Microdysgenesis in temporal lobe epilepsy
A quantitative and immunohistochemical study of white matter neurones

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Summary

Microdysgenesis is a microscopic cortical malformation considered to act as a substrate for seizures in some patients with generalized epilepsy. It is also recognized to involve the temporal lobe in a proportion of patients with intractable temporal lobe epilepsy, but the incidence of this abnormality, its relationship to mesial temporal lobe sclerosis and relevance to epileptogenesis remain unknown. This is partly due to a lack of well-defined quantitative pathological diagnostic criteria. To begin to address these issues, we have carried out a rigorous quantitative analysis, using three-dimensional cell counting methods, of several components of microdysgenesis in temporal lobectomy specimens. White matter, cortical and layer I neuronal densities (NDs) were measured using immunohistochemistry for the neuronal markers neuronal nuclear antigen and calbindin D-28-K. Patients with a seizure-free outcome (Class I) showed significantly more microdysgenetic features including higher white matter ND (P < 0.05), particularly of small (<10 µm diameter) neurones (P < 0.01), higher layer I ND (P < 0.05) and increased numbers of Cajal–Retzius-like calbindin-positive neurones (P < 0.05). We also demonstrated that white matter ND was independent of the degree of temporal lobe gliosis as assessed by quantitation of glial fibrillary acidic protein-immunoreactive cells. These findings suggest that microdysgenesis may be a significant lesion in temporal lobe epilepsy in terms of post-surgical prognosis.

Keywords: white matter neurones; microdysgenesis; epilepsy

Abbreviations: CR = Cajal–Retzius; CTL = caudal temporal lobe; GFAP = glial fibrillary acidic protein; ND = neuronal density; NeuN = neuronal nuclear antigen

Introduction

Meencke (Meencke and Janz, 1984) outlined the pathological appearances of microdysgenesis, a microscopic malformation, in post-mortem tissue from patients with generalized epilepsy. The constellation of subtle cyto-architectural abnormalities included heterotopic neurones in the molecular layer, an excess of neurones in the white matter and alterations in the cortical laminar architecture. The presence of similar pathology is recognized in temporal lobe surgical resections, suggesting a functional role for microdysgenesis in temporal lobe epilepsy (Hardiman et al., 1988, Armstrong, 1993; Kasper et al., 1999), although the importance of these features is debated (Emery et al., 1997; Mitchell et al., 1999). Indeed, the histopathological diagnosis of microdysgenesis and its relevance to epilepsy initially was regarded with scepticism (Lyon and Gaustaut, 1985; Meencke and Janz, 1985), largely because similar changes can be found in normal brains and in patients without epilepsy (Kaufman and Galaburda, 1989). Furthermore, many of the described features are open to subjective pathological interpretation with a lack of well-defined or quantitative criteria for a definitive diagnosis.

To address this, several studies have aimed to refine the diagnostic criteria for microdysgenesis, particularly with regard to the white matter component which is more amenable to quantitative analysis (Hardiman et al., 1988; Emery et al., 1997; Kasper et al., 1999). Undoubtedly, isolated single neurones are present in the white matter of normal brains, particularly the temporal lobe (Rojiani et al., 1996), and may represent remnants of subplate neurones or ‘misplaced’ cortical neurones (Chun and Shatz, 1989; Meyer et al., 1992). At the other end of the spectrum, a type of severe malformation associated with epilepsy, subcortical heterotopia, shows large nodular aggregates of malpositioned
cortical neurones in the white matter. Microdysgenesis may lie between these entities, but the point at which white matter neuronal numbers become abnormal and represent a significant malformation is not well defined. Previous quantitative studies of temporal lobe surgical resections have provided conflicting results in terms of clinicopathological correlation; in some higher white matter, neuronal numbers were associated with poorer post-surgical seizure outcome (Kasper et al., 1999), and in others with a better outcome (Hardiman et al., 1988).

There are several possible explanations for these inconsistent findings. Different methodologies have been employed, all using non-stereological cell counting techniques. It has been suggested that neuronal density (ND) in the white matter may be affected by the degree of gliosis or volume loss occurring in mesial temporal sclerosis (Emery et al., 1997). Furthermore, any regional variation in ND within temporal lobe white matter is unknown, and therefore the area selected for quantification in these studies may influence this measurement. Finally, in all previous studies on this topic, only the density of large ‘gangliod’ or pyramidal neurones (>10 or 12 μm diameter) was quantified, although white matter neurones are likely to be heterogeneous, of varying size and may all be significant both as a component of the malformation and to the generation of seizures.

In view of this, we undertook a stereological examination of 31 temporal lobectomy specimens from patients with refractory temporal lobe epilepsy and hippocampal sclerosis. We aimed to estimate the density of all white matter neuronal types in different anatomical regions of the temporal lobe and in relation to the degree of microscopic gliosis. We also aimed to correlate this white matter pathology with the presence of other microdysgenetic features. Finally, we correlated all these measurements with the clinical outcome.

Methods

Case selection and tissue processing
Thirty-one temporal lobe specimens were included in this study (17 right, 14 left side), selected at random from the pathology files at the National Hospital for Neurology and Neurosurgery, between the years 1994 and 1998, without knowledge of the clinical outcome. In all cases, the patients had suffered from medically intractable temporal lobe seizures, with a mean age at surgery of 36 years. Standard preoperative investigations, including MRI, were compatible with unilateral hippocampal sclerosis. This work is approved by the National Hospital of Neurology and Neurosurgery and Institute of Neurology Ethics Committee.

The temporal lobe specimens, comprising superior, middle and inferior gyri, were fixed in formalin for 5 days, sectioned in a coronal plane at intervals of 5 mm and processed routinely (mean seven slices per case). Serial 7 μm sections were stained with haematoxylin and eosin and Luxol fast blue/cresyl violet, and 20 μm sections were immunostained for the astrocytic marker, glial fibrillary acidic protein (GFAP; Dako, Cambridge, UK; 1: 400, counterstained with cresyl violet), the neuronal marker neuronal nuclear antigen (NeuN) (1 : 500; A60 Chemicon, Harrow, UK) and calbindin D-28-K (1 : 200; Sigma, Poole, UK) using standard immunoperoxidase techniques and microwave pre-treatments. In each case, the sections were evaluated to exclude neoplastic, inflammatory or neurodegenerative disease processes and severe cortical malformation, such as focal cortical dysplasia. Specimens of the adjacent hippocampus from each case were processed similarly, and hippocampal sclerosis of the classical type, with cell loss in CA1 and hilar subfields, was confirmed in all cases. Fifteen temporal lobe control specimens were also analysed with NeuN staining (mean age 52 years; nine right, six left side). Four of these specimens were temporal lobes removed at surgery and 11 were post-mortem specimens from neurologically normal patients. In none of the control post-mortem cases did routine examination of the brain or the temporal lobe disclose any significant pathology including hippocampal sclerosis. Tissue processing for the surgical controls was identical to the hippocampal sclerosis epilepsy cases, and details regarding the post-mortem delay and fixation times for the remaining controls are presented in Table 1. Immunostaining results with GFAP and calbindin were less satisfactory in post-mortem controls compared with the surgical material for quantitative analysis purposes, and only control NeuN sections were used in this study.

Cell counting methods

Cell densities were estimated using a three-dimensional cell counting technique and an optical dissector (Williams and Rakic, 1988). A Leica DMRB microscope (Leica, Heerbrugg, Switzerland) was fitted with a digital length gauge (Heidenhain MT12, Traunreut, Germany) to measure movement of the microscope stage through the depth of the section. An oil immersion lens (magnification ×100, aperture 1.4) was used to provide a narrow depth of field together with an eyepiece graticule for the counting box (100 μm square and depth 10 μm). Cells that came into focus either fully inside the counting box or touching one of three non-forbidden planes (right, top and upper sides) were counted. The counting box was divided further by a grid into boxes 10 μm wide to allow estimation of neuronal size during counting.

Estimation of white matter cell densities

The boundaries between the cortex and white matter were outlined on the GFAP/Nissl- and NeuN-stained sections with a fine ink line using the Luxol fast blue-stained section as a reference. The boundaries of the claustrum in the white matter of the superior temporal gyrus were also outlined with ink. Cells within the white matter were counted in parallel columns beginning two counting box widths away from the inked cortical boundary (to ensure exclusion of laminar VI
Table 1  Tissue processing for 15 control temporal lobe specimens

<table>
<thead>
<tr>
<th>Case control no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Cause of resection/death</th>
<th>Post-mortem delay (days)</th>
<th>Fixation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>M</td>
<td>Surgical resection; temporal lobectomy to treat brain swelling following acute cerebral trauma. No significant pathology</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>47</td>
<td>M</td>
<td>Surgical resection: DNT in the hippocampus</td>
<td>NA</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>F</td>
<td>Surgical resection: cavernous haemangioma in the hippocampus</td>
<td>NA</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>F</td>
<td>Surgical resection: cavernous haemangioma in the hippocampus</td>
<td>NA</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>85</td>
<td>F</td>
<td>PE</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
<td>M</td>
<td>MI</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>43</td>
<td>M</td>
<td>MI</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>49</td>
<td>M</td>
<td>MI</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>63</td>
<td>M</td>
<td>MI</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>F</td>
<td>Carcinomatosis</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>56</td>
<td>F</td>
<td>NR</td>
<td>NR</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>65</td>
<td>F</td>
<td>NR</td>
<td>NR</td>
<td>7</td>
</tr>
<tr>
<td>13</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>14</td>
</tr>
<tr>
<td>14</td>
<td>45</td>
<td>F</td>
<td>MI</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>67</td>
<td>F</td>
<td>MI</td>
<td>NR</td>
<td>14</td>
</tr>
</tbody>
</table>

Specimens 1–4 are surgical temporal lobe resections and 5–15 are from post-mortems of patients with no known underlying neurological illness. Underlying causes for resection or death, post-mortem delays and formalin fixation times are indicated. DNT = dysembryoplastic neuroepithelial tumour; PE = pulmonary embolus; MI = myocardial infarction; NA = not applicable; NR = not recorded.

Cells), moving systematically to the periventricular surgical resection margin (Fig. 1). Cells were counted in this direction as white matter neurones in normal brain (Meyer et al., 1992) and during development (Meyer et al., 2000) are known to be orientated radially with respect to the cortex. In NeuN-immunostained sections from cases and controls (Fig. 2), all positively labelling cells were counted in the white matter and categorized according to approximate nuclear size of greater or less than 10 µm diameter. Six columns of white matter were counted at random intervals across each slide in both cases and control sections, and the ND/mm³ calculated. This sampling strategy, in a pilot study of six cases, gave comparable ND estimates to counting all white matter neurones, with good repeatability on re-counting (Table 2). In GFAP/cresyl violet sections in epilepsy cases, both NDs (neurones identified as cells with vesicular nuclei, prominent nucleoli and Nissl-positive cytoplasm, irrespective of their size) and GFAP-positive astrocytic densities/mm³ were calculated.

In 13 cases, further measurements were carried out to evaluate any differences in ND between superficial and deep white matter. An NeuN-immunostained section from the caudal level of the temporal lobe resection (CTL), which included part of the claustrum, was selected from each case. This level was chosen as it more often had the greatest depth of white matter and provided an anatomically comparable region between cases. Both large and small neurones were counted within an area of 10 × 10 counting boxes, in the white matter core of the middle temporal gyrus and in a similar area of deeper, periventricular white matter (Fig. 1), and the ND compared.

Estimation of cortical neuronal densities
On the CTL section from all cases, estimation of cortical NDs was carried out. NeuN-immunopositive cells were counted in columns from the cortical–white matter boundary to the pial surface on the crest of the mid temporal gyrus as previously described (Thom et al., 2000). Four columns were counted per case and the mean ND for this region of cortex was estimated. Layer I (molecular layer) NDs were also estimated separately on NeuN-stained sections of CTL in cases and controls. The counting box was moved systematically along layer I, in a coronal plane, beginning in the sulcus and with one edge of the box maintaining contact with the pial border (Fig. 1). All immunoreactive cells were counted as before and the ND for this region of the temporal lobe then calculated.

Semi-quantitation of other microdysgenetic features
The number of neuronal clusters in the superficial cortex was also estimated on Nissl sections from CTL. Neuronal clusters, defined as three or more overlapping neurones (Fig. 3B), were counted in 20 consecutive fields along cortical layer II of the middle temporal gyrus using the eyepiece grid and a
myelinated in a coronal plane. The presence of abnormal tangential the sulcal depth and moving systematically around the gyrus, was counted along the middle temporal gyrus at the CTL number from each case. Cells were counted with a fi

A diagrammatic section of caudal temporal lobe, comprising superior, middle and inferior gyri, illustrating the direction of columns for three-dimensional cell counting of white matter neurones (long arrow). The boxes indicate the areas of white matter sampled for analysis of the difference between superficial (middle temporal gyrus core) and deep (periventricular) white matter NDs. Cortical layer I NDs were also quantified in this section in the middle temporal lobe gyrus, in a coronal direction beginning in the sulcus, as indicated by the short arrows.

×40 objective (total area = 5 × 10⁻² mm²), beginning in the sulcal depth and moving systematically around the gyrus, in a coronal plane. The presence of abnormal tangential myelinated fibres in the superficial cortex (Fig. 3C), as previously described (Thom et al., 2000), was also assessed on all the Luxol fast blue-stained sections of each case. The number of calbindin-immunoreactive cells in cortical layer I was counted along the middle temporal gyrus at the CTL level of each case. Cells were counted with a ×10 objective in 20 consecutive fields (total area 10 mm²) beginning in the sulcal depth and moving the eyepiece grid systematically around in the coronal plane (Fig. 3D).

Clinical evaluation and statistical methods
Post-surgical outcome was reviewed from the clinical notes. The data were analysed using SPSS software (version 9) for windows. Statistical methods included paired $t$-test (for comparison of mean ND in different regions of temporal lobe), Wilcoxon rank test, independent $t$-test (for comparison of ND between patient groups) and Pearson’s correlation.

Results

White matter neuronal densities
The mean temporal lobe white matter ND in Nissl-stained sections was 1010/mm³ with a wide variation between epilepsy cases (range 440–1751/mm³) (Table 3). NeuN-immunoreactive cells showed both nuclear and cytoplasmic positivity, many being small cells with little cytoplasm and nuclear diameter of <10 μm (Fig. 3A); these were not distinguishable from glial cells on the Nissl preparation. ND measurements with NeuN were significantly greater than with Nissl in all epilepsy cases (mean 2164/mm³, range 1212–3448/mm³) ($P < 0.001$) (Table 3 and Figs 2 and 5) although there was correlation between both measurements ($P < 0.01$). The proportion of small (<10 μm) to total NeuN-positive white matter neurones varied between 35 and 68% (mean 47%) in epilepsy cases.

In control cases, the mean white matter ND on NeuN sections was 1660/mm³ (range 620–2990 mm³) which was significantly lower than in epilepsy cases ($P < 0.05$), although there was overlap between the ND in both groups (Table 3). In control cases, NeuN also highlighted both large pyramidal and smaller (<10 μm) diameter neurones, the latter comprising 35–83% (mean 57%) of the total; this was not significantly different from epilepsy cases ($P = 0.07$). Higher mean NDs were present in the surgical than post-mortem controls, but this was not significantly different ($P = 0.19$). Although there was a trend for lower ND in post-mortem controls with longer post-mortem intervals and fixation times, this was not significant ($P = 0.16$ and 0.09). In controls and cases, there was no correlation with white matter ND and age of the patient.

We examined white matter ND variation at different coronal levels of the temporal lobe in the NeuN-stained sections. In the 31 cases, there was no significant difference in mean ND between temporal pole and caudal temporal lobe white matter ($P = 0.527$). This was also the case for separate analysis of small neurones of <10 μm diameter ($P = 0.9$). Using the Wilcoxon signed rank test for related samples, we confirmed that in individual cases, higher NDs were not observed in either the anterior or posterior part of the temporal lobe ($P = 0.72$). In addition, there was no significant difference between the mean density of NeuN-positive small ($P = 0.15$) or large neurones ($P = 0.13$) in superficial gyral core and deep periventricular white matter. However, using Wilcoxon signed rank test, in a significant number of cases (10 out of 13), higher densities of small neurones were present in the deep compared with the gyral white matter ($P < 0.05$), whereas large neurones were more evenly dispersed ($P = 0.16$). There were no differences in mean white matter ND between right and left temporal lobectomies ($P = 0.8$).
Fig. 2 (A) A coronal section from a temporal lobectomy specimen immunostained with NeuN neuronal marker which demonstrates good distinction between cortex and white matter (bar = 0.3 cm). Marked variations in white matter ND were observed between cases, varying from 1211/mm$^3$ (B) to 3448/mm$^3$ (C). Bar = 55 μm in B and C.

Table 2 Pilot study of sampling strategies and repeatability of measurements for white matter neuronal densities on six lobectomy specimens

<table>
<thead>
<tr>
<th>Area of white matter sampled (n = 6 cases)</th>
<th>All white matter</th>
<th>Six columns per section</th>
<th>Four columns per section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean boxes counted per case</td>
<td>7020</td>
<td>1400</td>
<td>950</td>
</tr>
<tr>
<td>Mean ND/mm$^3$</td>
<td>2084</td>
<td>2170</td>
<td>1890</td>
</tr>
<tr>
<td>Mean difference (SD)</td>
<td>–</td>
<td>–86 (–215)</td>
<td>194 (360)</td>
</tr>
<tr>
<td>Limits of agreement</td>
<td>–</td>
<td>–516 to 344</td>
<td>–526 to 914</td>
</tr>
<tr>
<td>Repeatability (RC)</td>
<td>–</td>
<td>436</td>
<td>–</td>
</tr>
</tbody>
</table>

Mean NDs calculated by counting six or four random (cortical to periventricular) white matter columns (see Fig. 1) were compared with NDs from analysis of counting the entire white matter. Stronger limits of agreement (mean difference in ND ± 2 SD) were achieved when counting six columns. Repeat ND measurements counting six columns were then carried out. RC (repeatability coefficient) = 2 SD of mean differences in ND between first and second measurement (Bland and Altman, 1986).

White matter gliosis

In all epilepsy cases, a variable degrees of astrocytic gliosis of the white matter was evident on GFAP immunostaining. However, there was no correlation between the degree of gliosis as measured by the density of GFAP-positive astrocytes in the white matter and the ND in either Nissl- ($P = 0.2$) or NeuN-stained sections ($P = 0.38$). In 10 epilepsy patients, there was evidence of obvious cortical nerve cell loss on NeuN-stained sections involving particularly the superficial cortical laminae (II and III) with a secondary gliosis. This was reflected in the wide range of middle temporal gyrus cortical NDs (28 716–53 750/mm$^3$) between cases, with a mean ND of 40 207/mm$^3$ (Table 3). There was no significant correlation between middle temporal gyrus
Fig. 3 (A) White matter NeuN-immunoreactive neurones demonstrated various morphologies including large pyramidal shaped cells and smaller neurones (bar = 10 µm). (B) Clusters of small neurones were observed in all cases in cortical layer II (Nissl, bar = 15 µm). (C) Abnormal bundles of myelinated fibres in the superficial aspects of cortical layer II were seen in six cases and often associated with neuronal clusters (Luxol fast blue, bar = 15 µm). (D) Calbindin-immunopositive neurone in layer I with the morphology of a Cajal–Retzius-like cell (bar = 15 µm).
Table 3 Results of quantitative analysis of temporal lobectomy specimens

<table>
<thead>
<tr>
<th>Groups</th>
<th>White matter ND (NeuN)/mm³</th>
<th>White matter ND (NeuN)/mm³</th>
<th>White matter ND (NeuN)/mm³</th>
<th>White matter ND (NeuN)/mm³</th>
<th>White matter ND (NeuN)/mm³</th>
<th>White matter ND (NeuN)/mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases (n = 31)</td>
<td>2164* (368)</td>
<td>992 (271)</td>
<td>1010 (343)</td>
<td>1040* (433)</td>
<td>9670 (3193)</td>
<td>41 598 (2717)</td>
</tr>
<tr>
<td>Seizure-free outcome (n = 17)</td>
<td>2359* (697)</td>
<td>1165 (386)</td>
<td>1170* (433)</td>
<td>9670 (3315)</td>
<td>43 396 (2717)</td>
<td>39 742 (2879)</td>
</tr>
<tr>
<td>Not seizure-free (n = 9)</td>
<td>1941* (368)</td>
<td>850 (271)</td>
<td>839* (197)</td>
<td>8319 (3677)</td>
<td>39 742 (8679)</td>
<td>8 436 (2952)</td>
</tr>
<tr>
<td>Control (n = 15)</td>
<td>1660* (368)</td>
<td>941 (550)</td>
<td>9559 (3819)</td>
<td>9670 (3819)</td>
<td>9556 (3819)</td>
<td>9556 (3819)</td>
</tr>
</tbody>
</table>

ND = neuronal density with Nissl staining and NeuN marker; AD = astrocytic density. Mean values (and standard deviation) are given. *Indicates significant differences between means in the seizure-free and non seizure-free group and † between epilepsy groups and controls (P < 0.05). Only cortical layer I and white matter NeuN ND measurements were carried out on control cases.

Cortical microdysgenetic features

In NeuN-stained sections, labelling of cells in layer I of the cortex was noted in all cases (Fig. 4A), including occasional large neurones and more numerous smaller neurones. In none of the cases were discrete clusters of these cells noted. The mean density of NeuN-positive cells in layer I of the middle temporal lobe gyrus was 10 436/mm³ (range 4117–16 400/mm³). There was no significant correlation between cortical layer I ND and white matter AD (P = 0.1). In addition, there was no significant correlation between cortical ND and temporal lobe white matter AD, although again there was a trend for lower cortical ND with higher AD (P = 0.1).

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Fig. 4 (A) Cortical layer I and the superficial part of layer II with frequent NeuN-immunopositive cells in layer I of the middle temporal lobe gyrus (bar = 35 µm). (B) Immunostaining for MAP2 protein also demonstrated a similar mixture of larger and smaller neurones in the temporal lobe white matter as seen on NeuN sections. The MAP2 marker (1 : 2000; Sigma, UK) also highlights the dendritic processes of these neurones and their neuronal morphology. Bar = 35 µm.
of small neurones in white matter in NeuN-stained sections, although this was not significant ($P = 0.08$). In control cases, mean layer I ND in NeuN sections was $9556/\text{mm}^3$ (range $2500–15\ 128/\text{mm}^3$) which, although lower, was not significantly different from epilepsy cases ($P = 0.4$) (Table 3).

Calbindin-immunoreactive layer I neurones were predominantly large neurones morphologically similar to Cajal–Retzius (CR) cells and appeared dispersed at regular intervals along the length of the layer, often in a subpial location (Fig. 3D). There was an impression of increased numbers of these cells in the sulci, a phenomenon also observed in the developing brain (Meyer et al., 1998), but no clusters of these neurones were noted. Quantitation showed variations in their number between cases, but there was no correlation with white matter ND ($P = 0.66$), layer I NeuN ND ($P = 0.25$) or cortical ND ($P = 0.234$). However, in six cases, abnormal cortical myelinated fibres were present running tangentially in layers I and II of the cortex (Fig. 3C) and there was a positive correlation between the presence of these fibres and the number of layer I calbindin-positive cells ($P < 0.05$). There was also a strong correlation between the numbers of neuronal clusters in cortical layer II and the number of calbindin-positive cells in layer I ($P < 0.001$).

**Clinical outcome**

The patients were followed-up for a minimum period of 2 years after surgery. Seventeen patients have been seizure free (Engel class I) for up to 5 years (mean 3 years). Nine patients are not seizure free, and in the remaining five patients sufficient follow-up information was not available. Those patients who were seizure free showed significantly higher white matter NeuN ND than those not ($P < 0.05$). In addition, white matter NDs were significantly different between the seizure-free group and controls ($P < 0.05$) but not between the non-seizure-free group and controls ($P = 0.4$) (Table 3). Furthermore, there was a significant difference in the presence of other microdysgenetic features in the seizure free compared with the non-seizure-free group, including density of small NeuN-positive white matter neurones ($P < 0.01$), layer I NeuN ND ($P < 0.05$), calbindin-positive neurones in layer I ($P < 0.05$) and cortical neuronal clusters in layer II ($P < 0.05$) (Table 3).

**Discussion**

The origin of neurones in the temporal lobe white matter is debated. They may represent remnants of the embryonic subplate (Chun and Schatz, 1989), some of the earliest migrating neurones which play a role in the establishment of normal cortical connections (Kostavic and Rakic, 1990; Molnar et al., 1998). Normal subplate neurones assume a variety of morphologies during development, including pyramidal and multipolar forms (Mrzljac et al., 1988; deAzevedo et al., 1997; Meyer et al., 2000). Then, following cortical maturation and expansion and loss of synaptic contacts, their numbers are reduced (Mrzljac et al., 1988). Alternatively, white matter neurones may represent an anatomical extension of layer VI (Meyer et al., 1992), akin to the normal ‘layer VII’ of rodent cortex (Clancy and Cauller, 1999), or the arrested migration of cortical neurones destined for the cortex, i.e. true ‘heterotopic neurones’. An excess number of white matter neurones is considered to be one of the characteristic features of microdysgenesis, a malformation of cortical development, although the distinction between microdysgenesis and normal white matter is ill-defined.

In our group of 31 patients with temporal lobe epilepsy, stereological cell counting revealed a wide variation in white matter ND in both conventional Nissl- and NeuN-immunostained sections, but with significantly higher white matter ND in the NeuN-immunostained sections. NeuN is a neurone-specific DNA-binding nuclear protein and is a sensitive and specific marker of mature neuronal cells (Wolf et al., 1996; Sarnat et al., 1998). We were also able to demonstrate using this antibody a significant difference in white matter ND between epilepsy cases and controls, although there was overlap between the groups as has been noted in previous studies (Hardiman et al., 1988; Emery et al., 1997). Although the majority of the control temporal lobes in this study were from post-mortem brains, we did not find a significant correlation between post-mortem interval or fixation time and ND in our cases and, in addition, ND in surgical and post-mortem controls was not significantly different. These observations make it unlikely that differences...
in tissue fixation and processing alone can explain the significantly higher white matter ND observed in the epilepsy group. Furthermore, although the mean age of patients was older in the controls than in the epilepsy patients, there was not a significant correlation between age and white matter ND in either group, making age-related neuronal loss also unlikely to explain our findings.

In the white matter, NeuN immunostaining demonstrated pyramidal-like cells as well as smaller neurones, which made up on average approximately half of all labelled white matter neurones in both epilepsy cases and controls. These smaller cells were not distinguishable from glial cells in Nissl-stained sections, as they lacked prominent nucleoli and distinct cytoplasm. Additional MAP2 immunostaining also labelled a similar subset of white matter neurones, many with processes, confirming their neuronal nature (Fig. 4B). However, in a previous semi-quantitative study of normal white matter, small non-pyramidal MAP2-positive neurones comprised only 10% of all neurones (Meyer et al., 1992). Previous quantitative studies in temporal lobe epilepsy have assessed only the large neurones in the white matter. Hardiman included cells with a nuclear diameter of 10 µm or greater and showed a range of 2 to >15 neurones/2 µm² in epilepsy patients using two-dimensional cell counting (Hardiman et al., 1988). Emery quantified cells with prominent nucleoli and nuclear diameter of >12 mm, finding NDs of 4.11 ± 1.86 per mm² in a similar patient group, acknowledging that they may have underestimated the real total (Emery et al., 1997).

In a more recent study, Kasper quantified cells with prominent nucleoli and nuclei larger than glial cells (Kasper et al., 1999) and found up to 10 neurones per high power field in temporal lobe epilepsy patients. Furthermore, in an earlier study, increased cellularity of white matter in complex partial seizures was attributed to hypertrophy of glial cell nuclei and an increase in their numbers (Krishnan et al., 1994); in the absence of specific neuronal markers being applied, it is possible that these cells also represented small neurones. Given that white matter neurones are likely to be a heterogenous population (deAzevedo et al., 1997), reflecting diverse origins and functional status, consideration of the density of all white matter neurones in the temporal lobe is likely to be of importance in the analysis of microdysgenesis. Further study to characterize their nature and connectivity more fully will be fundamental to our understanding of their possible contribution to epilepsy.

We analysed our data for any variation in ND occurring in different anatomical regions of white matter. This information was important to acquire as the ND measured may be dependent on the region removed surgically and the sections sampled for quantitative analysis. It has been demonstrated previously that temporal lobe white matter contains significantly more neurones than occipital and frontal lobe (Rojani et al., 1996). It has also been reported in epilepsy material that deeper white matter is more cellular than gyral white matter (Krishnan et al., 1994), whereas the neuronal number in normal white matter is considered to decrease with increasing distance from the cortical grey matter (Meeke 1983; Meyer et al., 1992). We found no variation in ND between the left and right sides or in a caudal–rostral axis, but we did identify an uneven distribution of smaller neurones, with higher neuronal densities in the deeper periventricular compared with the gyral core white matter in a significant number of cases. These findings suggest that future quantitative analysis of white matter microdysgenesis could be restricted to one coronal section of temporal lobe but should include both superficial gyral and deep periventricular white matter.

It has been suggested that increased ND observed in the white matter in temporal lobe epilepsy may be merely an epiphenomenon as a consequence of white matter atrophy secondary to epilepsy-induced damage (Emery et al., 1997). The pathological correlates of the atrophy and white matter signal change that may be observed in the temporal lobe in patients with temporal lobe epilepsy on neuroimaging are likely to be gliosis or myelin loss (Mitchell et al., 1999). Cortical and white matter gliosis is observed commonly in surgical resections. We estimated the severity of white matter gliosis by quantifying the density of GFAP-positive reactive astrocytes, but we found no correlation between this and white matter ND. This suggests that white matter neuronal ectopia is independent of the degree of secondary gliosis, which was also the conclusion from Kasper’s study (Kasper et al., 1999). In addition, our estimates of middle temporal gyrus cortical ND in these cases may also reflect neuronal loss and, indirectly, white matter fibre loss. Again though, we found no correlation between middle temporal gyrus cortical ND and white matter ND. Any simple relationship between cortical and white matter ND may be confounded by several factors; cortical neuronal numbers may be influenced by any existing migrational disorder and heterotopic white matter neurones may also be vulnerable to seizure-mediated cell loss. However, our quantitative findings support the hypothesis that higher white matter ND is not directly related to either the degree of gliosis or to cortical neuronal loss, both of which may correlate with macroscopic atrophy.

In patients with sufficient follow-up information available, we identified significantly more microdysgenetic features, including higher white matter and layer I ND, in patients with a seizure-free outcome. All patients in this study showed the histological features of classical hippocampal sclerosis. It is recognized that hippocampal sclerosis may co-exist with malformations of the temporal lobe (so-called ‘dual pathology’) including focal cortical dysplasia (Raymond et al., 1994), microdysgenesis (Armstrong et al., 1987; Armstrong, 1993) or undefined temporal lobe developmental malformations (Kuzniecky et al., 1999). Hardiman’s study also showed a more favourable post-operative outcome where microdysgenetic features were identified histologically in the temporal lobe (Hardiman et al., 1988), although in that study the presence or absence of co-existing adjacent hippocampal sclerosis is unknown.
We also noted significantly higher numbers of layer I calbindin-positive cells in the patients with a seizure-free outcome. The majority of these cells were large subpial horizontal and bipolar cells (Fig. 3D) with the morphology of CR cells (Marin-Padilla 1998; Meyer et al., 1999). The numbers of these cells strongly correlated with other abnormal developmental features in the superficial cortex, including the presence of neuronal clustering and a tangential fibre plexus. CR cells appear early in corticogenesis in the primordial plexiform layer (the origin of layer I) and include early calbindin- and calretinin-positive pioneer neurones and later reelin-secreting cells (Zecevic et al., 1999). The numbers of CR cells are tightly regulated during development, reelin protein being essential to the arrest of neuronal migration and formation of cortical laminae (Dulabon et al., 2000). As development proceeds, the number of CR cells is ‘diluted out’ with cortical expansion, and reelin synthesis is reduced (Meyer et al., 1998, 1999; Zecevic et al., 1999) with only a few cells persisting into adulthood. It is conceivable that the increased CR cells observed in these cases are of relevance to the microdysgenetic abnormalities observed. Calbindin-positive Cajal cells in mature temporal lobe neocortex may also have a functional role influencing pyramidal cell excitability through molecular layer ramifications (Ferrer et al., 1992). Increased numbers of CR cells have also been identified in the hippocampus in temporal lobe epilepsy (Blumcke et al., 1996,1999), which was also suggested as indicative of abnormal hippocampal development.

The incidence of microscopic malformations or microdysgenesis in temporal lobe epilepsy has been reported variably as between 10 and 15% (Kuzniecky et al., 1999), 16.7% (Nordberg et al., 1999) or in the majority of surgical specimens (Armstrong and Mizrahi, 1998); different pathological criteria used probably explain these differences. A more exact definition of microdysgenesis with quantitative criteria would give more consistent information regarding its incidence for future clinicopathological correlations. From our preliminary data, if we consider white matter ND > 2360/mm³, small white matter ND > 1170/mm³, layer I ND > 11 250/mm³ and layer I calbindin ND > 1.5/mm² as positive criteria for microdysgenesis, then in the present study group, 30% of patients with none of these criteria, 53% with 1–2 criteria and 67% with 3–4 criteria were seizure free following surgery. In the future, automated quantitative methods, such as image analysis, could be incorporated into routine practice to carry out these measurements and provide more accurate information regarding the incidence of microdysgenesis as well as potentially useful prognostic information for both patient and clinician.

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References
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