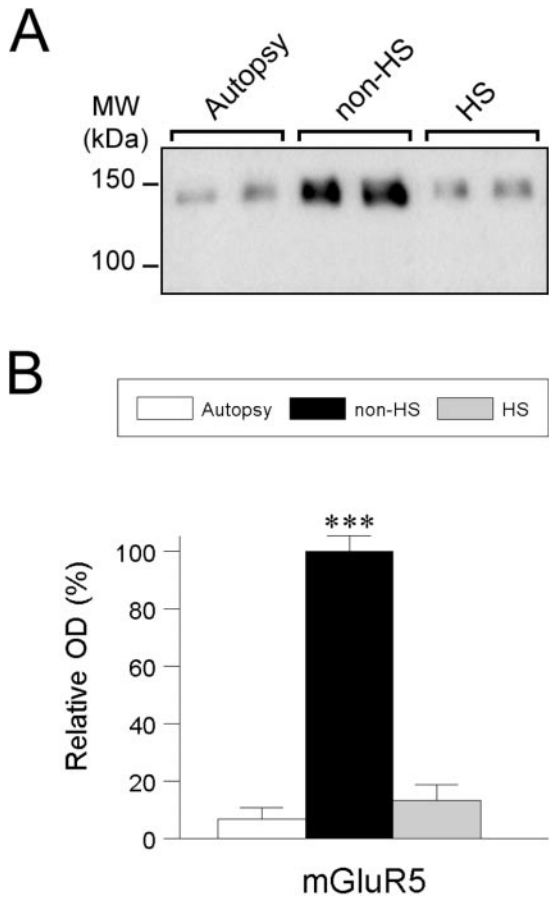

Fig. 6 Semi-quantitative immunoblot analysis of hippocampal mGluR5 IR. (**A**) Representative mGluR5 immunoblot loaded in duplicate with homogenates (10 μg/lane) from a non-epileptic autopsy control, a non-HS and an HS hippocampus stained with mGluR5 antiserum (Upstate's #06–451, diluted 1 : 40 000). Positions of molecular weight (MW) markers in kDa are indicated on the left. (**B**) Densitometric quantification of the mGluR5-immunoreactive band in the autopsy control (white bar), non-HS (black bar) and HS (grey bar) group. Values (mean ± SEM, $n = 6$) are expressed as percentage of the maximum optical density (OD) in the non-HS group. ***$P < 0.001$.

et al., 2000; but see Defagot *et al.*, 2002). Most likely, the inconsistency in detecting mGluR1α IR in hippocampal principal neurons is due to limits in sensitivity of methodologies used. Here we used 7 μm paraffin sections and antigen unmasking techniques based on microwave irradiation (Shi *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 mGluR1 α 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 (Ulas *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 gliotic 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 pharmacoresistant TLE patients suffer from a highly progressed state 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; Rodríguez-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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