


Increased expression of GABA_A receptor subunits associated with tonic inhibition in patients with temporal lobe epilepsy

Günther Sperk,¹ Susanne Pirker,² Elisabeth Gasser,¹ Anna Wieselthaler,¹ Anneliese Bukovac,¹ Giorgi Kuchukhidze,^{3,4} Hans Maier,⁵ Meinrad Drexel,^{1,6} Christoph Baumgartner,² Marin Ortler^{7,8} and  Thomas Czech⁹

Epilepsy animal models indicate pronounced changes in the expression and rearrangement of GABA_A receptor subunits in the hippocampus and in para-hippocampal areas, including widespread downregulation of the subunits $\alpha 5$ and δ , and upregulation of $\alpha 4$, subunits that mediate tonic inhibition of GABA. In this case-control study, we investigated changes in the expression of subunits $\alpha 4$, $\alpha 5$ and δ in hippocampal specimens of drug resistant temporal lobe epilepsy patients who underwent epilepsy surgery. Using *in situ* hybridization, immunohistochemistry and $\alpha 5$ -specific receptor autoradiography, we characterized expression of the receptor subunits in specimens from patients with and without Ammon's horn sclerosis compared to post-mortem controls. Expression of the $\alpha 5$ -subunit was abundant throughout all subfields of the hippocampus, including the dentate gyrus, sectors CA1 and CA3, the subiculum and pre- and parasubiculum. Significant but weaker expression was detected for subunits $\alpha 4$ and δ notably in the granule cell/molecular layer of control specimens, but was faint in the other parts of the hippocampus. Expression of all three subunits was similarly altered in sclerotic and non-sclerotic specimens. Respective mRNA levels were increased by about 50–80% in the granule cell layer compared with post-mortem controls. Subunit $\alpha 5$ mRNA levels and immunoreactivities were also increased in the sector CA3 and in the subiculum. Autoradiography for $\alpha 5$ -containing receptors using [³H]L-655,708 as ligand showed significantly increased binding in the molecular layer of the dentate gyrus in non-sclerotic specimens. Increased expression of the $\alpha 5$ and δ subunits is in contrast to the previously observed downregulation of these subunits in different epilepsy models, whereas increased expression of $\alpha 4$ in temporal lobe epilepsy patients is consistent with that in the rodent models. Our findings indicate increased tonic inhibition likely representing an endogenous anticonvulsive mechanism in temporal lobe epilepsy.

- 1 Department of Pharmacology, Medical University of Innsbruck, 6020 Innsbruck, Austria
- 2 Neurological Department, Klinik Hietzing, 1130 Vienna, Austria
- 3 Department of Neurology, Christian Doppler Klinik, Affiliated Member of the European Reference Network EpiCARE and Centre for Cognitive Neuroscience, Paracelsus Medical University of Salzburg, 5020 Salzburg, Austria
- 4 Neuroscience Institute, Christian Doppler Klinik, 5020 Salzburg, Austria
- 5 INNPATh GmbH-Institute of Pathology, 6020 Innsbruck, Austria
- 6 Institute of Molecular and Cellular Pharmacology, Medical University of Innsbruck, 6020 Innsbruck, Austria
- 7 Department of Neurosurgery, Klinik Landstrasse, Vienna Healthcare Network, 1030 Vienna, Austria
- 8 Department of Neurosurgery, Medical University of Innsbruck, 6020 Innsbruck, Austria
- 9 Department of Neurosurgery, Medical University Vienna, 1090 Vienna, Austria

Correspondence to: Professor Dr. Günther Sperk,
Department of Pharmacology, Medical University Innsbruck, Peter-Mayr-Str. 1a, 6020 Innsbruck, Austria.
E-mail: guenther.sperk@i-med.ac.at

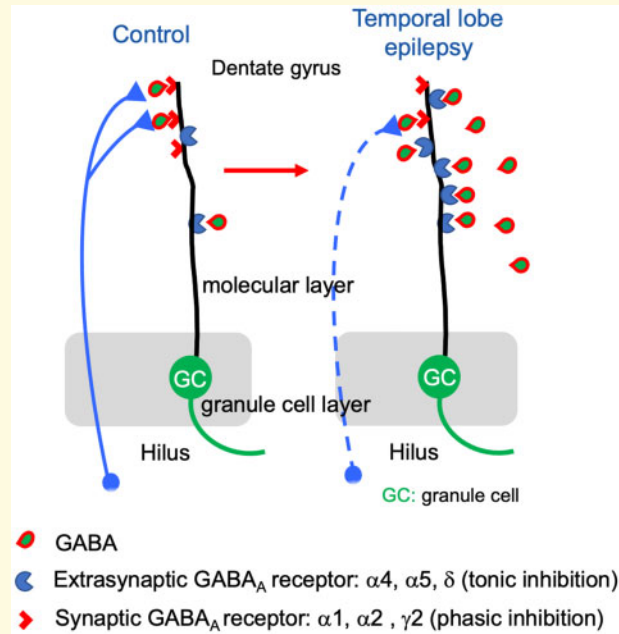
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Graphical Abstract



Abbreviations: AS = Ammon's horn sclerosis; NS = non-sclerotic; SE = status epilepticus; TLE = temporal lobe epilepsy

Introduction

γ -Aminobutyric acid (GABA) is the primary inhibitory transmitter in the mammalian brain. It acts through two different classes of receptors, GABA_A and GABA_B receptors. Whereas GABA_B receptors are metabotropic receptors, GABA_A receptors represent hyperpolarizing chloride channels. GABA_A receptors are assembled in a pentameric structure. Quite a large number of different possible subunit proteins ($\alpha 1$ -6, $\beta 1$ -3, $\gamma 1$ -3, δ , ϵ , θ) can mutually participate in the assembly of the pentameric receptor. Although in theory a huge number of different subunit combinations would be possible, the actual number of different receptors is not as extensive since their constitution follows certain rules. In general, GABA_A receptors contain two α -subunits ($\alpha 1$ -6), two β - ($\beta 1$ to $\beta 3$) and either one $\gamma 2$ - (less frequently $\gamma 1$) or one δ -subunit (rarely an ϵ or θ -subunit). This subunit composition crucially determines the physiological and pharmacological properties of the individual GABA_A receptor subtypes.^{1,2} GABA binds to the interface between the respective α - and β -subunits. Benzodiazepines and the hypnotic substance zolpidem are positive allosteric modulators of GABA_A receptors containing two α -subunits (either $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$), two β - together with one γ -subunit (not δ). Potent endogenous modulators of GABA_A receptors are neurosteroids, which act as positive allosteric modulators at the δ -subunit of the receptor.³

In the rodent dentate gyrus, the δ -subunit often assembles with $\alpha 4$ or $\alpha 5$ subunits.⁴⁻⁶ In the rat, these receptors are either extra- or peri-synaptically located and mediate *tonic inhibition*, in contrast to $\alpha 1$ -, $\alpha 2$ - and $\gamma 2$ -containing receptors that are positioned within the synapse and mediate *phasic inhibition*.⁶⁻⁹ Related to the extended distance of diffusion of GABA, extra-synaptic receptors containing the δ -subunit exert a higher affinity to GABA than $\gamma 2$ -subunit containing receptors.

Malfunctioning of GABA-ergic transmission can facilitate epileptic seizures. Thus, autoantibodies to the GABA-synthesizing enzyme glutamate decarboxylase in humans, *knock out* of the gene in mice,¹⁰⁻¹² and mutations of the $\alpha 1$, $\beta 3$ or $\gamma 2$ subunits of the GABA_A receptors are causatively associated with epilepsies.¹³ Furthermore, GABA_A receptor antagonists like pentylenetetrazol, bicuculline or picrotoxin are potent convulsive drugs, whereas GABA_A receptor agonists like benzodiazepines and barbiturates are anticonvulsive.¹⁴

Significant changes in the expression and assembly of individual GABA_A receptor subunits are seen in animal models of epilepsy. Presumably due to the strongly enhanced GABA release and related internalization, subunits $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 3$ and $\gamma 2$ become significantly downregulated in granule cells of the dentate gyrus during or subsequent to a SE e.g. induced by injection of kainic acid, but significantly recover already after a few days.^{15,16} This may be

the cause for the resistance to anticonvulsive treatment with benzodiazepines seen in some patients enduring a *status epilepticus* (SE).¹⁷ During the acute SE, also levels of δ -subunit mRNA and immunoreactivity (IR) significantly decline. These decreases, however, often persist for several months and are seen virtually in all epilepsy models.^{15,16,18–21} Also, $\alpha 5$ mRNA levels and IR were significantly decreased throughout all subfields of the hippocampus, including sectors CA1 and CA3, the subiculum, layers II and V/VI of the entorhinal cortex, and the perirhinal cortex in the same models.^{18,19,22} Downregulation of the δ -subunit is contrasted by marked and lasting increases in $\gamma 2$ (after the acute SE).^{16,19,22,23} Also, an expression of subunit $\alpha 4$ is markedly *increased* in the granule cell layer after a SE induced by kainic acid, trimethyltin or sustained electrical stimulation, as well as after electrical kindling in the hippocampus.^{15,16,18–20,24} Six weeks after a pilocarpine-induced status epilepticus, also increased subunit $\alpha 3$ -IR was seen in the molecular layer of the dentate gyrus²⁵ and in the same experiments $\alpha 5$ -IR was increased in the granule cell but not in the molecular layer of the dentate gyrus. And, after unilateral intrahippocampal injection of kainic acid, Bouillere et al.²⁶ observed increases in subunits $\alpha 1$, $\alpha 3$, $\alpha 5$ and $\gamma 2$ IR in the dispersed granule cell/molecular layer compared to the contralateral area. This may indicate that the changes are not driven by seizures but related to granule cell dispersion.

Since δ - and $\alpha 5$ -subunit containing receptors mediate tonic inhibition by GABA, it was suggested that the loss in these receptors may also reflect a loss in tonic inhibition in epileptic animals.^{21,22} A subsequent detailed study using immunogold labelling combined with whole cell electrophysiology by the same group, however,

demonstrated that subunit $\gamma 2$ -IR is partially translocated from the centre of the synapse to a more perisynaptic area of granule cell dendrites. Thus, in epileptic mice, $\gamma 2$ -containing receptors may not only mediate phasic inhibition at the synapse centre but may also partially compensate for the loss in δ -subunits at perisynaptic sites and participate in tonic inhibition.²⁷

Using human tissue, Shumate et al.²⁸ more than 20 years ago demonstrated that the potency of GABA to stimulate GABA_A receptors was considerably higher in granule cells of temporal lobe epilepsy (TLE) patients than in epileptic rats or their controls and that their sensitivity to zinc was increased both in TLE patients and epileptic rats.²⁸ These findings strongly indicated increased expression of GABA_A receptors in granule cells of TLE patients. The authors also suggested a possible shift from $\alpha 1$ containing receptors to receptors containing other α -subunits with a higher sensitivity to zinc than $\alpha 1$.²⁸

In contrast to rodents, there are only limited studies on changes in individual GABA_A receptor subunits in human epilepsies. In hippocampal tissue obtained at surgery from drug-resistant TLE patients changes in the expression of different subunits have been investigated by immunohistochemistry and *in situ* hybridization and were compared with post-mortem tissue. In addition to changes associated with Ammon's horn sclerosis, Loup et al.²⁹ found increased expression of subunits $\alpha 1$, $\alpha 2$, $\beta 2/3$ and $\gamma 2$ in the granule cell layer of patients with hippocampal sclerosis, but interestingly not in patients without hippocampal sclerosis, and we observed pronounced increases in the expression of $\alpha 3$ and of all three β -subunits in patients with and without hippocampal sclerosis. Stefanitis et al.³⁰ recently presented a detailed immunohistochemical study, investigating subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$,

Table 1 Characteristics of specimens used for the individual histochemical approaches

TLE patients	Number			Age		Duration of epilepsy	
	N	F	M	Mean \pm SEM	(Range)	Mean \pm SEM	
(Range)							
<i>In situ</i> hybridization							
TLE, lesional	29	11	18	36.2 \pm 1.92	(17–56)	24.1 \pm 2.31	(1–46)
TLE, non-lesional	13	5	8	34.4 \pm 4.03	(4–53)	13.8 \pm 2.33	(1–28)
Receptor autoradiography							
TLE, lesional	17	7	10	36.4 \pm 2.93	(17–56)	21.1 \pm 3.30	(1–46)
TLE, non-lesional	6	2	4	38.0 \pm 5.72	(8–53)	16.2 \pm 3.11	(9–28)
Immunohistochemistry							
TLE, lesional	24	14	10	41.1 \pm 1.94	(23–61)	23.8 \pm 2.87	(1–54)
TLE, non-lesional	3	2	1	38.3 \pm 8.76	(22–52)	11 \pm 6.23	(1–23)
Post-mortem controls	Number			Age		Post-mortem time	
	N	F	M	Mean \pm SEM	(Range)	Mean \pm SEM	(Range)
<i>In situ</i> hybridization	28	9	19	49.9 \pm 2.7	(24–95)	14.8 \pm 1.15	(5–24)
Receptor autoradiography	23	7	16	51.4 \pm 2.80	(33–95)	14.8 \pm 1.26	(5–24)
Immunohistochemistry	12	1	11	54.7 \pm 5.20	(28–95)	12.4 \pm 0.97	(8–20)
Post-mortem, died during SE	4	3	1	72.0 \pm 8.46	(55–88)	10.6 \pm 1.48	(6.5–13)

F, female; M, male; N, total number of sample. Note that some specimens were used for *in situ* hybridization, receptor autoradiography and immunohistochemistry.

$\beta 2/3$ and $\gamma 2$ in seven nuclei of the amygdala and in the entorhinal cortex of patients with and without hippocampal sclerosis. They observed decreased expression for all these subunits in all nuclei investigated except for subunit $\gamma 2$. The study revealed an increased number of $\gamma 2$ -positive dendrites in most amygdaloid nuclei, although labelling of the neuropil was often reduced.

In our present study, we focussed on the dentate gyrus of TLE patients with and without Ammon's horn sclerosis and investigated possible changes in the expression of subunits $\alpha 4$, $\alpha 5$ and δ , subunits that in the rat mediate tonic inhibition of GABA. We collected hippocampal specimens from TLE patients at the epilepsy centres in Vienna and Innsbruck, conducted *in situ* hybridization, immunohistochemistry and $\alpha 5$ -specific receptor autoradiography for these subunits, and compared the results with those obtained in post-mortem tissue.

Materials and methods

Patients

Hippocampal tissues were obtained at surgery from drug resistant TLE patients. Approval for the study was obtained from the Institutional Boards of the Medical Faculties of the Universities of Vienna and Innsbruck according to the Helsinki Declaration, and informed consent was obtained from all patients providing specimens. Presurgical evaluation of TLE patients included detailed clinical examinations, prolonged video-electroencephalographic (video EEG) monitoring with scalp and sphenoidal electrodes and neuropsychological tests.^{31–33} Neuroimaging studies included high-resolution magnetic resonance imaging (MRI) and interictal single photon emission computed tomography (SPECT) with [^{99m}Tc]hexamethyl-propylene-amine-oxim (HMPAO). In patients with normal MRI scans or divergent findings from these non-invasive studies, invasive electrophysiological recordings from chronically indwelling subdural grid electrodes were obtained. Based on an accurate localization of the seizure onset zone derived from these investigations, patients were referred to selective amygdalohippocampectomy, anteromedial temporal lobe resection or *en bloc* temporal lobe resection.

Surgical specimens were examined by routine pathology and were grouped into those with hippocampal sclerosis ($n=51$) or without sclerosis ($n=22$). Tissue with hippocampal sclerosis originated from patients with selectively damaged hippocampus as assessed by MRI and confirmed by the presence of distinct hippocampal sclerosis at the neuropathological examination. In these patients, seizures presumably arose from the medial temporal region. Patients without obvious Ammon's horn sclerosis were defined as 'non sclerotic'. The mean age of patients with Ammon's horn sclerosis was 38.4 ± 1.36 (range 17–61) years that of patients without sclerosis was 36.1 ± 2.86 (range 22–55) years. The mean duration of

epilepsy was 22.9 ± 1.80 years (range 1–54) and 12.2 ± 1.85 (range 1–28) years in patients with and without hippocampal sclerosis, respectively. All patients were taking antiepileptic drugs in mono- or polytherapy. The majority of patients received a combination of two drugs (72.2%); 19.4% were on monotherapy and 8.3% of the patients were treated with three or four anticonvulsive drugs. The most frequent antiepileptic drugs applied were carbamazepine (45% of all drugs used), lamotrigine (10%), valproate (23%), clobazam (9.5%) and oxcarbazepine (7.2%). In Table 1, we show the distribution of age, gender, duration of epilepsy for patients included in the individual histochemical studies.

Post-mortem controls

For controls, 31 hippocampi were obtained at routine autopsy post-mortem from patients without known history of neurological or psychiatric disease. Each brain was studied by a neuropathologist to confirm the absence of a brain lesion. The mean age at autopsy was 48.9 ± 2.24 years (range 24–76) and the mean post-mortem time prior to fixation of the specimen was $13.2 \text{ h} \pm 01.00 \text{ h}$ (range 5–24 h) (see Table 1 for specifications in individual experiments). Causes of death were pneumonia, lung cancer, pharynx cancer, larynx cancer, breast cancer, liver cancer, liver cirrhosis, melanoma, pulmonary embolism, cardiovascular arrest, leukaemia or renal failure. In addition, we obtained post-mortem hippocampal tissues from four patients without history of epilepsy that had died during or after a SE (mean age: 72.0; range: 55–85 years; the SE was associated with cardiac arrest, theophylline intoxication, lung edoema, lymphoma). These specimens were included for comparing effects of an acute SE with those of chronic epilepsy with recurrent seizures.

Sample preparation

Surgical specimens were rinsed in 50 mM phosphate buffered saline (PBS, pH 7.4) at 4°C, photographed for documentation and sectioned perpendicularly to the hippocampal axis into 5 mm thick blocks. Those obtained from the hippocampal body (middle segment) were included in the study. At autopsy, the whole hippocampus was removed from the brain, placed in ice cold PBS and sectioned in the same way as surgical tissue in a cold room. Tissue blocks were either processed for immunocytochemistry or *in situ* hybridization by fixation in paraformaldehyde (PFA) or by immediate snap freezing, respectively. Some specimens were split for processing them in both ways. For immunohistochemistry, tissue blocks were immediately immersed in 4% PFA/PBS for 4–5 days followed by stepwise immersion in sucrose (concentrations ranging from 5% to 20%) over 2 days. Specimens fixed by this procedure and unfixed brain samples selected for *in situ* hybridization were immersed

in -70°C cold isopentane for 3 min and then put in a freezer at -70°C . After allowing the isopentane to evaporate for 24 h, tissue samples were sealed in vials and kept at -70°C . Prior to immunohistochemistry, The PFA-fixed specimens were cut in a Zeiss Microtome (Microm HM500 OM Cryostat; Carl Zeiss, Vienna, Austria), $40\mu\text{m}$ thick sections were collected and kept in PBS/0.1% sodium azide at 5°C for up to 2 weeks. For *in situ* hybridization, $20\mu\text{m}$ thick sections were cut and mounted immediately on poly-lysine coated slides (Menzel-Gläser, Braunschweig, Germany) and stored at -20°C .

In situ hybridization

In situ hybridization was performed as described previously.^{16,32} We used the following oligo-DNA probes that were complementary to selected DNA sequences of the human prepro-mRNAs: for subunit $\alpha 4$ the oligo-DNA probe was complementary to bases 6086–6129 (gene bank accession number NM_001204266.2, CCAACATCAACAACCTCTCACTTCCCTGCATTCACCCTTACCTCC, 44 bases), that for subunit $\alpha 5$ to bases 462–508 (gene bank accession number: XM_017022056.1, CGAAGTCTGTTGTCGTAGCCATCCAAGAGCCCATCCAAGATCTGCTGGT, 47 bases), that for the δ -subunit was complementary to bases 3740–3742 (gene bank accession number: XM_011541194.3; CCCACCTCCTCCCACGCTCTGATCCGATCCTGCAGAGC, 39 bases) and for β -actin we used an oligonucleotide complementary to bases 1464–1501 (gene bank accession number: NM_001101.5; GGC AAG GGA CTT CCT GTA ACA ACG CAT CTC ATA TTT GG, 38 bases) of the respective prepro-mRNA sequences.

The probes were custom synthesized (Microsynth, Balgach, Switzerland) and labelled on their 3' end with [^{35}S] α -thio-deoxyadenosine 5' triphosphate (1300 Ci/mmol; New England Nuclear, Wilmington, Germany) by reaction with terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany) as described in detail previously.^{16,32} For *in situ* hybridization of β -actin mRNA, we used [α - ^{33}P]deoxyadenosine 5' triphosphate (3000 Ci/mmol; SCF-203H; Hartmann Analytic, Braunschweig, Germany) applying the same procedure for DNA labelling.

Frozen sections from controls ($n=29$), sclerotic ($n=29$) and non-sclerotic ($n=14$) specimens, and specimens from non-epilepsy patients who died after a SE ($n=4$) were rapidly immersed into ice cold 2% PFA, PBS for 10 min, rinsed in PBS, transferred to 0.25% acetic anhydride in 0.1 M triethylamine hydrochloride for 10 min (room temperature), dehydrated by ethanol series and delipidated with chloroform. They were then hybridized in $50\mu\text{l}$ hybridization buffer, containing about 50 fmol ($0.8\text{--}1 \times 10^6$ cpm) labelled oligonucleotide probe at 42°C for 18 h. The hybridization buffer consisted of 50% formamide, $4 \times \text{SSC}$ ($1 \times \text{SSC}$ is 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 500 $\mu\text{g}/\text{ml}$ salmon sperm DNA, 250 $\mu\text{g}/\text{ml}$ yeast tRNA, $1 \times \text{Denhardt's}$ solution (0.02%

ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), 10% dextran sulphate, and 20 mM dithiothreitol (all from Sigma, Munich, Germany). At the end of the incubation, we rinsed the slides twice in $1 \times \text{SSC}$, 50% formamide (Merck, Darmstadt, Germany). They were then washed four times in 50% formamide in $1 \times \text{SSC}$ (42°C , 15 min) and briefly rinsed in $1 \times \text{SSC}$ followed by water. We then dipped the sections in 70% ethanol, dried them and exposed them to BioMax MR films (Amersham Pharmacia Biotech, Buckinghamshire, UK), for 7–14 days and then developed the films with a Kodak D19 developer. The sections were then counter stained with cresyl violet, dehydrated, cleared in butyl acetate and cover slipped with Eukitt (Merck, Darmstadt, Germany). Control experiments were performed using the respective sense probes.

Receptor autoradiography

We used the $\alpha 5$ -specific inverse GABA_A agonist [^3H]L-655,708 as ligand for specifically labelling $\alpha 5$ -containing GABA_A receptors in our specimens³⁴ and examined the same slice-mounted tissue sections ($20\mu\text{m}$, kept at -20°C) as used for *in situ* hybridization ($n=24$ controls, 19 with and eight without hippocampal sclerosis, two post-mortem with SE). We washed the sections four times in fresh ice-cold 10 mM Tris, 1 mM EDTA, pH 7.4 (Tris/EDTA) buffer for 30 min and then incubated them in Tris/EDTA containing 2 nM methoxyl [^3H]L-655,708 (Amersham, Buckinghamshire, Great Britain, catalog number: 708N) and $10\mu\text{M}$ zolpidem (Tocris, catalog number: 0655) to inhibit low affinity binding to other α -subunit containing receptors at 4°C for 2 h. Non-specific binding was assessed by incubating the sections with the same solution containing $10\mu\text{M}$ flunitrazepam. Labelled sections were rinsed twice in Tris/EDTA for one min, dipped in distilled water, dried in cold air and then exposed together with [^{14}C] micro-scales (Amersham International, Amersham, UK) to Hyperfilm-B (RMN 9 Bmax 1562545) films (Amersham International, Amersham, UK) for 10 weeks. The films were developed with Kodak D-19 developer (16%) for 1 min, briefly rinsed in water and subsequently fixed for three min. The films were then digitalized using a Canon 9000 Mark II scanner.

Immunohistochemistry

Antibodies

The antibodies were originally raised to fusion proteins derived from DNA constructs of the maltose binding protein gene with specific rat sequences of $\alpha 4$ - and δ -subunits and transcribed in *Escherichia coli*, and to an $\alpha 5$ -subunit specific peptide coupled to keyhole-limpet haemocyanin.³⁵ They were first characterized in the rat brain³⁵ and then further characterized in the monkey brain by using synthetic peptides corresponding to human/monkey

sequences of $\alpha 5$ and δ subunits for displacing antibody binding.³⁶

We performed incubations on 40 μm free-floating sections obtained from PFA-fixed hippocampal specimens from TLE specimens with hippocampal sclerosis and from post-mortem controls. The sections were first incubated in 0.6% H_2O_2 , 20% methanol in 50 mM Tris-HCl buffered saline pH 7.2 (TBS) for 20 min to reduce endogenous peroxidase activity and then treated with target retrieval solution (pH 6.0 and pH 10; Dako, Vienna, Austria, 70°C, 20 min). We then incubated the sections in 10% normal goat serum (GIBCO #16210-072, obtained through Fisher Scientific, Vienna, Austria) in TBS/0.2% Triton X100 and 0.1% sodium azide for 90 min and subsequently with the respective primary antiserum at dilutions of 1:100 ($\alpha 4$ and $\alpha 5$) or 1:200 (δ) at 4°C for 48–72 h. The sections were then processed by the Vectastain ABC standard procedure using 1:200 dilutions of Vectastain PK4001 goat biotinylated anti-rabbit antibody (Vector Laboratories Cat# PK-4001, obtained through Szabo-Scandic, Vienna, Austria). Incubations with the biotinylated secondary antibodies and subsequent incubations with the ABC reagent (a mixture of avidin–biotin–horseradish peroxidase complex; 1:100) were done at room temperature for 60 min. The resulting complex was labelled by reacting the peroxidase with 0.05% 3,3'-diaminobenzidine (Sigma, Munich, Germany) and 0.005% H_2O_2 (30%, Merck, Darmstadt, Germany) in TBS for 4 min. After each incubation step (except pre-incubation with 10% blocking serum), three 5 min washes with TBS were included. All buffers and antibody dilutions, except those for washing after target retrieval solution and peroxidase treatment, and the reaction with diaminobenzidine, contained 0.2% Triton X-100. We included normal horse or goat serum (10%) in all antibody-containing buffers. The sections were finally mounted on glass slides, air-dried, dehydrated, and cover-slipped (VWR International, Vienna, Austria). No labelling was observed when the primary antisera were omitted. For light microscopy a Zeiss Axiscope 5 microscope equipped with an Axiocam 105 digital camera (Carl Zeiss, Vienna, Austria) was used. Digital images were obtained by a Openlab 5.5.0 Imaging Software, Improvion, Coventry, UK.

Nissl stains were performed as described previously.³⁷

Densitometric analyses

Analysis of mRNA expression, receptor binding, immunohistochemistry and of Nissl stains

Autoradiographic films were digitized and opened in NIH ImageJ (version 1.46; U.S. National Institutes of Health, Bethesda, ML, USA; <http://imagej.nih.gov/ij/>). *In situ* hybridization signals for subunit $\alpha 4$ and δ mRNAs, and that of Nissl stains were quantified in the granule cell layer and those for $\alpha 5$ mRNA in sectors CA1 and CA3

and in the subiculum (in addition to the granule cell layer). Evaluation of $\alpha 5$ -specific receptor binding was done in the molecular layer of the dentate gyrus using films from [^3H]L-655,708 receptor autoradiographs. For evaluating the dentate granule cell layer, line selections (20 pixels width) were drawn perpendicular to the layer of interest and a density profile plot (grey values) was created using the function 'Analyze—Plot profile'. All other areas including the molecular layer for evaluating $\alpha 5$ -specific receptor autoradiographs were evaluated in the same way using circular selections. For evaluating immunohistochemical labelling digitalized immunohistochemical images were used and circular selections of the dentate molecular layer were quantified. Background measures were done adjacent to the brain section. Values for relative optical densities (RODs) were calculated from grey values according to the following formula: $\text{ROD} = \log[256/(255 - \text{gray value})]$. In the dentate gyrus, ROD values were obtained from two to three different measures obtained at a distance of about one-third of the entire granule cell/molecular layer blade to its borders and from its middle part. In the other hippocampal subfields measures were taken randomly at sites with equal distance from each other and the borders of the area. Background ROD values were obtained at the site of the respective section with lowest densities. ROD values were averaged and the background ROD value was subtracted.

Statistical analysis

Data are presented as of % controls (mean \pm SEM). Statistical analyses were conducted using GraphPad Prism 5.0a for Macintosh (GraphPad Software, San Diego, CA, USA) and Microsoft Excel data analysis software. Gaussian distribution was assessed for all groups by the d'Agostino & Pearson omnibus normality test using the GraphPad Software. When two groups were compared an unpaired two-tailed Student's *t*-test was applied depending whether a Gaussian distribution was observed, assuming $P < 0.05$ as statistically significant. When more than two groups were compared, an analysis of variance (ANOVA) was done. The alpha level was set at $P < 0.05$ for multiple comparisons and a Dunnett's multiple comparison *post hoc* test was performed for determining between-group differences among multiple sets of data and controls. *F*-values and degrees of freedom (df) are presented. For assessing correlations between age and ROD values and post-mortem times (PMT) and RODs, the Pearson correlation factor (*r*) and respective *P*-values were determined using the GraphPad Software.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

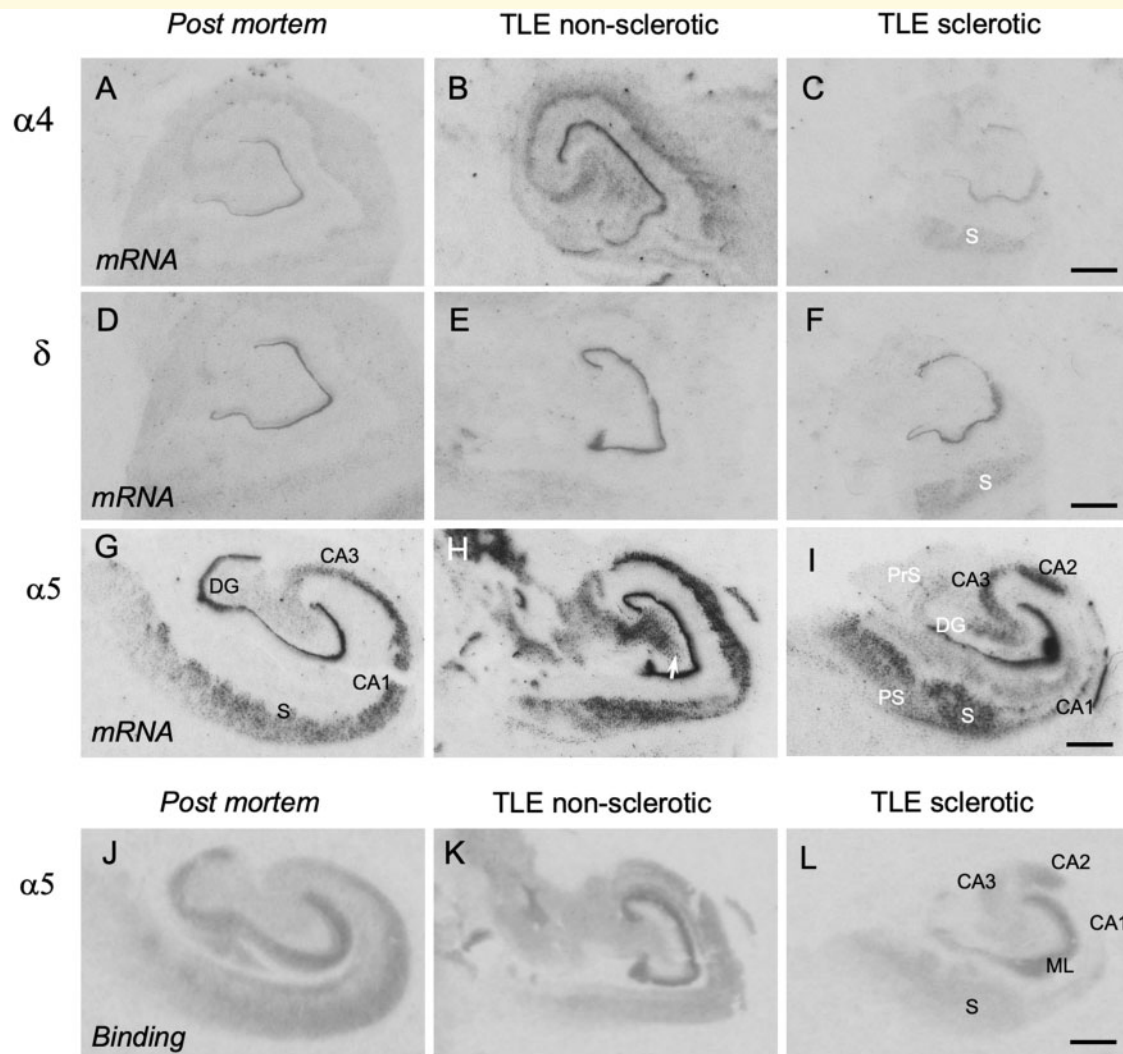


Figure 1 GABA_A receptor subunit mRNAs and $\alpha 5$ receptor autoradiography in TLE patients. Autoradiograms after *in situ* hybridization subunit $\alpha 4$ (A, B), δ (D, E) and $\alpha 5$ (G, H) mRNAs and receptor autoradiography for $\alpha 5$ -containing receptors (J, K). Shown are images of the hippocampus of autopsy controls and for non-sclerotic and sclerotic TLE specimens as indicated on top of the figure. Note the pronounced expression of $\alpha 5$ mRNA throughout all hippocampal subfields. It correlates well with the receptor autoradiography using the $\alpha 5$ -specific ligand [³H]L-655,708. DG, dentate gyrus (granule cell layer); PrS, presubiculum; PS, parasubiculum; S, subiculum. Scale bars in C, F, I and L for all images: 200 μ m.

Results

Characteristics of epilepsy specimens

Table 1 shows a summary of the clinical data of the patients included in the different histochemical procedures. In agreement with our previous studies,^{31,32} and those of many others,^{38,39} we observed in most cases with Ammon's horn sclerosis pronounced cell losses in the hilus of the dentate gyrus, sectors CA1 and CA3, accompanied by less severe in the granule cell layer, sector CA2 and in the subiculum. Owing to the surgical procedure, only small parts of the CA1 sector were preserved in most TLE

tissue specimens. In nineteen patients with hippocampal sclerosis dispersion of granule cells was observed.⁴⁰ In these patients, the granule cell layer was broader than in other TLE patients or controls. In four of these specimens, it was bilaminar. None of the post-mortem controls or non-sclerotic specimens showed comparable dispersions of granule cells. We assessed cell losses in the granule cell layer by determining ROD values of the granule cell layer of Nissl-stained sections and observed reductions by $45.6 \pm 3.50\%$ ($n=16$; $P < 0.0001$ in ANOVA with Dunnett's Multiple Comparison test) and $14.3 \pm 8.89\%$ ($n=8$, not significant) in specimens with and without Ammon's horn sclerosis, respectively. These estimates were in striking agreement with previously performed cell counts

Table 2 Changes in subunit $\alpha 5$ mRNA expression in subfields of the hippocampus of TLE patients without Ammon's horn sclerosis

	Control	TLE	
ROD (% of control \pm SEM)			
Granule cells	100.0 \pm 5.69 (30)	187.3 \pm 9.20 (14)	$P = 3.68 \cdot 10^{-8}$
CA3	100.0 \pm 11.55 (9)	186.6 \pm 33.31 (9)	$P = 0.025$
CA1	100.0 \pm 9.46 (9)	190.2 \pm 22.16 (9)	$P = 0.007$
Subiculum	100.0 \pm 11.46 (9)	170.7 \pm 18.45 (9)	$P = 0.005$

Due to the variable neurodegeneration in specimens with Ammon's horn sclerosis only non-sclerotic samples were used for the analysis of hippocampal subfields. The data show ROD values expressed as mean of controls \pm SEM. Autoradiographs obtained after *in situ* hybridization of the respective mRNAs were done by ImageJ (<http://imagej.nih.gov/ij/>). For statistical analysis, the two-tailed Student's *t*-test was applied.

in our TLE specimens revealing losses in granule cells by $55 \pm 2.0\%$ and $10 \pm 4.1\%$ in sclerotic and non-sclerotic specimens, respectively.³⁷

In situ hybridization of GABA_A receptor subunit mRNAs

Figure 1 shows photomicrographs from autoradiographs after *in situ* hybridization for subunits $\alpha 4$, $\alpha 5$ and δ . Quantitative assessments for the granule cell layer are shown in Fig. 2, and in Table 2 data on $\alpha 5$ mRNA expression in other subfields of the hippocampus. In post-mortem controls, we observed weak signals for $\alpha 4$, and δ -subunit mRNAs (Fig. 1A and D), but considerably stronger and significantly more abundant ones for $\alpha 5$ (Fig. 1G). All three subunits were unambiguously expressed in the dentate granule cell layer. Signals for $\alpha 4$ and δ were faint in the pyramidal cell layers and in the subiculum. In contrast, besides the dentate granule cells there was high expression of $\alpha 5$ mRNA in the pyramidal cell layers of CA1 to CA3, in the subiculum and in the pre- and parasubiculum (Fig. 1G). One-way ANOVA revealed statistically significant differences in the expression of all three subunit mRNAs in TLE patients compared with post-mortem controls: $\alpha 4$, $F(2,68) = 15.9094$, $P = 2.148E-06$; $\alpha 5$, $F(2,69) = 33.8106$, $P = 5.82E-11$; δ , $F(2,65) = 28.6391$, $P = 1.205E-9$. Using the Dunnett's *post hoc* analysis, there were statistically significant increases in mRNA expression both in patients with (mean % of control \pm SEM): $\alpha 4$: 151.7 ± 9.55 ($n = 29$); $\alpha 5$: 140.1 ± 6.65 ($n = 28$) and δ : 180.9 ± 10.10 ($n = 27$); $P < 0.001$ for all three subunit mRNAs) and without Ammon's horn sclerosis (mean % of control \pm SEM): $\alpha 4$: 174.3 ± 13.54 ($n = 13$); $\alpha 5$: 182.7 ± 8.98 ($n = 14$) and δ : 184.8 ± 9.62 ($n = 14$); $P < 0.001$ for all three subunit mRNAs compared with post-mortem controls ($n = 29$, 30 and 27 for $\alpha 4$, $\alpha 5$ and δ , respectively) (Fig. 2). Subunit mRNAs were not significantly altered in specimens obtained from patients who died after or during a SE (mean % of control \pm SEM: $\alpha 4$: 85.1 ± 18.83 ; $\alpha 5$:

107.3 ± 11.89 and δ : 130.8 ± 15.28 ; $n = 4$, $P < 0.05$ for all three subunit mRNAs).

In addition to the enhanced expression in the granule cell layer, $\alpha 5$ mRNA levels were markedly increased throughout all subfields of the hippocampus in non-sclerotic specimens (Figs 1H and 2B, Table 2), including sectors CA1 to CA3 and the subiculum. In specimens with Ammon's horn sclerosis, markedly increased expression of the $\alpha 5$ subunit in the granule cell layer and in the subiculum was observed. The increase in $\alpha 5$ subunit was weaker in sector CA3 and the pre- and parasubiculum in sclerotic than in non-sclerotic specimens and, due to extensive neurodegeneration, absent in sector CA1 (Fig. 1I). Faint labelling, presumably of interneurons was observed in the hilus of the dentate gyrus of controls (Fig. 1G). This labelling was significantly enhanced in the non-sclerotic samples, and virtually absent in the sclerotic specimens, presumably reflecting the degeneration of hilar interneurons (Fig. 1H and I).

We also investigated β -actin mRNA as a housekeeping gene. β -actin mRNA was expressed throughout the pyramidal layers CA1 to CA3 of the hippocampus including the subiculum and parasubiculum and was somewhat less prominent in the granule cell layer and the hilus of the dentate gyrus (Supplementary Fig. 1). This distribution was consistent with that observed by Ohnuma et al.⁴¹ in brains of Schizophrenia patients. In patients with hippocampal sclerosis less mRNA expression was observed throughout the hippocampus and, due to neurodegeneration, was virtually absent in sector CA1. Expression of β -actin mRNA was, however, unchanged in non-sclerotic specimens compared with controls (Supplementary Fig. 1).

Analysis of correlation of *in situ* hybridization data with age and post-mortem time of control specimens

The mean age of the controls at death (48.6 ± 2.17 ; $n = 30$) was significantly ($P < 0.0001$) higher than the mean age of the patients at surgery (34.8 ± 1.61 ; $n = 41$). To investigate a possible bias by the higher age and also of potential degradation of mRNAs during prolonged PMT of our control specimens, we investigated for possible correlations between the patients' age at death and measured ROD values, and of the mean time between death and dissection with RODs. These data are shown in Fig. 3. Whereas there was no correlation between PMT and ROD values obtained in *in situ* hybridization autoradiographs (Fig. 3D and E; Pearson's correlation $r = 0.03$ to 0.21 for the different subunit mRNAs), we observed a statistically non-significant tendency (P -values 0.31 to 0.67) for correlations between the detected mRNA levels of all three GABA_A receptor subunit mRNAs in the granule cell layer and the patients' age at

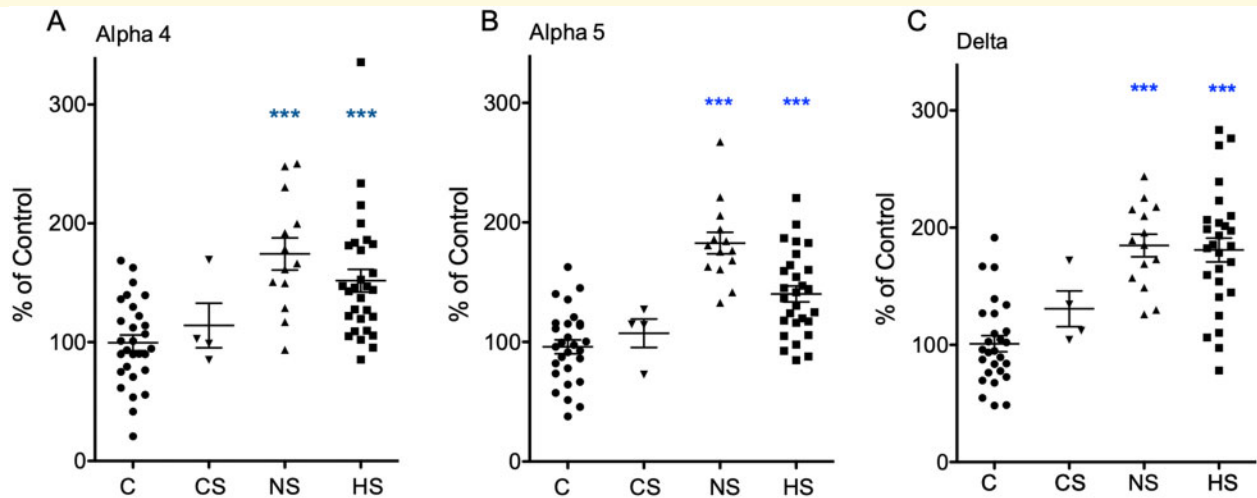


Figure 2 Change GABA_A receptor subunit mRNA levels in TLE patients. Shown is a quantitative assessment of *in situ* hybridization data for $\alpha 4$ (A), $\alpha 5$ (B) and δ (C) mRNAs in the granule cell layer of C, post-mortem controls; CS, post-mortem specimens of patients that died after *status epilepticus*; NS, non-sclerotic specimens; and HS, specimens with hippocampal sclerosis. The data show ROD values and are presented as % of mean ROD of controls. Statistical significance (one-way ANOVA, with Dunnett's multiple comparison *post hoc* test): *** $P < 0.001$.

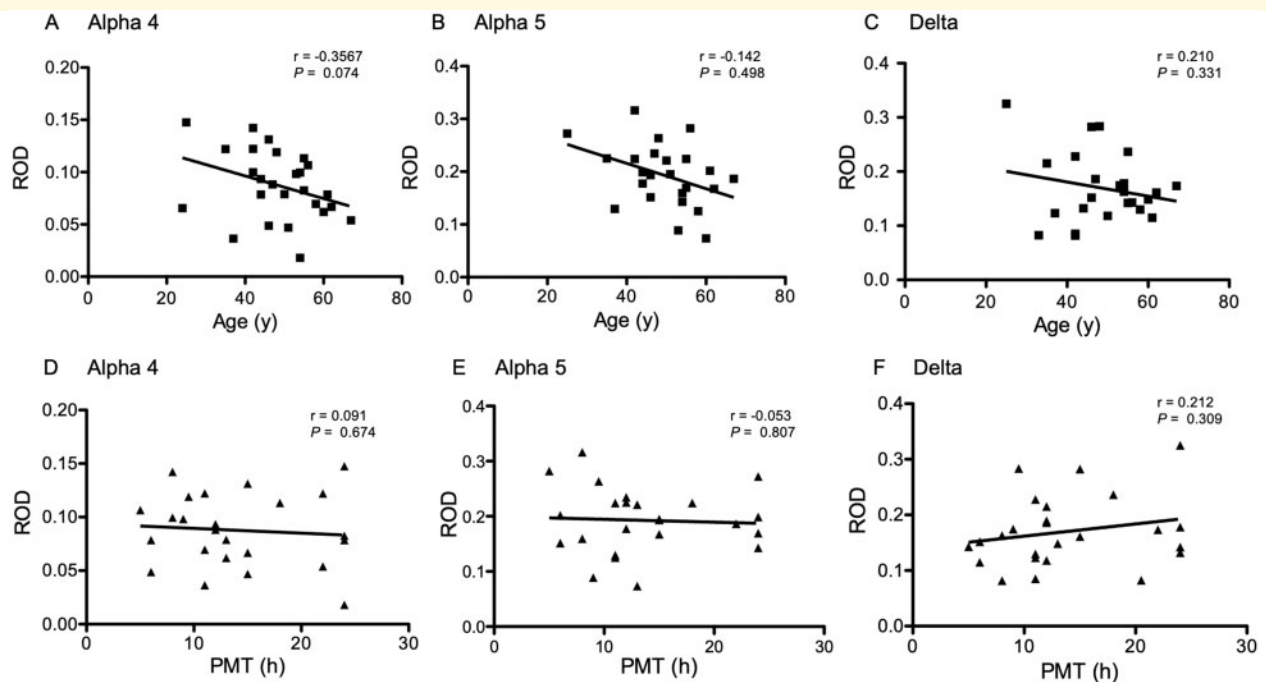


Figure 3 Correlation of age and post-mortem times with mRNA levels in autopsy controls. (A–C) Correlation of age, (D–F) correlation of PMT with mRNA levels in autopsy controls. Shown are ROD values obtained in receptor autoradiographs. Note that mRNA levels of all three subunits show a tendency for a correlation with age, which, however was statistically not significant (see Pearson's correlation coefficients r , and P -values). There was no correlation of mRNA levels with PMT.

death (Fig. 3A–C; Pearson's correlation $r = 0.14$ – 0.36). To further assess the possible dependence of the *in situ* hybridization signals and the age at death, we re-evaluated our analysis of mRNA levels in hippocampal samples after excluding autopsy samples obtained above an age of 49. The mean age of the resulting group of

controls was 39.13 ± 1.94 years ($n = 16$). Also when compared to this adjusted control group ($n = 13$ – 16), the mRNA levels of all three receptor subunits were still significantly higher in TLE specimens ($\alpha 4$: $P = 6.41E-04$, $df = 2, 52$; $\alpha 5$: $3.36E-07$, $df = 2, 52$; δ : $1.32E-04$, $df = 2, 50$); sclerotic specimens (mean % of control \pm

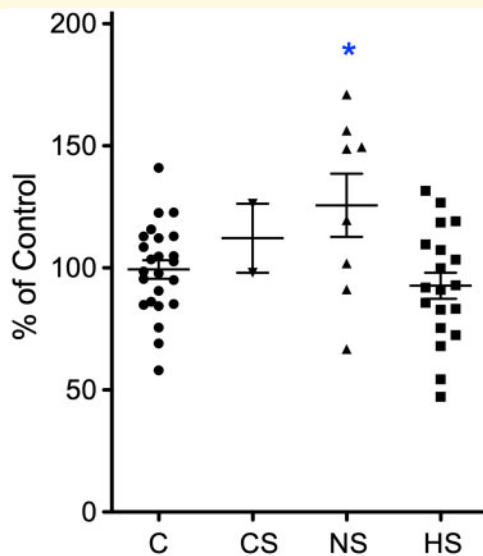


Figure 4 Change in $\alpha 5$ -specific receptor binding TLE patients. Shown is a quantitative assessment of receptor autoradiographs of the granule cell layer using the $\alpha 5$ -specific ligand [^3H]L-655,708. C, post-mortem controls; CS, post-mortem specimens of patients that died after SE; NS, non-sclerotic specimens; and HS, specimens with hippocampal sclerosis. The data points represent ROD values and are presented as % of mean ROD of controls. Statistical significance (one-way ANOVA, with Dunnett's multiple comparison *post hoc* test): * $P < 0.05$.

SEM, $n = 27\text{--}29$): $\alpha 4$: 145.1 ± 9.13 , $P < 0.05$ (Dunnett's *post hoc* test), $\alpha 5$: 136.6 ± 8.59 , $P < 0.01$ and δ : $156.9 \pm 12.19\%$, $P < 0.05$, as well as in non-sclerotic specimens (mean % of control \pm SEM, $n = 13\text{--}14$): $\alpha 4$: 166.7 ± 12.94 , $P < 0.01$ (Dunnett's *post hoc* test), $\alpha 5$: 179.5 ± 8.38 , $P < 0.001$ and δ : 163.8 ± 9.01 , $P < 0.01$. This analysis indicates that neither PMT nor the age of controls significantly influenced the outcome of subunit mRNA levels.

Subunit $\alpha 5$ -specific receptor autoradiography

To characterize expression of the $\alpha 5$ -subunit containing receptors, we performed receptor autoradiographs for the $\alpha 5$ -specific inverse agonist GABA_A agonist [^3H]L-655,708 (Figs 1J and K and 4). In post-mortem controls, specific labelling was observed in the molecular layer of dentate gyrus, throughout the pyramidal layer and in the subicular complex (Fig. 1J). This distribution correlated well with the distribution observed by *in situ* hybridization and by immunohistochemistry. Using one-way ANOVA for evaluating ROD values in the molecular layer, a statistically significant difference between [^3H]L-655,708 binding in controls, and sclerotic and non-sclerotic TLE specimens was seen [$F(2,48) = 5.5176$, $P = 0.0069$]. Using Dunnett's *post hoc* analysis, there were statistically

significant increases in the binding in the molecular layer of non-sclerotic specimens (mean $125.6 \pm 12.39\%$ of control, $P < 0.05$) but (presumably due to loss of granule cells) not in the sclerotic TLE samples (mean $92.7 \pm 5.35\%$ of control) (Figs 1K and 4).

Immunohistochemistry

In post-mortem controls, $\alpha 4$ -IR was faint or not detectable throughout the hippocampus (Fig. 5A). In the sclerotic TLE specimen, we detected $\alpha 4$ -IR in the molecular layer of the dentate gyrus, in sector CA3, in the subiculum and parasubiculum (Fig. 5B). Consistent with our observation in the brains of macaques,³⁶ $\alpha 5$ -IR seemed to be considerably more abundant than that for $\alpha 4$ and δ . It was strong in the dentate molecular layer, sector CA3 and weaker in sector CA1 and in the subiculum (Fig. 5C). In the hippocampus from TLE patients with sclerosis, we observed rather strong $\alpha 5$ -IR in the dentate molecular layer, in sector CA3, in the subiculum and in the pre- and parasubiculum (Fig. 5D). In contrast, subunit δ -IR was only detectable in the molecular layer of post-mortem controls. In the tissues from TLE patients (with hippocampal sclerosis), δ -IR was enhanced in the subicular complex (subiculum and presubiculum), in addition to faint labelling in sectors CA3 and CA2 (Fig. 5F). Using the ImageJ program, we observed statistically significant increase in ROD values in the dentate molecular layer of TLE patients with Ammons' horn sclerosis compared to post-mortem controls for all three subunit IR [as % of controls \pm SEM (n): $\alpha 4$, control 100.0 ± 18.28 (8), TLE 189.1 ± 14.82 (8), $P = 0.0023$, $df = 14$; $\alpha 5$, control 99.9 ± 7.40 (18), TLE 135.6 ± 4.81 (24), $P = 0.00034$, $df = 40$] and δ control 100.0 ± 8.51 (13), 148.1 ± 5.35 (16), $P < 0.0001$, $df = 27$. Data followed a Gaussian distribution in all groups except for controls of δ subunit.

Discussion

This study revealed three major results:

1. The expression of GABA_A receptor subunit $\alpha 5$ is highly abundant in the granule cell and molecular layers of the dentate gyrus. Whereas in the rat it is mainly restricted to the hippocampal sectors CA1 and CA3, the subiculum and entorhinal cortex,^{18,35} we observed in humans also high concentrations of the subunit in granule cell/molecular layer of the dentate gyrus. Since the $\alpha 5$ subunit mediates tonic inhibition, this may have important functional consequences on the dentate gyrus that is gating excitatory input from the entorhinal cortex.

2. In contrast to a profound downregulation of subunits $\alpha 5$ and δ in most hippocampal and para-hippocampal areas in rodent models of TLE, we observed profound upregulation of both subunits in the dentate granule cell layer and for $\alpha 5$ also throughout the

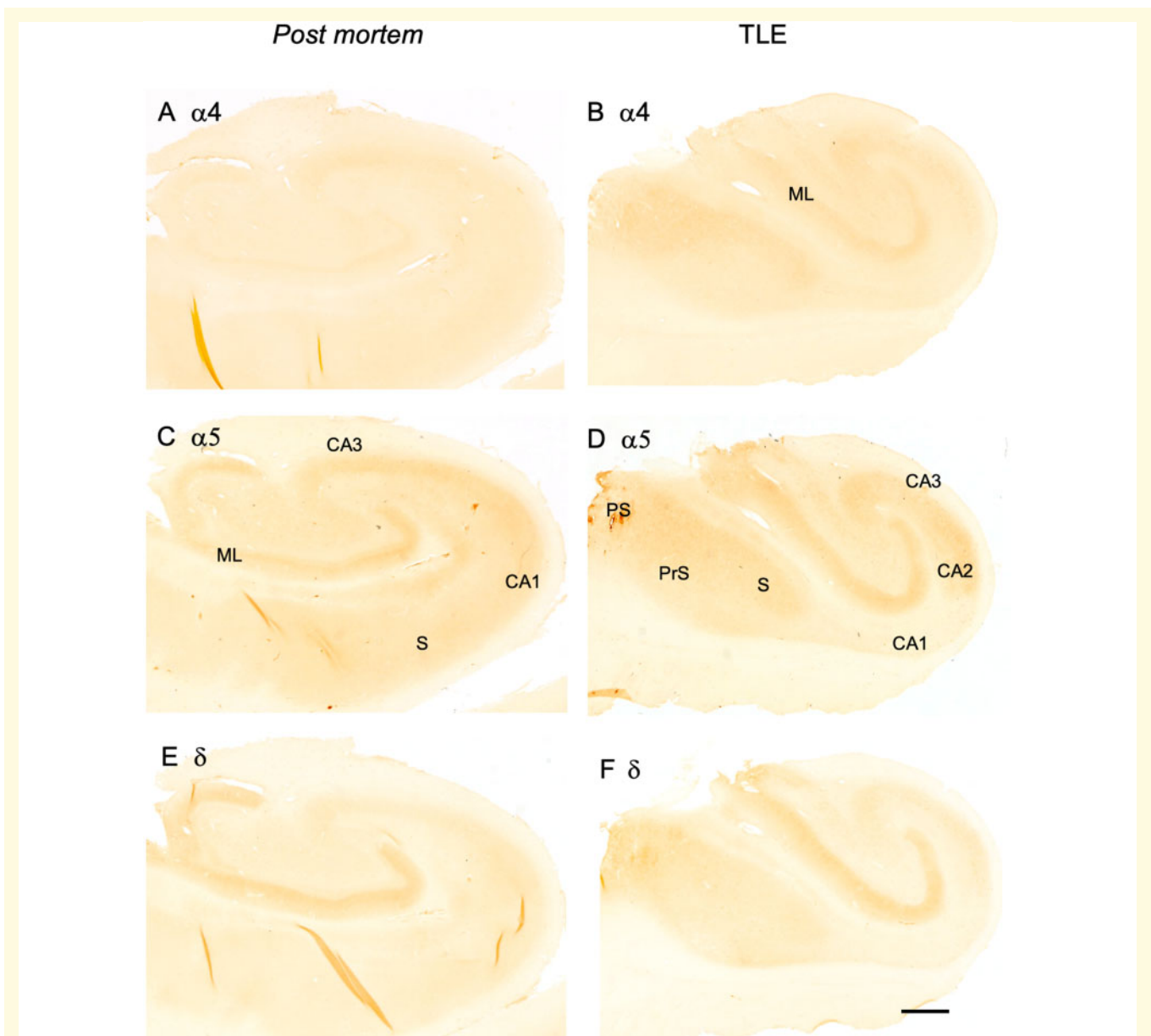


Figure 5 Change in GABA_A receptor subunit immunoreactivities in TLE patients without Ammon's horn sclerosis. Immunoreactivities for subunits $\alpha 4$ (A, B), $\alpha 5$ (C, D) and δ (E, F) are shown for post-mortem controls (A, C, E) and patients without Ammon's horn sclerosis (B, D, F). RODs of immunoreactivities were quantified in the molecular layer of the dentate gyrus of sclerotic specimens using the NIH ImageJ program. Significant increases were observed for all three subunit IR, as % of controls \pm SEM (n): $\alpha 4$, control 100.0 ± 18.28 (8), TLE 189.1 ± 14.82 (8), $P = 0.0023$, $df = 14$; $\alpha 5$, control 99.9 ± 7.40 (18), TLE 135.6 ± 4.81 (24), $P = 0.00034$, $df = 40$ and δ control 100.0 ± 8.51 (13), 148.1 ± 5.35 (16), $P < 0.0001$, $df = 21$. Data followed a Gaussian distribution in all groups except for controls of δ subunit. Scale bar in F for all images: 250 μ m.

hippocampal formation of TLE patients. Consistent with the data from rodent epilepsy models, expression of the $\alpha 4$ -subunit was also increased in the dentate gyrus of TLE patients.

3. In post-mortem specimens from patients who died after a SE, we observed no changes in the expression of $\alpha 4$ and $\alpha 5$ subunits, in spite of a modest (statistically not significant) increase in expression of δ (Fig. 2). Although the sample of these patients was small, the observation

indicates that upregulation of GABA_A receptor subunits may require more sustained and recurrent stimulation by epileptic seizures.

Our data are based on *in situ* hybridization, immunohistochemistry and receptor autoradiography (for subunit $\alpha 5$), thus characterizing the expression of the receptor subunits on mRNA and protein level. The regional distribution of the subunits and changes in their expression are consistent for all three methods. *In situ* hybridization with

radiolabeled oligonucleotide probes allows quantitative assessment of the signal. A draw-back of our histochemical studies may, however, be instability of mRNAs and proteins especially in the autopsy specimens often originating from older patients than the TLE specimens and by possible degradation of mRNA during the time between death and dissection of the brains. We therefore tested our *in situ* hybridization data for these variables. Although we observed statistically not-significant, age-dependent decreases in mRNAs levels of all three subunits, we detected no statistically significant (negative) correlation between PMT and *in situ* hybridization signals. Furthermore, we re-examined our data set after excluding post-mortem controls with age of 50 years and higher. This evaluation confirmed our results revealing also significantly increased expression of all three mRNAs in TLE patients when compared to controls of equal age.

Our data, however, could be also confounded by variable cell losses in TLE patients. Thus, in sclerotic TLE specimens about 46% were lost, whereas cell loss was minimal in non-sclerotic specimens (about 16%). We decided not to correct our mRNA data for these cell losses, since they would (perhaps artificially) further exaggerate the observed increases in subunit expression. On the other hand, the failure of a significant increase in [³H]L-655,708 binding in the dentate molecular layer of sclerotic specimens could be due to the loss in granule cells in these sections.

Furthermore, molecular parameters associated with neurotransmission are often increased in their expression and may lead to the apprehension that recurrent seizures cause general unspecific overexpression of these components. Thus, opposite to respective animal models, in human TLE most GABA_A and GABA_B receptor subunits become upregulated. On the other hand, expression of subunit $\alpha 1$ is decreased both in animal models and in human TLE.²⁹ Also expression of some neuropeptides is not uniformly increased. For example, neuropeptide Y expression is not significantly altered in granule cells/mossy fibres of TLE patients although it is prominently upregulated in respective TLE animal models.⁴² Furthermore, immunoreactivities of the neuropeptides secretoneurin and chromogranin A are not significantly altered while levels of chromogranin B and dynorphin are drastically increased in granule cells/mossy fibres of TLE patients.^{31,43} Therefore, in spite of the fact that epilepsy may put a high unspecific drive upon the expression GABA_A receptor subunits, this expression is to some extent also diverse. And, concomitant overexpression of different GABA_A receptor subunits may also be purposive for establishing formation of functioning receptors. Furthermore, expression of β -actin mRNA was not increased in TLE specimens compared to controls.

The histochemical data on the increased expression of the $\alpha 5$ -subunit in TLE patients is in striking accordance with a recent elegant PET study by.⁴⁴ The authors investigated the uptake of [¹¹C]Ro15-4513, a partial inverse

benzodiazepine agonist for $\alpha 5$ subunit-containing GABA_A receptors in MRI-negative (non-lesional) TLE patients and non-epilepsy controls. [¹¹C]Ro15-4513 binds to $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ - and to $\alpha 5$ -containing receptors. Using voxel-by-voxel bandpass spectral analyses, they were able to separate slow and high frequency signals (V_S and V_F , respectively). These signals differentiate between $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ -subunit-containing receptors (V_F) and $\alpha 5$ -containing receptors (V_S).⁴⁵ Strikingly, the $\alpha 5$ -selective component of the signal was increased in the hippocampus, temporal lobe, anterior cingulate gyri and the piriform cortex (*area tempestas*) of TLE patients, whereas $V_F:V_S$ ratios corresponding to $\alpha 1$ -, $\alpha 2$ - and $\alpha 3$ -containing receptors were reduced compared to those in healthy controls, suggesting a possible downregulation of receptors containing the respective α -subunits. The later finding corresponds to the previously observed loss in [¹¹C]flumazenil binding (presumably preferring $\alpha 1$, $\alpha 2$, $\alpha 3$ opposed to $\alpha 5$ containing receptors) close to the seizure focus which correlated with increased ictal activity.^{46,47} Taken together, these data indicate a loss in subunits $\alpha 1$, $\alpha 2$ or $\alpha 3$ in the seizure focus, whereas receptors containing $\alpha 5$ are upregulated. McGinnity et al.⁴⁴ therefore interpreted their data as a shift from $\alpha 1$ -, $\alpha 2$ - or $\alpha 3$ - to $\alpha 5$ -containing receptors.

Besides overexpression of $\alpha 5$ -subunits in the hippocampus of TLE patients, there is also previous evidence for similar changes in the expression of subunits $\alpha 4$ and δ in brains of epilepsy patients. Thus, Joshi et al.⁴⁸ recently investigated subunit $\alpha 4$ expression in cortical tissue obtained at surgery from children with focal cortical dysplasia II, tuberous sclerosis or ganglioglioma associated epilepsies and performed voltage-clamp measurements in xenopus oocytes injected with membranes obtained from these cortical samples. Using Western blotting, the authors observed increased expression of the $\alpha 4$ -subunit (interestingly colocalizing with $\gamma 2$) compared to post-mortem controls. Potentiation of GABA-evoked currents by the neurosteroid allopregnanolol was, however, reduced. On the other hand, they found significant potentiation of GABA currents by the imidazobenzodiazepine Ro15-4513, which acts on $\alpha 4\beta\delta$ containing receptors in membranes obtained from patients but not of controls, again supporting the expression of $\alpha 4/\delta$ -containing receptors in the cortex of epilepsy patients. These findings are in perfect agreement with earlier findings by Scimemi et al.⁴⁹ reporting a tonic current that is modulated by neurosteroids in granule cells of TLE patients and thus likely mediated by $\alpha 4/\delta$ containing receptors.

It is interesting to note that, in contrast to rodents, in the epileptic human brain, also $\alpha 5$ and δ are upregulated besides $\alpha 4$. Increased expression of subunits $\alpha 4$, $\alpha 5$ and δ mediating tonic inhibition goes in hand with upregulation of β -subunits, $\alpha 2$ and $\gamma 2$, but not $\alpha 1$ or $\alpha 3$ in the granule cell/molecular layer of the dentate gyrus.^{29,32} This opens the possibility, that in addition to an almost general overexpression of receptor subunits, also partial reorganization of GABA_A receptors to functioning receptors may

take place in granule cells, constituting of receptors containing $\alpha 4$ and/or $\alpha 5$, one of the major β -subunits $\beta 2$ or $\beta 3$ and δ or $\gamma 2$. All these possible combinations can facilitate tonic inhibition. In spite of the fact that some interneurons are lost in TLE, both animal and human studies show compensatory increases in GABA-ergic transmission in surviving interneurons.^{50–54} GABA even released from distant sites then can act upon high affinity GABA_A receptors responding already at low GABA concentrations with tonic inhibition.⁵⁵ On the other hand, GABA_A receptors mediating phasic inhibition (containing $\alpha 1$, $\alpha 2$, βx and $\gamma 2$) by GABA released within the synapse may in part become deprived from their presynaptic innervation due to loss of interneurons.

It is worth to emphasize that the PET study by McGinnity et al.⁴⁴ and our histochemical data demonstrate an abundant expression of $\alpha 5$ -containing GABA_A receptors in the human hippocampus including the dentate gyrus. In rodents, $\alpha 5$ -containing GABA_A receptors, although abundant on dendrites of pyramidal neurons of hippocampal sector CA3, the subiculum and the entorhinal cortex, are expressed only at low concentrations in the molecular layer of the dentate gyrus.^{18,36,37} In rodents stimulation of $\alpha 5$ -containing receptors in sector CA1 impairs the performance in hippocampus-dependent learning and memory tests^{56,57} and the reverse $\alpha 5$ -specific GABA_A agonist PWZ-029 improves the performance of rats in the object recognition test.⁵⁸ In their PET study, McGinnity et al.⁴⁴ showed a negative correlation between $\alpha 5$ -selective binding and the performance in a cognitive test battery also in human controls and MRI negative TLE patients.⁴⁴ Interestingly, the $\alpha 5$ -selective PET signal V_S was, however, *negatively* correlated with interictal intervals reflecting an association of increased [¹¹C]Ro15-4513 uptake with decreased seizure activity and points to a possible anticonvulsive action mediated by $\alpha 5$ -containing receptors. Respective receptors located on granule cell dendrites could importantly contribute to such a mechanism. GABA_A receptors overexpressing the $\alpha 5$ -subunit on granule cell dendrites may substantially contribute to such an anticonvulsive action. $\alpha 5$ -receptor agonists may therefore specifically augment tonic inhibition by GABA on granule cell receptors. A clinical use of such compounds, however, may be obstructed by possible negative effects on cognition mediated by $\alpha 5$ -containing receptors in CA1 and CA3.

Conclusion

In conclusion, our present study demonstrates that $\alpha 5$ -containing GABA_A receptors, in contrast to the rat, are highly expressed also in the molecular layer of the dentate gyrus and that their expression is significantly upregulated throughout the hippocampus in (lesional and non-lesional) TLE patients. We also show increased expression of $\alpha 4$ and δ in the dentate gyrus, which coincides with increased

expression of all three β -subunits,³² ultimately allowing the assembly of $\alpha 5\beta\delta$ or $\alpha 4\beta\delta$ receptors, or of receptors containing $\gamma 2$ instead of δ . Extra-synaptic GABA_A receptors (containing $\alpha 4$, $\alpha 5$ and/or δ) exert high affinity to their transmitter and therefore can respond well to volume transmission.^{4,6,8,9} They may sense even low concentrations of GABA spilled-over during seizures, from even distant sites and support tonic inhibition at the molecular layer of the dentate gyrus and in other parts of the hippocampal formation. Increased expression of GABA_A subunits mediating tonic inhibition, thus, may be part of an endogenous anticonvulsive mechanism contributing to inhibition or cessation of acute seizures.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interest

The authors report no competing interests.

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