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Selective changes in $GABA_A$ receptor subtypes in white matter neurons of patients with focal epilepsy

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Mapping the distribution of $GABA_A$ receptor subtypes represents a promising approach to characterize alterations in cortical circuitry associated with neurological disorders. We previously reported subtype-selective changes in $GABA_A$ receptor expression in the grey matter of patients with focal epilepsy. In the present follow-up study, we focused on the subcortical white matter in the same tissue specimens obtained at surgery from 9 patients with temporal lobe epilepsy (TLE) and hippocampal sclerosis, 12 patients with TLE associated with neocortical lesions and 5 patients with frontal lobe epilepsy; post-mortem tissue from 4 subjects served as controls. The subunit composition and distribution of three major $GABA_A$ receptor subtypes were determined immunohistochemically with subunit-specific antibodies. In all cases, a majority of neurons in the white matter was distinctly labelled, allowing detailed visualization of their dendritic arborization and revealing a differential, cell type-specific expression pattern of α-subunit variants. In controls, α1-subunit staining was most prominent, displaying a gradient that decreased with depth, in parallel with the density of NeuN-positive cells. Subsets of pyramidal cells were α3-subunit-positive, and α2-subunit-labelled neurons were rare. In 19 of the 26 patients with focal epilepsy, no changes were detected as compared with controls. In five patients with TLE, striking changes in the dendritic arborization of a subset of white matter neurons were seen with the α1-subunit antibody. In two further patients with TLE, we observed a disorganized dendritic network immuno-positive for the α1-subunit, cell clusters selectively expressing the α2-subunit and small neuronal aggregates that expressed all subunits and appeared to connect to neighbouring white matter neurons. All seven patients with anomalies in the white matter had a selective reduction in α3-containing $GABA_A$ receptors in the superficial layers of the grey matter. These results demonstrate a distinct organization of $GABA_A$ receptors in human white matter neurons, consistent with an inhibitory network that is likely to be integrated functionally with the overlying grey matter. The altered dendritic morphology and changes in $GABA_A$ receptor expression in the white matter of a subset of patients with focal epilepsy are suggestive for a rewiring of neuronal circuits.

Keywords: cerebral cortex; epilepsy; GABA; human; white matter

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Abbreviations: FLE = frontal lobe epilepsy; GAD = glutamic acid decarboxylase; GAT-1 = GABA membrane transporter-1; HS = hippocampal sclerosis; MAP2 = microtubule-associated protein 2; mMCD = mild malformation of cortical development; NeuN = neuron-specific nuclear protein; PBS = phosphate-buffered saline; TLE = temporal lobe epilepsy

Introduction

The white matter subjacent to the cerebral cortex in adult human brain contains considerable numbers of neurons situated among the fibre tracts (Ramón y Cajal, 1900; Kostovic and Rakic, 1980; Meyer *et al*., 1992). At present, little is known about the role of these cells both in normal brain function and in neurological disease. White matter neurons, also referred to as interstitial neurons, consist of a heterogeneous population of pyramidal cells and inhibitory interneurons (Kostovic and Rakic, 1980; Schiffmann *et al*., 1988; Meyer *et al*., 1992; Smiley *et al*., 1998). The white matter neurons subjacent to the mammalian cortex are derived from the embryonic subplate, a transient structure critical for cortical development (Allendoerfer and Shatz, 1994; Bystron *et al*., 2008). In the late pre-natal and early post-natal period, many of these subplate neurons undergo programmed cell death, leaving the surviving neurons distributed throughout the subcortical white matter (Chun and Shatz, 1989; Kostovic and Rakic, 1990). Recently, Torres-Reveron and Friedlander (2007) have shown in the mature rodent that these neurons are functionally connected with the neocortical network. Similarly, in the human white matter, a possible functional role for these neurons may be assumed based on their axonal projections (Mrzljak *et al*., 1988) and their synaptic inputs (Kostovic and Rakic, 1980).

The pathological significance of changes in white matter neurons for human epilepsy remains controversial (Eriksson *et al*., 2005*b*; Kasper, 2005). An increase in the density of these neurons was reported in patients with primary generalized epilepsy (Meencke, 1983; Meencke and Janz, 1984), but this finding could not be confirmed (Opeskin *et al*., 2000). Excessive numbers of white matter neurons have been reported in patients with temporal lobe epilepsy (TLE) although there was overlap between the epilepsy and the control groups (Hardiman *et al*., 1988; Emery *et al*., 1997; Kasper *et al*., 1999). In patients with TLE associated with hippocampal sclerosis (HS), Kasper *et al*. (2003) reported increased white matter neuronal density in 5 of 24 cases. Using design-based stereology in surgical specimens, an increase in the density of white matter neurons in patients with TLE was observed in one study (Thom *et al*., 2001), whereas no significant difference in number or density was found in another study (Bothwell *et al*., 2001). Finally, Thom *et al*. (2005), in a post-mortem stereological investigation of patients with poorly controlled seizures, did not report a significant increase in the density of white matter neurons in patients with verified HS. Interestingly, a combined histopathological and PET study in patients with TLE showed a correlation between an increased number of white matter neurons and increased white matter binding of ^{11}C-flumazenil, an antagonist at the benzodiazepine/$GABA_A$ receptor complex (Hammers *et al*., 2001, 2002). Previous investigations have suggested that $GABA_A$ receptor signalling, which mediates both synaptic and tonic inhibition (Scimemi *et al*., 2006), is impaired in the neocortical grey matter in patients with TLE (Arion *et al*., 2006; Loup *et al*., 2006).

The aim of this study was to examine whether alterations in the expression of $GABA_A$ receptor subunits observed in the hippocampus and neocortex of patients with focal epilepsy (Loup *et al*., 2000, 2006) also extend to subcortical white matter neurons. Nineteen $GABA_A$ receptor subunits have been identified in the mammalian CNS (Simon *et al*., 2004), and these assemble into pentameric receptors to form distinct subtypes, most of which include at least one of each of the α, β and γ subunit class (Sieghart and Sperk, 2002; Fritschy and Brünig, 2003). Here, we utilized an immunohistochemical approach to characterize (i) the distribution and the density of neurons stained for the neuronal marker NeuN; and (ii) the neuronal expression of five major $GABA_A$ receptor subunits (α1, α2, α3, β2/3 and γ2) in the white matter of patients with medically refractory focal epilepsy and controls. The present investigation was based on the same tissue sections as the earlier study on grey matter, in which we demonstrated a marked reduction in α3-containing $GABA_A$ receptors exclusively in the superficial neocortical layers in a subset of patients with TLE (Loup *et al*., 2006).

Methods

Patient selection

Detailed information on patient selection, intra-operative electrocorticography and tissue collection has been provided previously (Loup *et al*., 2006). Briefly, brain tissue from 26 patients with medically intractable focal epilepsy was obtained in collaboration with the neurosurgical units of the University Hospitals of Zurich, Geneva and Strasbourg. All procedures were undertaken with the informed consent of patients or their legal next of kin and were approved by the ethics committees of the respective institutions in accordance with the Declaration of Helsinki. Based on histopathological findings, in conjunction with neuroimaging and electro-clinical data, we classified the patients into groups comprising those with frontal lobe epilepsy (FLE) ($n = 5$, mean age at surgery ± SD: 22.6 ± 12.3 years, range 5–34 years) and those with TLE. The latter group was further subdivided into those with HS ($n = 9$, mean age at surgery ± SD: 39.6 ± 14.4 years, range 12–56 years) and those with neocortical lesions (non-HS) ($n = 12$, mean age at surgery ± SD: 28.3 ± 12.2 years, range 9–49 years). Table 1 provides a summary of relevant clinical data for each patient. Patients with isolated focal cortical dysplasia, that is, with no other apparent structural changes, were excluded.

Brain tissue consisting of neocortical grey matter and the underlying white matter was from resections performed for strictly therapeutic reasons (Loup *et al*., 2006). The samples originated from the anterolateral temporal neocortex ($n = 21$), the frontopolar area, the frontal lateral region and the orbital part of the inferior gyrus of the

Table 1 Summary of clinical data and experimental results

Patient/sex/ age (years)	Location/ side	Age at onset	Duration of epilepsy	Seizure frequency at surgery	Seizure type	Medication at time of surgery	Histopathology	Post-surgical follow-up (months)	Engel outcome	α3-subunit changes in grey matter	α-subunit changes in white matter neurons
1/F/48	T, R/L	–	–	–	–	–	Control	–	–	No	No
2/M/60	T/F, R/L	–	–	–	–	–	Control	–	–	No	No
3/M/69	T, L	–	–	–	–	–	Control	–	–	No	No
4/M/73	T, L	–	–	–	–	–	Control	–	–	No	No
5/M/5	F, R	1	4	20–30/night	CPS	LTG PHT VGB	Mild subpial gliosis	71	IV	No	No
6/F/15	F, R	7	8	1–5/night	CPS SGS	BBC MSM CLB	Venous angiitis	92	IV	No	No
7/M/27	F, R	23	4	3/month	CPS SGS	LTG VPA	Astrocytoma	86	I	No	No
8/M/34	F, R	12	22	20/month	CPS SGS	LTG VPA OXC	Glial scar	85	III	No	No
9/M/32	F/T, L	3	29	4–10/month	CPS SGS	CBZ CZP	Glial scar	71	II	No	No
10/F/56	T, R	12	44	10/month	SPS CPS	OXC CZP LEV	HS	56	I	No	No
11/F/49	T, L	18	31	15/month	CPS SGS	CBZ VGB	HS	62	I	No	No
12/M/56	T, L	15	41	6/month	CPS SGS	LTG CZP PRM	HS	51	I	No	No
13/F/12	T, R	1	11	4/month	SPS CPS SGS	STM	HS	64	I	No/Yes[a]	No
14/F/35	T, R	8	27	7/month	CPS SGS	CBZ	HS, mMCD Type II	76	I	Yes	α1, α2, α3
15/M/38	T, R	1	37	8/month	SPS CPS SGS	CBZ LTG	HS, mMCD Type II	56	I	Yes	α1, α2, α3
16/M/25	T, R	15	10	5/month	CPS SGS	OXC LTG	HS	40	I	Yes	α1
17/F/40	T, R	5	35	2/month	CPS SGS	CBZ	HS	60	I	Yes	α1
18/M/45	T, L	5	40	4/month	CPS SGS	CBZ CLB	HS	56	I	Yes	No
19/M/16	T, R	9	7	4/month	SPS CPS SGS	OXC VPA	Ependymoma	84	I	No	No
20/M/15	T, R	6	9	3/month	CPS	CBZ	Oligodendroglioma	42	I	No	No
21/M/49	T, L	32	17	8/month	CPS SGS	CBZ	Cavernoma	39	II	No	No
22/F/31	T, L	29	2	5/month	CPS	CBZ VPA	Cavernoma	43	I	No	No
23/M/43	T, L	11	32	4/month	SPS CPS	CBZ PHT	DNT	96	I	No	No
24/F/9	T, R	8	1	4/month	CPS	CBZ	DNT	56	I	No	No
25/M/32	T, R	15	17	5/month	SPS CPS SGS	CBZ	Glial scar	64	I	No	No
26/M/27	T, R	18	9	5/month	CPS	CBZ	Oligodendroglioma	81	I	Yes	α1
27/M/22	T, R	15	7	8/month	CPS SGS	CBZ TGB	DNT, FCD Type IB	48	II	Yes	α1
28/M/40	T, L	24	16	4–12/month	CPS SGS	VPA TGB	Ganglioglioma, FCD Type IB	65	III	Yes	No
29/M/34	T, R	30	4	2/month	CPS	CBZ	Cavernoma	49	I	Yes	α1
30/F/20	T, L	18	2	4/month	CPS	CBZ	DNT	48	I	Not done	No

a In tissue from this patient, densitometric measurement of α3-subunit staining in the superficial layers showed no change in one sample and decreased staining in the other.
Classification after Palmini and colleagues for mMCD and FCD (Palmini *et al.*, 2004). Classification after Engel for outcome (Engel, 1987).
BBC = barbexaclone; CBZ = carbamazepine; CLB = clobazam; CPS = complex partial seizures; CZP = clonazepam; DNT = dysembryoplastic neuroepithelial tumour; F = frontal; FCD = focal cortical dysplasia; HS = hippocampal sclerosis; LEV = levetiracetam; LTG = lamotrigine; mMCD = mild malformation of cortical development; MSM = mesuximide; OXC = oxcarbazepine; PHT = phenytoin; PRM = primidone; SGS = secondary generalized seizures; SPS = simple partial seizures; T = temporal; TGB = tiagabine; VGB = vigabatrin; VPA = valproic acid.

frontal lobe. Control autopsy tissue (mean post-mortem interval ± SD: 11.2 ± 3.4 h, range 8–16 h) was from four subjects (mean age ± SD: 62.5 ± 11.1 years, range 48–73 years) with no known history of a neurological or psychiatric disorder. The left, and, in two cases, also the right hemispheres were cut into coronal slabs of 1–1.5 cm thickness. Tissue blocks of the anterolateral temporal neocortex were dissected that corresponded to the areas removed in those patients undergoing surgery for epilepsy.

Tissue preparation and immunohistochemistry

Tissue blocks were rinsed immediately after surgical resection or after dissection at autopsy in phosphate-buffered saline (PBS) at pH 7.4, and then immersion-fixed for 6–8 h at 4°C under constant agitation in a mixture of 4% freshly dissolved paraformaldehyde and 15% saturated picric acid in 0.15 M phosphate buffer at pH 7.4 (Somogyi and Takagi, 1982) or in 4% paraformaldehyde alone. The fixed tissue was then pre-treated using a modified antigen-retrieval method based on microwave irradiation (Loup *et al*., 1998). Tissue blocks were cryoprotected in 10, 20 and 30% sucrose in PBS over a period of 3–4 days, frozen at –28°C in isopentane and stored at –80°C. Series of 40 μm-thick sections were subsequently cut in a cryostat with the plane of section oriented perpendicularly to the pial surface or the main gyral axis and collected in ice-cold PBS. Each series of 40 μm-thick sections consisted of 18–20 sections. Of these, five consecutive sections were stained for the five $GABA_A$ receptor subunits and two additional adjacent sections were stained with antibodies against the neuron-specific nuclear protein NeuN (Wolf *et al*., 1996) and for Nissl with cresyl violet. Thus the distance between the first section of each series of sections was between 720 and 800 μm.

The following subunit-specific antibodies were used: mouse monoclonal antibodies bd-24 and bd-17 recognizing the human $GABA_A$ receptor α1-subunit and both the β2 and β3-subunits, respectively (Schoch *et al*., 1985; Ewert *et al*., 1990), and polyclonal guinea-pig antisera recognizing the α2, the α3 or the γ2-subunit. The specificity of these antibodies has been demonstrated previously (Fritschy and Möhler, 1995; Loup *et al*., 1998; Waldvogel *et al*., 1999). The dilutions of the subunit-specific antibodies were: α1-subunit (monoclonal antibody bd-24), 0.14 μg/ml; α2-subunit (affinity-purified), 1.3 μg/ml; α3-subunit (crude serum), 1:3000; β2/3-subunit (monoclonal antibody bd-17), 3.8 μg/ml; and γ2-subunit (crude serum), 1:1500. Further antibodies used were NeuN 1:1000 (MAB377; Chemicon, Temecula, CA, USA), GAD 1:2000 (glutamic acid decarboxylase GC 3008; BIOMOL, Exeter, UK) and GAT-1 1:3000 (GABA membrane transporter-1; DiaSorin, Stillwater, MN, USA). Series of free-floating sections were pre-incubated in 1.5% H_2O_2 in PBS for 10 min at room temperature to block endogenous peroxidase activity, washed three times for 10 min in PBS and processed for immunoperoxidase staining (Hsu *et al*., 1981) as described previously (Loup *et al*., 1998, 2000).

Digital images were obtained with a high-resolution camera (AxioCam; Zeiss, Jena, Germany) and Zeiss camera software (AxioVision version 4.5). Images were adjusted for brightness and contrast only (Adobe Photoshop version 8.0; Adobe Systems Incorporated, San José, CA, USA) and not manipulated in any other manner. Final figures were prepared with Adobe Illustrator (version 10.0; Adobe Systems Incorporated, San José, CA, USA).

White matter neuron counts

Neurons were counted throughout the white matter using NeuN-stained sections from controls (temporal lobe; $n = 4$), FLE ($n = 4$), HS ($n = 8$) and non-HS cases ($n = 10$). For cell counting, a 12.5 × 12.5 mm ocular grid consisting of 10 × 10 squares and corresponding to an area of 0.098 mm^2 (312.5 × 312.5 μm) at ×40 objective magnification was positioned parallel to the grey–white matter border and then moved vertically downward in stepwise fashion to the lowermost boundary of the white matter available. In cases where the grey–white matter border was not readily identifiable, adjacent sections stained for the α2-subunit were used that revealed the border between Layer VI and the underlying white matter as an abrupt change in staining intensity. Serial sections were also examined to ensure that the neurons observed in the white matter were not part of tangentially cut grey matter from adjacent cerebral gyri. Two counts per section were performed in two sections from each specimen. All nuclear profiles within the grid were counted, except for those crossing the top and right edges. The vertical extent of the white matter was greatest in autopsy control tissue, whereas it was variable and dependent on the type of pathology and operative procedure in the surgical cases. The results were expressed as mean number of neurons per 0.1 mm^2. In 4 of the 30 cases, the quality of the resected tissue precluded accurate counts.

White matter neuron counts were analysed for statistical significance using the Kruskal–Wallis test (non-parametric analysis of variance; GraphPad Prism, San Diego, CA, USA).

Results

Distribution and density of white matter neurons in controls and in patients with focal epilepsy

We first examined the white matter subjacent to temporal neocortex of control autopsy specimens, which exhibited large numbers of neurons that were identified with NeuN immunolabelling. As described previously using microtubule-associated protein 2 (MAP2) staining (Meyer *et al*., 1992; Anderson *et al*., 1996) or Nissl staining and the Golgi technique (Kostovic and Rakic, 1980), the density of labelled neurons was highest in the white matter immediately below the grey matter and then decreased with increasing distance from the grey–white matter border (Figs 1A–C, 2B, E and H). The gradient in neuronal density was confirmed by counting NeuN-immunoreactive neurons at fixed intervals, beginning at the grey–white matter border and continuing to deep within the white matter (Fig. 3A). At higher magnification, we observed the white matter neurons to be distributed with a predominantly radial orientation with respect to the axis of their apical dendrites (Fig. 2B, E and H). Furthermore, the NeuN-positive somata were generally largest the closer they were to the grey–white matter border, and became smaller and more irregularly shaped in the deep white matter (Fig. 2B, E and H).

Similar numbers of NeuN-immunoreactive neurons were visualized in the white matter from patients with focal epilepsy. Furthermore, the gradient in the density of neurons from

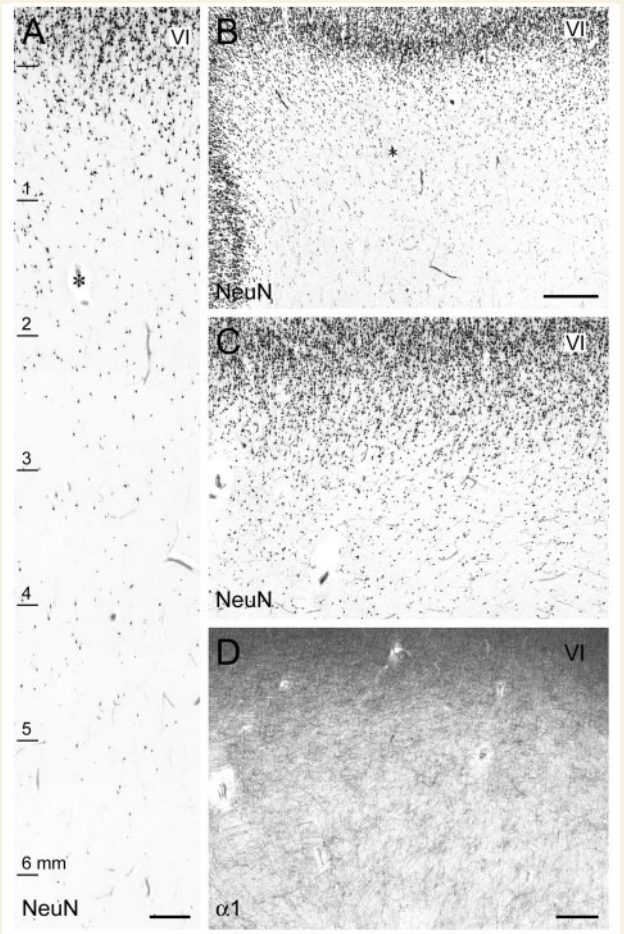

Figure 1 Distribution of neurons and GABA$_A$ receptor α1-subunit immunoreactivity in the white matter subjacent to temporal neocortex in two control subjects. (**A**–**C**) NeuN-stained sections. (**A** and **B**) The density of labelled neurons is highest in the white matter immediately below Layer VI and diminishes with increasing distance from the grey–white matter border. Note that (**A**) represents a segment of (**B**) at higher magnification, with the corresponding vessel marked with an asterisk. (**C** and **D**) Adjacent sections stained for NeuN (**C**) and the α1-subunit (**D**). GABA$_A$ receptor α1-subunit staining parallels the distribution of NeuN-positive white matter neurons, with greatest intensity in the white matter beneath Layer VI and diminishing with increasing depth from the grey–white matter border. All panels in all figures are oriented with the grey matter on top. Scale bar: (**A**) 300 μm; (**B**) 1 mm; (**C** and **D**) 500 μm.

superficial to deep white matter was conserved in the three different patient groups: those with FLE, those with HS and those with non-HS (Fig. 3B–D, respectively). Thus, at each level of depth, no significant difference was observed in the density of NeuN-positive neurons between the patient groups and the control group ($P > 0.05$) (Fig. 3). In two of the cases with histopathologically confirmed HS, we did, however, observe striking changes in the organization of white matter neurons (Figs 7A and 8F). Thus, NeuN-labelled neurons were irregularly distributed throughout the white matter and often formed clusters composed of very few cells surrounded by spaces devoid of neurons (Fig. 7A). The radial neuronal orientation was largely lost, and neurons with larger and smaller soma sizes were now intermingled (Fig. 7A). In addition, small neuronal aggregates were present with ill-defined borders (Fig. 8F). As there were no cyto-architectural abnormalities in the overlying grey matter in these two cases with HS, the changes observed in the white matter were classified as mild malformation of cortical development Type II (mMCD Type II) according to Palmini *et al*. (2004), corroborating the initial neuropathological diagnosis.

GABA$_A$ receptor subtypes in control white matter neurons

We then determined the distribution of the GABA$_A$ receptor subunits α1, α2, α3, β2/3 and γ2 in the white matter from sections adjacent to those stained for the neuronal marker NeuN (Figs 1, 2 and 4). For each subunit, we observed similar staining patterns in the temporal and frontal lobe white matter of all autopsy cases. As reported for the overlying grey matter of the same specimens (Loup *et al*., 2006), the five subunit antibodies revealed distinct distribution patterns and labelling intensities. Whereas the β2/3 and γ2-subunits are included in most GABA$_A$ receptor subtypes, the α-subunit variants represent largely distinct subtypes with a characteristic pharmacological profile and distribution pattern (Fritschy and Brünig, 2003). Likewise, in control white matter, we found a specific and unique distribution for each of the α-subunit variants, with the α1-subunit being most abundant (Figs 1D, 2A, D and G). At low power magnification, α1-subunit staining was highest in the superficial white matter and then decreased with increasing depth from the grey–white matter border (Fig. 1D). This pattern of distribution was even more obvious at higher magnification (Fig. 2A, D and G). Because of the extensive dendritic network formed by the α1-subunit-labelled neurons near the border with Layer VI, the individual cell bodies could not always be identified (Fig. 2A). With increasing depth, neurons became less numerous, so that the soma and dendrites of α1-subunit-positive neurons were distinctly outlined (Fig. 2D and G). The distribution pattern and the density of α1-subunit-labelled neurons appeared largely similar to those of the NeuN-stained neurons when comparing adjacent sections (Fig. 2).

The α1-subunit antiserum revealed different types of neurons, among which the most abundant were pyramidal-like cells with a long apical dendrite ascending towards the grey matter and with few basal dendrites (Figs 2D, 4A and B). As shown in Fig. 2D, the pyramidal cells generally displayed a regular arrangement with radially aligned apical dendrites. There were also neurons resembling pyramidal cells, according to their somatodendritic morphology, but which lacked a main apical dendrite (Fig. 2G). These neurons were often located in the deep white matter, millimetres below the grey–white matter border (Fig. 2G). An additional, prominent cell type was a small, intensely stained neuron with short, fine dendrites (Figs 2G and 4B). Based on their strong resemblance to the GAD-positive cells in adjacent

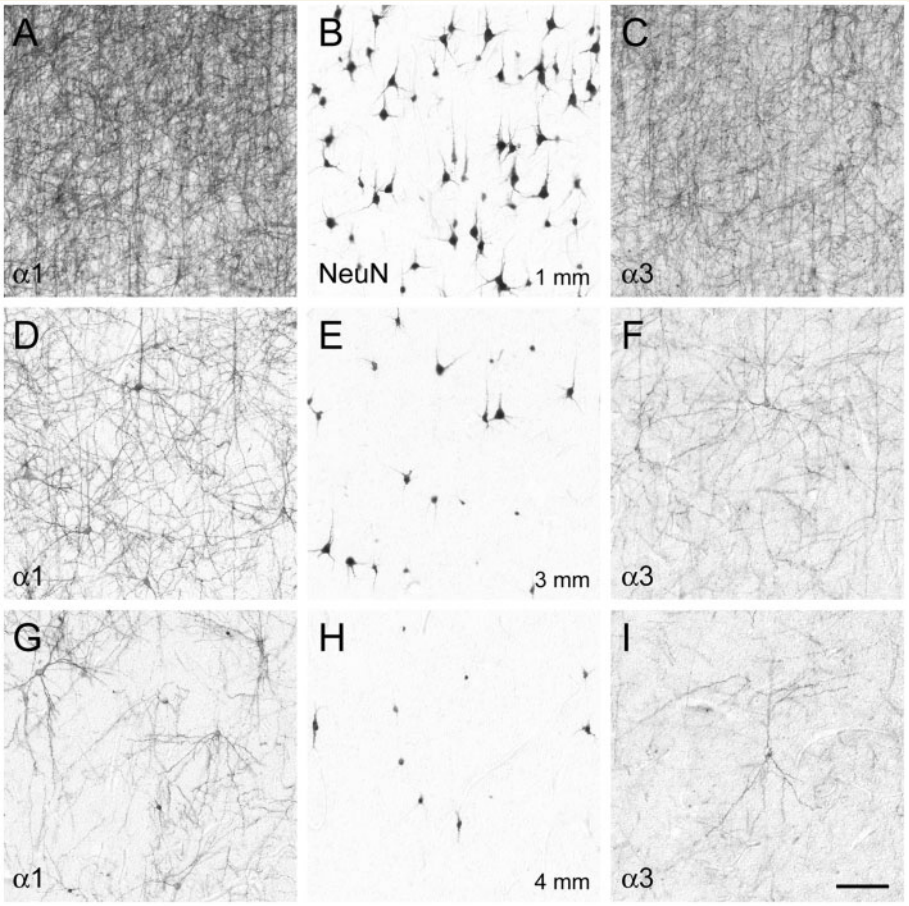

Figure 2 Radial distribution of neurons and differential expression of $GABA_A$ receptor subunit immunoreactivity in control white matter. (**A–I**) Adjacent sections stained for the α1-subunit (left column, **A**, **D** and **G**), NeuN (middle column, **B**, **E** and **H**) and the α3-subunit (right column, **C**, **F** and **I**) show that the density of labelled neurons and the intensity of staining are greatest in the white matter near the border with Layer VI and diminish with increasing depth. (**A–C**) At 1 mm from the grey–white matter border, numerous NeuN-immuno-positive neurons are present, many of which exhibit an apical dendrite directed towards Layer VI (**B**). The extensive dendritic network formed by the α1-subunit-positive neurons partially occludes many of the labelled somata (**A**). The staining pattern for the α3-subunit is similar to that of the α1-subunit, but staining is less intense (**C**). (**D–F**) At 3 mm from the grey–white matter border, neurons are less numerous (**E**). Immunoreactivity for the α1-subunit outlines the soma and dendrites, revealing a regular arrangement with radially aligned apical dendrites (**D**). In comparison, fewer neurons, but also showing a radial distribution, are stained for the α3-subunit (**F**). (**G–I**) At 4 mm from the grey–white matter border, fewer neurons are present (**H**). Neurons stained for the α1-subunit (**G**) are more numerous than neurons stained for the α3-subunit (**I**). Scale bar: 100 μm.

sections (Fig. 4C) and their size and dendritic arborization, these neurons were classified as putative GABAergic interneurons.

A distribution pattern similar to that for the α1-subunit was found for the α3-subunit, with more neurons labelled close to the border to Layer VI, and progressively fewer labelled neurons with increasing depth in the white matter (Fig. 2C, F and I). Staining for the α3-subunit was, however, less intense, reflecting the smaller number of neurons labelled. Furthermore, the α3-subunit antiserum labelled only neurons with pyramidal cell morphology (Figs 2C, F, I and 4D–F). In contrast, the α2-subunit antiserum revealed few, and faintly labelled, pyramidal cells that were mostly located close to the border to Layer VI (Fig. 4G–I). Typically, these neurons had either a fusiform cell body, with dendrites that penetrated Layer VI (Fig. 4G and H), or a horizontally oriented cell body with two dendritic trunks parallel to the grey–white matter border (Fig. 4I). At greater depth, the white matter was almost devoid of α2-subunit-positive neurons. Finally, the β2/3 and γ2-subunits, which are part of most $GABA_A$ receptor subtypes, exhibited a comparable labelling pattern to that of the α1-subunit (see Fig. 5A and B). A similar co-distribution of the α1, β2/3 and γ2-subunits was also observed in our previous study in the grey matter (Loup *et al*., 2006).

$GABA_A$ receptor subtypes in white matter neurons of patients with focal epilepsy

The white matter of patients with FLE, HS or non-HS was analysed for changes in $GABA_A$ receptor subunit immunoreactivity. In the

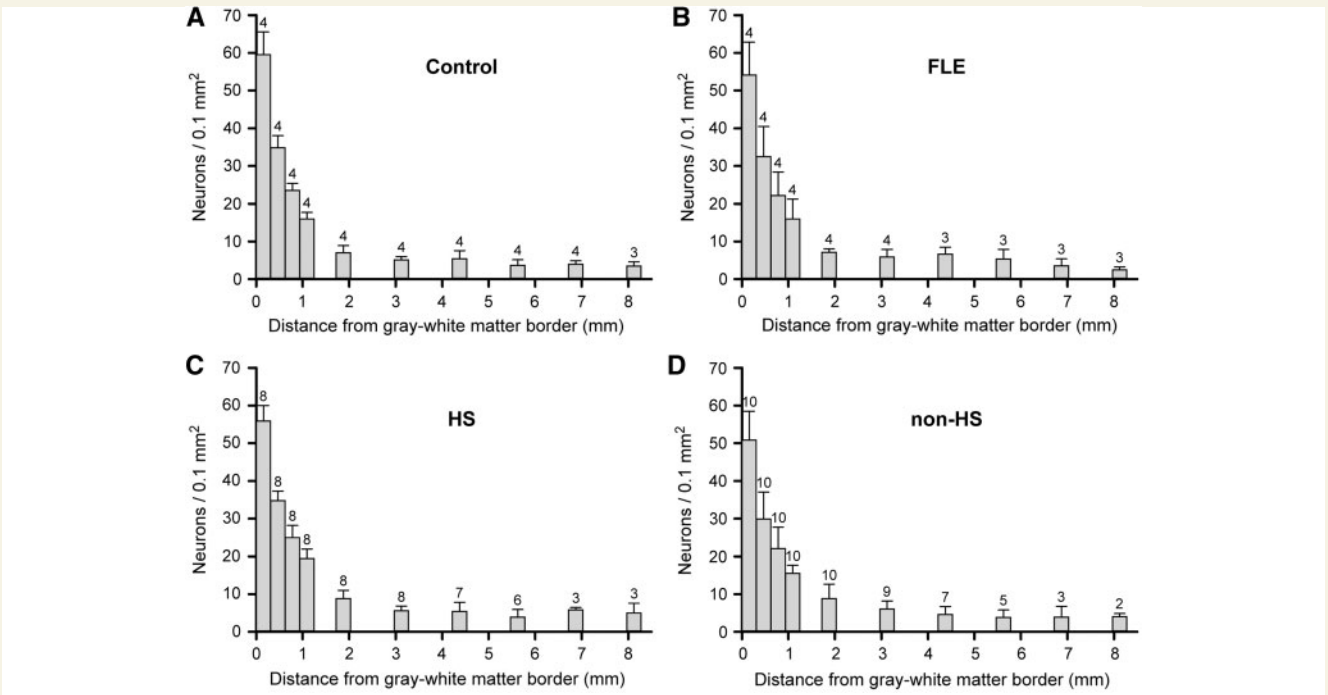

Figure 3 Neuronal cell counts (mean ± SD) in 4 controls (**A**), 4 FLE (**B**), 8 hippocampal sclerosis (**C**) and 10 non-HS (**D**) patients showing a decreasing gradient in neuronal density as a function of increasing depth in the white matter for all four groups. Sections stained for the specific neuronal marker NeuN were used. The position of the counting grid relative to the grey–white matter border is indicated on the *x*-axis. The value above each bar refers to the number of subjects from whom tissue samples still included white matter at the depth indicated.

patients with a circumscribed lesion in the neocortex (Table 1), the tissue samples used for study were from the periphery of the lesion. The results were classified into three main categories. In the first, which included 19 of 26 patients, the staining pattern for each of the five subunits appeared unchanged in white matter neurons when compared with controls. This was the case in the 5 patients with FLE, in 5 of the 9 patients of the HS group and in 9 of the 12 patients of the non-HS group (Table 1; Fig. 5). Thus, the gradient in staining intensity from superficial to deep white matter was maintained in parallel with the gradient in neuronal density (Fig. 3). Furthermore, the radial distribution of the pyramidal cells with their vertically oriented apical dendrites was also conserved (Fig. 5A–D). As in control tissue, staining patterns were largely similar for the α1, β2/3 (Fig. 5A and B) and γ2-subunits (not shown), and staining was more intense for the α1 than the α3-subunit (Fig. 5C and D). Moreover, at the cellular level, staining for the three α-subunit variants again revealed that the α1-subunit was expressed both in pyramidal-like cells and in putative GABAergic interneurons (Fig. 5E), the α3-subunit exclusively in pyramidal-like cells (Fig. 5F) and the α2-subunit in only few pyramidal cells that were mostly located close to Layer VI (Fig. 5G).

In the second category, comprising five patients, two with HS and three with non-HS, we observed selective changes in GABA$_A$ receptor α1-subunit staining in white matter neurons (Table 1 and Fig. 6) without apparent changes in their arrangement and density, as shown in adjacent sections stained for NeuN (Fig. 3). Strikingly, neurons expressing the α1-subunit, but not the α2 or α3-subunits, exhibited several long and fine, radiating or predominantly horizontal dendrites (Fig. 6A–C). Depending on their orientation in the horizontal plane, it was sometimes possible to observe processes in cross-section (Fig. 6A and C). These alterations affected a subset of α1-subunit-positive neurons, as unchanged pyramidal cells with a radially oriented apical dendrite were also seen (Fig. 6A and B). Thus, the predominantly horizontal dendritic trajectories of the morphologically altered neurons contrasted sharply with the radially arrayed apical dendrites of normal appearing pyramidal cells, manifesting as a marked disruption in the radial organization of α1-subunit-positive white matter neurons (compare Figs 2, 5 and 6).

The third category consisted of two patients with HS and mild mMCD Type II, in which there were pronounced changes in GABA$_A$ receptor subunit immunoreactivity affecting all subunits (Figs 7 and 8). In particular, the α1-subunit was prominently expressed in large numbers of irregularly distributed white matter neurons, many of which had lost their radial orientation (Fig. 7B and C). Comparison of adjacent sections labelled for NeuN and the α1-subunit showed that the distribution pattern and the density of α1-subunit-labelled neurons closely paralleled those of NeuN-stained neurons (Fig. 7A and B). As in controls, α1-subunit immunoreactivity intensely outlined the soma and dendrites of white matter neurons, but unlike controls, also revealed a dense and disorganized dendritic network (Figs 7B, C and 8A). Whereas staining for the α1-subunit was present in both

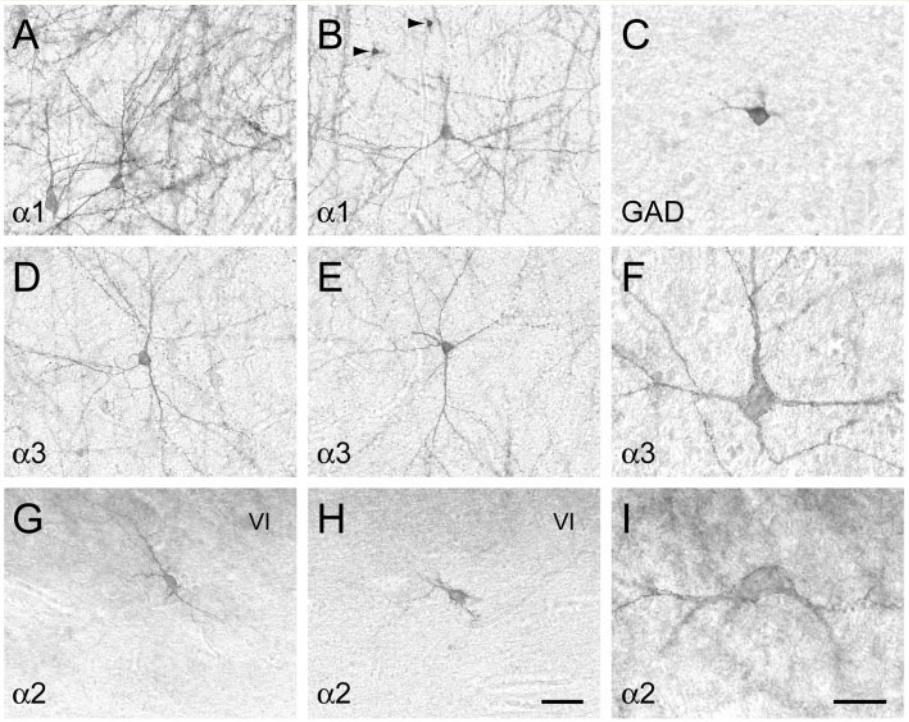

Figure 4 Differential cellular distribution of $GABA_A$ receptor subunit immunoreactivity in white matter neurons from control subjects. (**A** and **B**) The α1-subunit is expressed in pyramidal cells and in small and intensely stained putative GABAergic interneurons (arrowheads). (**C**) High power magnification of a GAD-stained interneuron. (**D–F**) The α3-subunit is present in pyramidal-like cells only. (**G–I**) The α2-subunit is expressed in very few neurons, near Layer VI, which either have dendrites penetrating Layer VI (**G** and **H**) or horizontally oriented dendritic branches parallel to the grey–white matter border (**I**). Scale bar: (**A**, **B**, **D**, **E**, **G** and **H**) 50 μm; (**C**, **F** and **I**) 25 μm.

pyramidal-like cells and putative GABAergic interneurons (Fig. 7C), staining for the α3-subunit was present exclusively in pyramidal-like cells (Fig. 7D), as noted in control tissue. In addition, most of the small neuronal clusters identified with NeuN stained intensely for the α1-subunit (Fig. 7B and C), only faintly for the α3-subunit, and not at all for the α2-subunit (data not shown). Interestingly, other cell clusters contained neurons that selectively expressed the α2-subunit, with intense immunoreactivity that outlined the soma and numerous, long dendrites (Fig. 7E and F). These α2-subunit-positive cells tended to appear in linear arrays of two or three clusters (Fig. 7E). These findings stood in contrast to the staining pattern in control white matter, where virtually no neurons were α2-subunit-positive, except for a few located close to Layer VI (compare Figs 4G–I and 7E and F). Finally, within the white matter of these two patients we observed small aggregates of neurons displaying subunit-specific $GABA_A$ receptor immunoreactivity, which was strong for the α1-subunit (Fig. 8A and C) and the α2-subunit (Fig. 8B and G), and faint for the α3-subunit (Fig. 8D). A particularly striking observation was the abundant dendritic overlap between the neurons located in the aggregates and those in the neighbouring white matter, as revealed by α1-subunit staining (Fig. 8C). At higher magnification, these aggregates, with ill-defined borders, consisted of differentiated neurons that looked remarkably normal, except that they were oriented randomly, without any evidence for cortical lamination (Fig. 8F). The staining pattern of the aggregates was largely similar to that of the overlying grey matter, as shown for the α2-subunit (Fig. 8G; see also Loup *et al*., 2006). Furthermore, we detected strong GAT-1 immunoreactivity in numerous, randomly oriented so-called cartridges (Fig. 8E), which represent the axon terminals of GABAergic chandelier cells that synapse on the axon initial segment of pyramidal cells (DeFelipe and Gonzalez-Albo, 1998).

A correlation in the expression of $GABA_A$ receptor subtypes between the grey and the white matter of patients with focal epilepsy

In a previous publication using the same tissue sections as in the present study, we reported a selective reduction of α3-containing $GABA_A$ receptors in the superficial layers of the grey matter in a subset of the 26 patients with focal epilepsy (Loup *et al*., 2006). As can be seen in Table 1, of the seven patients in which changes in $GABA_A$ receptor subunit staining were observed in the white matter, all exhibited concomitant changes in α3-subunit staining in the overlying grey matter [Fig. 6 in the present study and Fig. 4D–F in Loup *et al*. (2006) are from the same patient]. In contrast, in only three of the remaining 19 patients with no

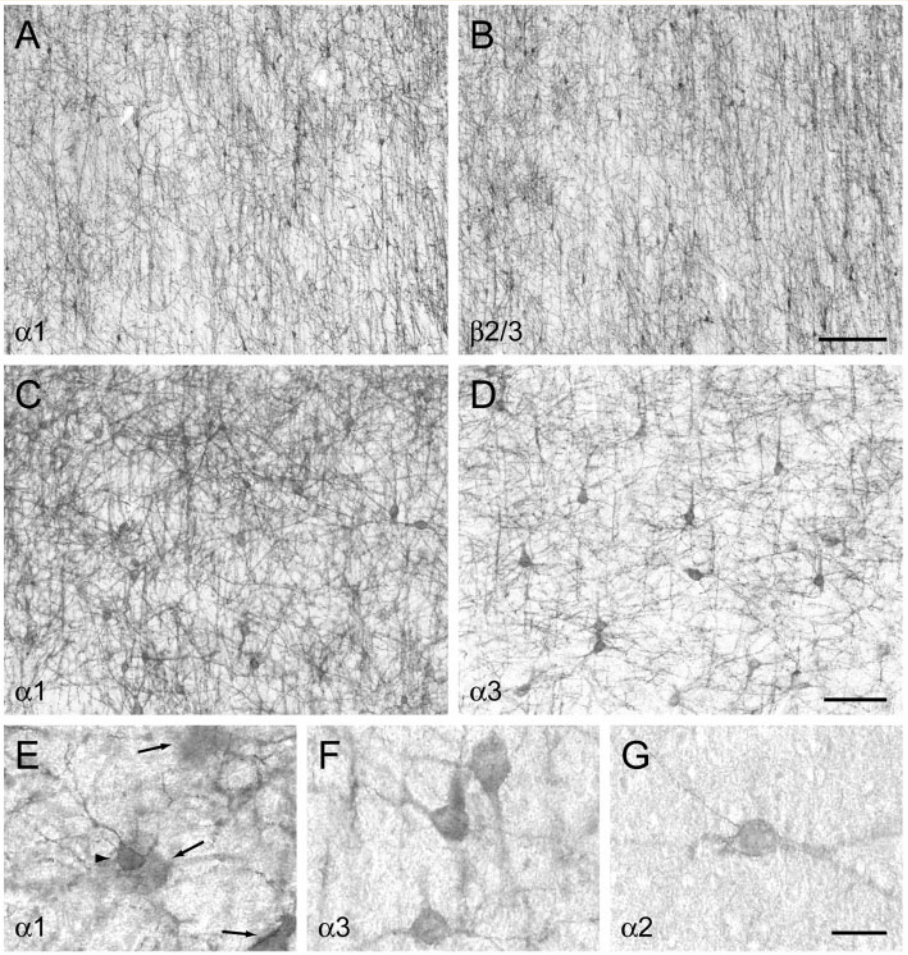

Figure 5 Distribution of GABA$_A$ receptor subunit immunoreactivity in white matter neurons from two patients with focal epilepsy. (**A** and **B**) Adjacent sections showing similar staining for the α1-subunit (**A**) and the β2/3-subunit (**B**) from a patient with FLE. The distribution pattern of these subunits is comparable with that seen in controls. (**C** and **D**) Adjacent sections stained for the α1 and α3-subunit from a patient with non-HS. Staining for the α1-subunit (**C**) is more intense than for the α3-subunit (**D**) with the overall pattern for both subunits essentially unchanged in comparison with controls. (**E–G**) High magnification images of three adjacent sections from the same patient as in (**C**) and (**D**). (**E**) The α1-subunit is expressed in several pyramidal cells (arrows) and in a small and intensely stained putative GABAergic interneuron (arrowhead). (**F**) The α3-subunit is present in pyramidal-like cells only. (**G**) α2-subunit staining showing a pyramidal cell with a horizontally oriented soma and dendrites running parallel to the grey–white matter border. Scale bar: (**A** and **B**) 200 μm; (**C** and **D**) 100 μm; (**E–G**) 25 μm.

apparent changes in the white matter was there a decrease in α3-subunit staining in the overlying grey matter.

Discussion

Three main findings emerge from the presented data. First, in human white matter we observed a differential and neuron-specific expression pattern of five major GABA$_A$ receptor subunits (α1, α2, α3, β2/3, γ2). Moreover, staining for the α1 and α3-subunits revealed a decreasing gradient in the density of neurons from the superficial to the deep white matter, and a radial distribution of pyramidal cells. Second, in 7 of 26 patients with focal epilepsy, we detected either selective changes in α1-subunit labelling, revealing altered dendritic morphology in a subset of white matter neurons in 5 patients or alterations in all five subunits in the 2 patients with HS and mMCD Type II. Third, in these seven patients we observed that the alterations in the white matter were correlated with decreased α3-subunit staining in the overlying grey matter. Importantly, as discussed previously (Loup *et al*., 2006), it is unlikely that factors such as clinical history, including drug regimens or staining artefacts biased our results.

GABA$_A$ receptor subtype expression in the normal human white matter

Immunohistochemical staining for α1, α2, α3, β2/3 and γ2 GABA$_A$ receptor subunits permitted an extensive morphological characterization of white matter neurons. Based on previous

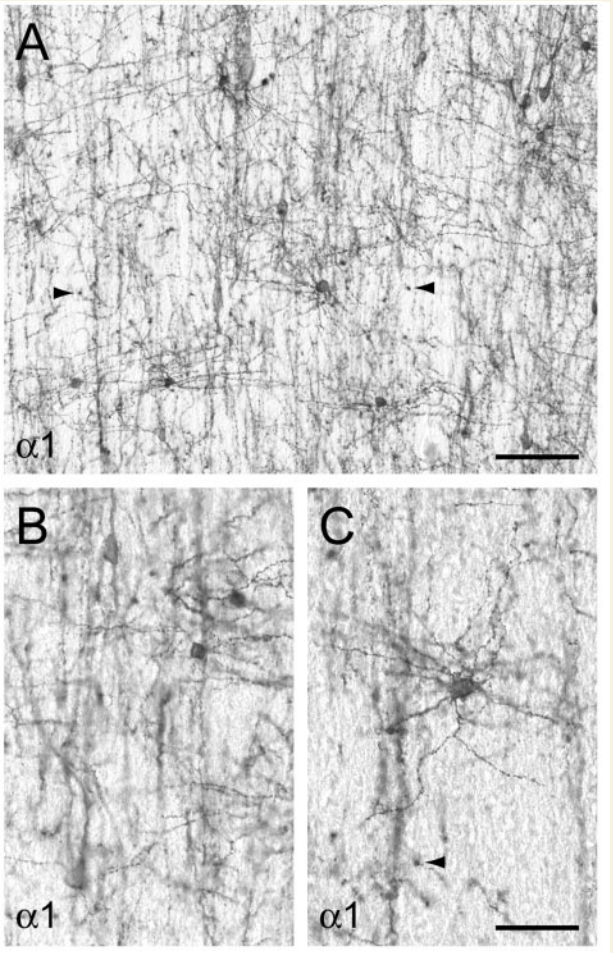

Figure 6 Disruption in the radial organization of white matter neurons immunoreactive for the $GABA_A$ receptor α1-subunit in a patient with hippocampal sclerosis. (**A–C**) Many α1-subunit-labelled neurons display fine dendrites with an altered, predominantly horizontal orientation, while other α1-subunit-labelled neurons exhibit a radially oriented apical dendrite similar to that seen in control pyramidal cells. The horizontal dendrites can be followed over long distances, or are tangentially sectioned, appearing as irregular dots (arrowheads). Scale bar: (**A**) 100 μm; (**B** and **C**) 50 μm.

studies indicating that these subunits account for ~90% of all $GABA_A$ receptors in rodent brain (Sieghart and Sperk, 2002), and by comparing NeuN and $GABA_A$ receptor subunit immunostaining (Figs 1 and 2), we can conclude that we have visualized the majority of the neurons present in the human white matter. Furthermore, the dendritic arborization of individual white matter neurons was revealed in great detail, as we used high affinity antibodies in conjunction with an antigen-retrieval procedure that enhanced specific immunostaining and reduced non-specific background (Loup *et al*., 1998). Based on the size and shape of the soma and the dendritic pattern, we identified different morphological types of white matter neurons and determined their topological organization.

One of our most striking observations was the differential distribution in white matter neurons of the α1, α2 and α3-subunits, which form distinct receptor subtypes (Fritschy and Brünig, 2003). A remarkable cell-type specificity in the expression of $GABA_A$ receptor α-subunit variants was found in the temporal and frontal lobes from both control and patient tissue samples. A previous study examining $GABA_A$ receptor subunit immunoreactivity in the grey matter had reported the presence of a few α1-subunit-positive neurons at the border between the grey and the white matter in the human visual cortex (Hendry *et al*., 1994). When comparing $GABA_A$ receptor subunit architecture and organization in grey matter (Loup *et al*., 2006) versus white matter, both similarities and differences emerge. As in the grey matter, the most abundantly expressed subunit was α1, which was present in pyramidal cells and GABAergic interneurons whereas the α2 and the α3-subunits were present in pyramidal cells only. Likewise, the $GABA_A$ receptor subunits β2/3 and γ2, components of most $GABA_A$ receptor subtypes, showed a largely similar staining pattern to that of the α1-subunit. However, in contrast to the grey matter where α2-subunit labelling was pronounced, the underlying white matter in the same tissue sections showed surprisingly few neurons expressing this subunit.

A number of approaches using Nissl staining and the Golgi technique (Kostovic and Rakic, 1980), MAP2 staining and the Golgi technique (Meyer *et al*., 1992), MAP2 staining (Anderson *et al*., 1996; Beasley *et al*., 2002) and NeuN staining (Eastwood and Harrison, 2003) have shown a gradual decrease in the density of subcortical white matter neurons with increasing depth in normal human brain. Our data with α1-subunit-labelled neurons and NeuN-stained neurons confirm this distribution. Moreover, depending on the size of the control tissue samples, we were able to systematically examine the white matter down to a depth of 8 mm and the cell counts obtained are in the same range as reported previously in the white matter subjacent to motor cortex (Meyer *et al*., 1992). White matter neurons have also been counted in the temporal, frontal and occipital lobes in normal human brain (Rojiani *et al*., 1996), but differences in tissue processing preclude a meaningful comparison with our results. Finally, the radial distribution of white matter neurons that we observed with the α1 and α3-subunits was also noted with MAP2 staining (Meyer *et al*., 1992).

Several lines of evidence suggest that the $GABA_A$ receptors in white matter neurons are functional components of GABAergic circuits. First, the $GABA_A$ receptor subunits in the combinations characteristic for functional receptors were expressed by white matter neurons (present study). Second, the pre-synaptic component of the GABAergic synapses, the inhibitory interneurons, are present in white matter, as demonstrated in human brain on the basis of their morphology (Meyer *et al*., 1992) or with antibodies for GABA (Schiffmann *et al*., 1988), GAD (present study) or markers for specific classes of GABAergic interneurons such as neuropeptide Y (Chan-Palay *et al*., 1985) or calretinin (Gabbott *et al*., 1997). Third, symmetrical synaptic junctions consistent with GABAergic synapses are present in the white matter of both the developing as well as the adult human and also monkey telencephalon (Kostovic and Rakic, 1980, 1990). Fourth, GABA and the $GABA_A$ receptor subunits α1 and β2/3 are expressed in subplate neurons and their subunit-labelled dendrites form symmetrical synapses, as reported for developing primate occipital

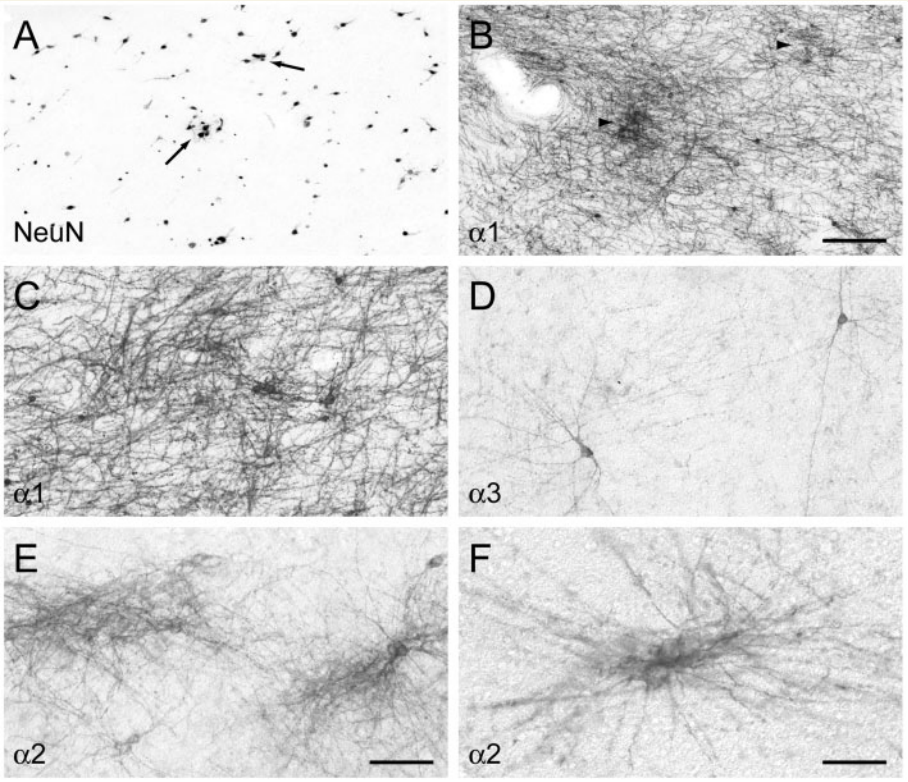

Figure 7 Subunit-specific alterations in GABA$_A$ receptor immunoreactivity in the white matter from a patient with hippocampal sclerosis and mMCD Type II. (**A**) A NeuN-stained section displaying neurons that are irregularly arranged throughout the white matter, either standing alone or in small cell clusters (arrows) surrounded by cell-free spaces. The radial neuronal orientation is largely lost. (**B** and **C**) α1-subunit staining reveals many labelled white matter neurons, either standing alone, or within cell clusters (arrowheads). The radial organization is disrupted and the dendritic network is dense and disorganized. (**D**) Section adjacent to (**C**) showing two α3-subunit-labelled pyramidal-like cells. Note that staining is much less intense for the α3 than the α1-subunit. (**E** and **F**) α2-subunit staining outlines the soma and dendrites of neurons forming the small cell clusters. Scale bar: (**A** and **B**) 200 μm; (**C–E**) 100 μm; (**F**) 50 μm.

cortex (Meinecke and Rakic, 1992). In this respect it is of interest that white matter neurons have been shown to represent remnants of subplate neurons (Chun and Shatz, 1989; Kostovic and Rakic, 1990). The implications of their persistence in adult brain and their role in mature cortical function are, however, only beginning to be addressed (Torres-Reveron and Friedlander, 2007).

Altered GABA$_A$ receptor subtype expression in the white matter of patients with focal epilepsy

Previous morphological studies of the white matter from patients with TLE focused mainly on neuronal cell counts using routine neuropathological protocols or immunohistochemistry for NeuN or MAP2 (Hardiman *et al*., 1988; Emery *et al*., 1997; Kasper *et al*., 1999, 2003; Bothwell *et al*., 2001; Thom *et al*., 2001, 2005). Even though the majority of these investigations found excessive numbers of white matter neurons in patients with TLE compared with controls, there was considerable overlap between the two groups. In our study, we observed no increase in the density of subcortical white matter neurons down to a depth of 8 mm in patients with focal epilepsy. Although we agree that cell number or density is an important parameter, we believe that the alterations in neuronal morphology and receptors, as described here, represent a further indicator that may provide insight into the underlying pathogenesis of epilepsy. Recently, a new technique was reported (Eriksson *et al*., 2005*a*), which allows the correlation of quantitative MRI parameters with histopathological measures in patients with epilepsy (Eriksson *et al*., 2007). GABA$_A$ receptor subunit staining in human brain may serve as a valuable adjunct in this approach.

Interestingly, nuclear imaging studies have shown that patients with focal epilepsy often exhibit increased white matter binding of ^{11}C-flumazenil, an antagonist at the benzodiazepine/GABA$_A$ receptor complex, notably around the ventricles (Hammers *et al*., 2001, 2002, 2003, 2005). In a few of these patients who had undergone surgery, a correlation was noted between increased white matter flumazenil binding and increased number of white matter neurons (Hammers *et al*., 2001, 2002, 2003). Our data in two patients suggest a further possibility, namely, an increase in the number of GABA$_A$ receptors expressed by individual neurons. Although the tissue available to us did not extend to the periventricular region, GABA$_A$ receptor expression

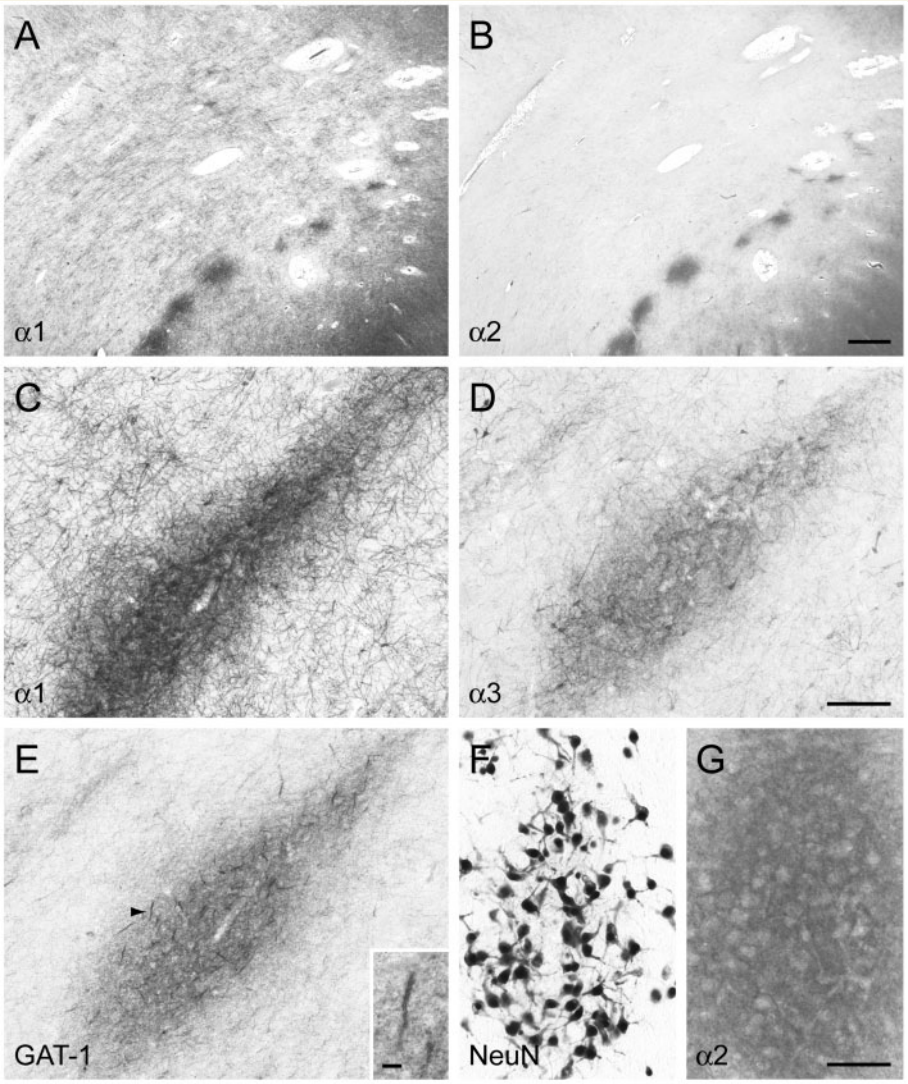

Figure 8 Subunit-specific $GABA_A$ receptor immunoreactivity in neuronal aggregates in the white matter of two patients with hippocampal sclerosis and mMCD Type II. (**A** and **B**) The neuronal aggregates exhibit strong staining for the α1-subunit (**A**) and the α2-subunit (**B**). (**C–E**) Higher magnification images of a neuronal aggregate. Adjacent sections stained for the α1-subunit (**C**), the α3-subunit (**D**) and GAT-1 (**E**). Labelling is intense for the α1-subunit (**C**) and faint for the α3-subunit (**D**). Note the intricate α1-subunit-positive dendritic network between the neuronal aggregate and the neighbouring white matter neurons. (**E**) GAT-1 staining reveals numerous, randomly oriented so-called cartridges, which represent axon terminals of chandelier cells that form synapses on the axon initial segment of pyramidal cells. Inset shows a high magnification image of two cartridges (arrowhead). (**F** and **G**) Adjacent sections stained for NeuN (**F**) and the α2-subunit (**G**). Aggregates consist of differentiated neurons that are randomly oriented. Intense α2-subunit labelling is present in the neuropil. The lightly stained round structures represent pyramidal cell somata. Scale bar: (**A** and **B**) 1 mm; (**C–E**) 200 μm; inset 15 μm; (**F** and **G**) 100 μm.

may also be altered at deeper levels of the white matter, as suggested by these ^{11}C-flumazenil binding studies (Hammers *et al*., 2002, 2003, 2005).

In our study, we found no changes in staining for five major $GABA_A$ receptor subunits in 19 of 26 patients across all three groups with focal epilepsy, that is, FLE, HS and non-HS (Table 1). We did not examine subunits with less prominent expression, such as the α4, α5 or δ-subunits, which are altered in rodent models of epilepsy (Fritschy *et al*., 1999; Houser and Esclapez, 2003; Peng *et al*., 2004; Scimemi *et al*., 2005), as the corresponding antisera are ineffective in human brain tissue (α5-subunit, F. Loup and J.M. Fritschy, unpublished data) or, to our knowledge, not yet available for immunohistochemistry in human samples. In the other seven patients, all with TLE, we observed a normal distribution of white matter neurons in five patients and an irregular distribution of white matter neurons with microscopic neuronal aggregates corresponding to mMCD Type II (Palmini *et al*., 2004) in two patients.

In the five patients with a normal neuronal distribution, α1-subunit staining revealed aberrant dendritic arborization in a subpopulation of white matter neurons, a finding that was not apparent with NeuN staining. Based on their size and morphology,

these neurons appear to be pyramidal cells, but with horizontally rather than radially oriented dendrites, suggesting a disruption of neuronal polarization (Crino and Eberwine, 1997). Similar changes in white matter neurons have been described in patients with focal epilepsy using Lucifer Yellow microinjections (Belichenko *et al*., 1994). As modelling studies predict significant changes in neuronal firing pattern as a consequence of altered dendritic morphology (Mainen and Sejnowski, 1996), it is conceivable that abnormal dendritic orientation could predispose towards aberrant synaptic connectivity in the white matter.

In the two patients with HS and mMCD Type II, the question arises as to the origin and the degree of synaptic interaction of the small aggregates of randomly oriented neurons. With respect to their morphology and $GABA_A$ receptor expression, these microscopic assemblies of cells may represent heterotopic white matter neurons, that is, neurons that did not reach their cortical targets while migrating to the grey matter (Sarnat, 1991; Mischel *et al*., 1995). Alternatively, they could be remnants of subplate neurons that failed to undergo programmed cell death (Chun and Shatz, 1989). Our findings of small cell clusters that selectively and strongly expressed the α2-subunit, whereas control white matter was practically devoid of α2-subunit-positive cells, are more consistent, however, with defective neuronal migration.

Heterotopic neurons appear to be integrated functionally with neurons in neighbouring areas as evidenced in animal models of cortical malformation (Chevassus-au-Louis and Represa, 1999; Tschuluun *et al*., 2005; Ackman *et al*., 2009), in functional imaging studies of patients with nodular or band heterotopia (Richardson *et al*., 1998; Pinard *et al*., 2000; Mai *et al*., 2003; Tyvaert *et al*., 2008), and with carbocyanine dye tracing in patients with nodular heterotopia (Hannan *et al*., 1999). Our data also support this view. First, the particularly striking dendritic overlap between the neurons located in the aggregates and those in the neighbouring white matter is indicative of functional interconnectivity. Second, the pyramidal cells in the aggregates expressed all five $GABA_A$ receptor subunits studied here and GAT-1 staining revealed axon initial segment, which is known to be targeted by a particular class of GABAergic interneuron, the chandelier cells (DeFelipe, 1999). As chandelier cells are parvalbumin-positive, but parvalbumin-immunoreactive cell bodies are not present in human white matter (Alonso-Nanclares *et al*., 2005), it seems likely that the somata of these interneurons are located in the grey matter.

Further support for a functional circuit encompassing grey and white matter is provided by the correlation we observed when comparing the grey matter (Loup *et al*., 2006) and the white matter (present study) on the same tissue sections. In all seven patients where morphological changes and altered $GABA_A$ receptor subunit expression were detected in the white matter, we found a concomitant reduction in α3-containing $GABA_A$ receptors in the superficial layers of the overlying grey matter. This pronounced correlation could reflect a pathogenetic process affecting both grey and white matter during a critical period of development, or a reaction to a primary insult leading to subsequent re-organization.

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