1 Title : Microscopic Maiformations in Temporal Lobe Epilepsy : An Immunohistochemical and Quantitative Study.

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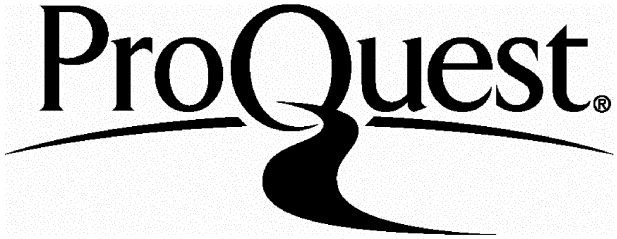
MD Thesis



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Commonly used abbreviations in text :

FCD	Focal cortical dysplasia
MD	Microdysgenesis (syn. Mild cortical dysplasia)
HS	Hippocampal sclerosis (syn. Ammon's horn sclerosis)
TLE	Temporal lobe epilepsy
CB	Calbindin
PV	Parvalbumin
CR	Calretinin
NPY	Neuropeptide Y

2 Abstract

In a large series of 413 epilepsy surgical resections from the National Hospital, hippocampal sclerosis was the commonest pathology identified in 60% of cases. Microdysgenesis is a complex microscopic cortical malformation recognised in association with hippocampal sclerosis in epilepsy, but its incidence, relationship to hippocampal sclerosis and functional relevance to epileptogenesis remain unknown. This is largely due to a lack of well-defined diagnostic criteria. To address this a stereological quantitative analysis of several components of microdysgenesis was carried out. White matter, cortical and layer I neuronal densities (ND) were measured in 31 surgical temporal lobectomies using immunohistochemistry for neuronal marker NeuN. Patients with seizure-free post-operative outcomes showed significantly more microdysgenetic features, including high white matter ND ($P<0.05$) or layer I ND ($P<0.05$). Mean white matter ND of 2164/mm^3 were observed in epilepsy patients. There was no correlation between layer I and white matter ND suggesting that they may represent separate developmental abnormalities. Abnormal patterns of cortical myelination were also identified in microdysgenesis. Cyto-architectural abnormalities in analysis of 206 hippocampal specimens included disorganisation of dentate granule cells in 40% and cytoskeletal abnormalities in residual hilar cells in 55%. Granule cell disorganisation correlated with the degree of hippocampal neuronal loss suggesting an acquired rather than developmental lesion. Stereological quantitation of granule cells showed an overall cell loss, but greater numbers in regions of disorganisation, which may indicate enhanced neurogenesis. Quantitation of Cajal-Retzius cells showed increased numbers in hippocampal sclerosis and microdysgenesis compared to controls. Abnormalities in the cyto-architectural distribution of inhibitory interneurones were observed in microdysgenesis and focal cortical dysplasia in epilepsy, which may represent adaptive changes. In summary, the findings suggest that microdysgenesis may be a significant lesion in temporal lobe epilepsy in terms of post-surgical prognosis and quantitation allows a more precise definition. Increased Cajal-Retzius cells in hippocampal sclerosis and microdysgenesis may indicate a common developmental process involving the reelin pathway.

3 Introduction and background to study

3.1 Temporal lobe epilepsy: Clinical features and surgical treatments

Epilepsy is one of the commonest serious neurological conditions with an annual incidence in the UK of 40-70/100,000 (Sander and Shorvon, 1996). The first descriptions of epilepsy appear as long as 4000 years ago with historical medical literature depicting patients suffering typical generalised convulsions, although the organic nature of epilepsy was only recognised relatively recently (Scaravilli, 1997). Epilepsy is of course not a single disease, but is a predisposition to have seizures, which may be due to a variety of causes. It is classified according to the type of seizure, the cause of the seizures and other clinical features (International League Against Epilepsy, 1989).

The epilepsies as a group of syndromes worldwide affect more than 50 million people. Epileptic seizures are convulsive or non-convulsive episodes characterised by synchronous paroxysmal discharges arising from a group of neurones. They can affect all age groups and may be the result of acute or chronic cerebral illness. They can be common manifestations of diseases early in life, as mitochondrial disorders, or appear in age-related disorders such as Alzheimer's disease. Important causes of epilepsy are structural cerebral abnormalities, hypoxia and trauma. Furthermore seizures themselves can cause brain injury and neuronal damage, for example following status epilepticus. Brain injury following seizures is likely to be the result of excessive activation of glutamate receptors. In addition, seizures themselves activate genes, encoding for example neurotrophic factors, that may result in structural neuronal re-arrangements at the synaptic level. As a result, seizure-induced brain injury can predispose to further seizures and set up a vicious cycle of ongoing seizures and brain damage.

An increased interest in the study of epilepsy over the last two decades has occurred as a result of parallel advances in neuroimaging, neurosurgical treatments, neurophysiology and molecular neurobiology. Neuropathology plays an integral role in advancing the understanding of epilepsy by correlating information from these disciplines. In addition, with the increasing use of surgical treatments for epilepsy, the opportunity to study and describe the cellular changes in the well-

preserved tissues resected and the possible cellular mechanisms contributing to epilepsy has arisen. In many cases the more subtle lesions are invisible with current MRI techniques, so called 'radio-occult' lesions, and pathology has become the 'gold standard' in the identification of such microscopic cortical abnormalities. This has resulted in a growing number of publications in this field and it has become critical that universal terminology is adopted for the pathological lesions identified in order to further scientific understanding and the clinical practice in dealing with them. The major aim of this work has been the further analysis of microdysgenetic abnormalities observed in temporal lobe epilepsy.

Temporal lobe epilepsy (TLE), is a partial or localisation-related epilepsy which indicates that the seizures arise from a particular region, in this instance the temporal lobe. As with other types of epilepsy, they may be idiopathic (no cause identified), symptomatic (an underlying structural abnormality is seen) or cryptogenic (features suggest there is an underlying structural abnormality, but this cannot be identified). The majority of localisation-related epilepsies are symptomatic or cryptogenic in contrast to generalised epilepsies. Common clinical histories in temporal lobe epilepsy are an onset in the first decade of life and, in some cases, a history of febrile convulsions. The seizures may remit for several years until adolescence and then often become intractable to medical treatments. Secondary generalised seizures may occur. Aura and complex partial seizures are common, with oro-alimentary automatisms and post-ictal amnesia. EEG shows ictal activity with 5-7/s rhythmic spikes in the basal temporal electrodes (Fish, 1997). Temporal lobe hypometabolism is shown on PET and hypoperfusion on SPECT. In mesial temporal lobe epilepsy, memory disturbance is seen on contralateral intra-carotid sodium amytal injection. In a proportion of cases atrophy of mesial temporal lobe structures, including the hippocampus, is seen on imaging. This type of epilepsy most often has a favourable outcome following epilepsy surgery (Fish, 1997).

Epilepsy is often associated with malformations of cortical development. Other common clinical manifestations of malformations being mental retardation, autism, and neuro-psychiatric syndromes. It is estimated that cortical malformations may account for 20% of all epilepsies (Crino et al., 2002) and may be associated with any seizure type, including generalised, complex partial, myoclonic seizures, infantile spasms, etc. Satisfactory control of epilepsy may be difficult to attain in patients with malformations, despite poly-pharmacy, and surgical resection may be

necessary. Overall, surgery is successful in less than 50% of cases (Crino et al., 2002, Sisodiya 2000). In a proportion of malformations of cortical development in epilepsy, surgical treatment is, however, often appropriate and indicated. Such surgical procedures are often carried out at tertiary referral neurological centres where the necessary pre-operative investigations to determine the optimal surgical approach and strategy are available (Crino et al., 2002).

It is a conservative estimate that, of the 300,000 children and adults in the UK with active seizures, a third are refractory to treatment and of these 10% would benefit from epilepsy surgery; currently insufficient numbers of surgical procedures are being carried out (Shaefi and Harkness, 2003). In the treatment of refractory temporal lobe epilepsy, randomised studies have shown that surgical treatment is preferable to continued treatment with AEDs, with their inherent side effects, and also reduces the long-term risk of SUDEP (Engel et al., 2003). Pre-surgical evaluation for potential candidates includes investigations confirming that any structural lesion identified on MRI may be the source of the seizures. The *epileptogenic zone* is defined using invasive monitoring including depth electrodes, subdural strips and grid; it may extend beyond the borders of the visualised lesion. Any adjacent or overlapping 'eloquent' cortex may be simultaneously mapped with functional MRI studies. Surgical strategies include lesionectomy with or without perilesional normal cortex, for example, for the treatment of low grade glial-neuronal tumours and cavernomas. The presence of a second cortical lesion should always be excluded pre-operatively as this may affect surgical management and outcome.

Surgical treatments of patients with temporal lobe epilepsy and hippocampal sclerosis include temporal lobectomy. Operative procedures include a standard *en bloc* resection of the anterior temporal lobe versus selective resection of the amygdala and hippocampus. Whilst the former approach may result in an increased incidence in post-operative neuropsychological deficit, minimally invasive procedures may result in poorer outcome in terms of seizure control and it is recognised that resection of 3-3.5cm of the hippocampus is required to achieve the optimal outcome. Mortality from this procedure is rare and post-operative complications rare, including quadrantanopia and post-operative depressive symptoms.

It is necessary to measure the impact of surgery on the control of seizures to identify those patients with certain types of lesions who are likely to benefit from surgery. The Engel system (Engel et al.,1993) is widely used and distinguishes patients who are seizure-free from those who have occasional seizures, a marked improvement in seizures and those who have no worthwhile improvement. This classification system is set out in Appendix 1. Most rigorous epilepsy surgical series favour a minimum follow up period of 2 years (Sisodiya, 2000) although it is recognised that later post-operative seizure recurrences may occur.

3.2 Classification of neuropathological lesions in temporal lobe epilepsy

The demonstration of neuropathological changes in the hippocampi from patients with epilepsy in 1880 by Sommer (Sommer, 1880) was one of the first indications that this region was important in the generation of seizure activity. In the clinical investigation of patients with one of the commonest forms of epilepsy, temporal lobe epilepsy (TLE), the epileptogenic focus is typically localised in the mesial temporal structures; these include the amygdala and hippocampus (MTLE). With the increasing use of surgical treatments for patients with intractable MTLE, as anterior temporal lobectomy and amygdalohippocampectomy (Bruton 1988, Blumcke et al.,1999c, Armstrong 1993, Mathern et al., 1995a), examination of the resected tissue has enabled the neuropathologist to provide confirmatory and diagnostic information which may have a bearing on the patients expected post-operative seizure outcome. In addition, however, these resected tissues also provide a very valuable resource and unique opportunity for the *in vitro* study of the disease mechanisms in epilepsy using a combination of morphological, molecular and electrophysiological techniques (Blumcke et al., 1999c).

The commonest neuropathological lesion identified in temporal lobectomy series in patients with MTLE is hippocampal sclerosis (HS), or Ammon's horn sclerosis (AHS), which is seen in approximately 42%-63% of cases (Bruton 1988, Blumcke et al., 1999c). Other major pathologies can be grouped under 'lesion associated TLE' (Blumcke et al., 1999c) which are seen in 17%-33% of specimens (Bruton 1988, Blumcke et al., 1999c) and include vascular malformations, malformations of cortical development and glio-neuronal tumours (Wolf et al., 1993). In 9-12%

of patients (Blumcke et al., 1999c, Bruton 1988) more than one epileptogenic pathology is present, these being referred to as 'dual pathology' cases.

Common pathologies recognised in temporal lobectomy specimens :

I. **Hippocampal sclerosis,**

II. **Lesion-associated epilepsy (including tumours, vascular and cortical malformations)**

III. **Inflammatory, traumatic, hypoxic-ischaemic lesions**

IV. **Conditions and lesions as a sequel of seizure**

V. **Dual pathology**

The table below lists the incidence of the more commonly identified lesions in two large reported series of temporal lobectomies carried out for TLE :

NEUROPATHOLOGICAL LESION	UNIVERSITY OF BONN Based on 541 temporal lobe resections. (Blumcke et al., 1999)	INSTITUTE OF PSYCHIATRY, LONDON Based on 234 temporal lobe resections. (Bruton, 1988)
TLE without lesions	**49.7%**	**74.3%**
Hippocampal sclerosis	37.2%	45.7%
Uncertain/no pathology	12.5%	28.6%
TLE with focal lesions	**50.3%**	**25.7%**
Tumours	25.3%	11.5%
Malformations	13.9%	5.6%
Vascular malformations	5.7%	1.3%
Gliosis /others	5.4%	7.3%

3.3 Normal cortical development

Malformations of cortical development therefore form a significant proportion of pathologies identified in epilepsy tissue. In order to understand the pathogenesis of these lesions it is essential that the steps and mechanisms governing normal cortical development are clarified. In the last decade major advances have been made in this area. The majority of neurones will migrate from their place of origin to relocate in a predetermined distal site. Migration of neurones was predicted to

occur as long ago as the 19th century by early pioneers of developmental neurobiology including Ramon y Cajal and His (Bentivoglio & Mazzarello, 1999). Studies of cortical development continue to be important to reveal the bio-mechanisms controlling cell migration and neurodevelopment and also to further the understanding of human diseases caused by abnormal neuronal migration. Conversely, major advances in developmental neurobiology have in fact been made through the identification of mutations in genes in severe human cortical malformations, as lissencephaly and grey matter heterotopias; these genes have been subsequently shown to have roles in controlling neuronal migration.

There are two distinct pathways or modes of neuronal migration. Radial migration is important for the columnar organisation of the cortex and gives rise primarily to the excitatory glutamatergic neurones. Tangential migration of inhibitory interneurones into the cortex occurs from the ganglionic eminences.

3.3.1 Stages In formation of the cortex: pre-plate, cortical plate and subplate

In man neuronal migration begins around the 6th week of gestation. The first stage in the development of the cortex and the formation of cortical layers involves the formation of a preplate or primordial plexiform layer (PPL) above the ventricular zone (VZ). The VZ is the proliferative zone or the germinal matrix of the telencephalon (Gleeson and Walsh, 2000). The preplate is composed of Cajal Retzius cells, first generated projection neurones (pioneer cells) and the subplate neurones, which are born slightly later. The Cajal-Retzius cells occupy a position near the subpial surface and secrete reelin protein. Post mitotic cells from the VZ then migrate along a glial scaffold to form a cortical plate within the preplate, separating the Cajal-Retzius cells from the subplate cells. This process divides the preplate into the marginal zone (to become the molecular layer in the adult cortex) and the distinct subplate beneath the cortical plate (to become integrated in the white matter in the adult cortex). As more cells arrive in the cortical plate a systematic set of layers (from 5 to 2) is generated in an 'inside-out' fashion with later-born cells migrating past older neurones (Marin-Padilla 1998). Laminar identity of neurones is probably decided before migration from the ventricular zone begins (Rakic, 1988) and is likely to involve interactions between migrating and migrated neurones (Cotter et al., 1999). In addition, it is likely that there is

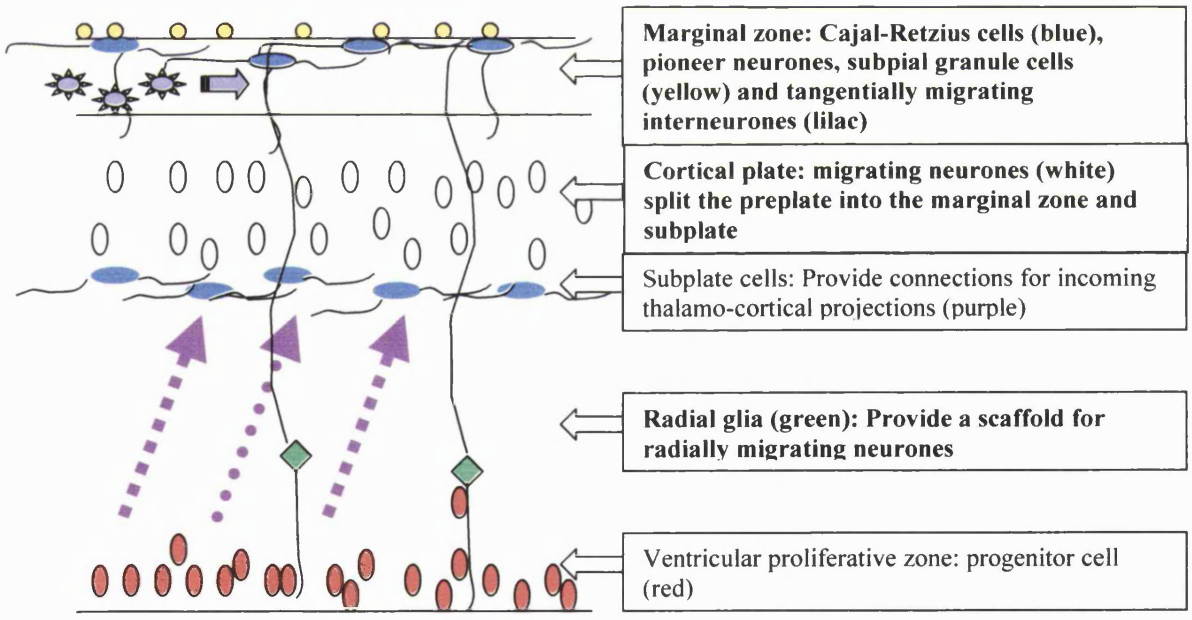
some overlap between layers and cell birth-date may not be the only determinant of laminar fate (Hevner et al., 2002). The migrating cells express genes linked to cytoskeletal motility (e.g. *Filamin 1, DCX, Lis1 and cdk5/p35*) and involved in neuronal-glial binding (e.g. Astrotactin 1 and integrin α3 and neuregulins) (Adams et al., 2002). The reelin pathway signals migrating neurones to dissociate from the radial glia into their correct lamina. Normal radial migration is therefore highly dependent upon a specialised scaffold of cells that spans the cerebral wall, the radial glia. These specialised cells, visualised with both Golgi impregnation techniques and immunohistochemical methods, send short processes to the ventricular wall and a long ascending process with several branches into the subpial region. Migrating cortical neurones closely associate with the glial processes through adhesion molecules. The radial glia subsequently transform into astrocytes.

Initially it was considered that glia and neurones arise from separate progenitor cell,s but now it is considered that they arise from the same multipotential 'precursor cell' (Lillien et al.,1998). More recent work suggests that radial glial cells themselves represent many of the neuronal precursors in the developing cortex (Fishell and Kriegstein, 2003). Exogenous growth factors influence the survival and proliferation of early neuronal precursor cells including fibroblast growth factor (FGF-2), epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and brain derived neurotrophic factor (BDNF). There is a complex cascade of these extracellular signals that act in synchrony at different times during early development and influence differentiation and cell survival in the CNS (Cotter et al., 1999).

Movement of neurones along glial fibres has been studied using video microscopy (Hatten, 2002). An adhesion junction between the migrating cell and glial fibre occurs with extension of a leading process. Microtubules hold the nucleus in a dorsal position in the cell. Microtubules also extend into the leading process and vesicles move along this and actin also extends into the leading process. Radial migration of neurones is thus a process more akin to a dendritic extension than a growth cone in axon extension. Astrotactin (Astn1) and neuregulin are important adhesion molecules in this process. Loss of Astn 1 leads to slowing of migration and defects in dendritic systems in animal models (Adams et al., 2002).

During development the subplate is known to comprise morphologically heterogeneous neurones, including GABAergic populations as Calbindin positive neurones and NPY positive cells. The subplate cells are the first to send axons outside the cortex to the ganglionic eminence. Inhibitory neurones migrate into the cortex via the subplate. Subplate cells also make connections with thalamo-cortical projections prior to their entry into the cortex. The deeper subplate neurones therefore serve as transient synaptic targets for thalamo-cortical projections during cortical development. (Allendorfer and Shatz, 1994). Cortical afferents also make transitory synaptic contacts with subplate cells. Deeper GABAergic cells in the subplate may be important in the establishment of callosal cortical connections and these cells may arise from the ganglionic eminences (Del Rio et al., 2000).

Diagram showing the Development of the Neocortex



3.3.2 Cajal-Retzius cells

Cajal-Retzius cells (CRC) were recognised by neuroanatomists in the 19th century. Cajal in 1891 described slender horizontal cells in the marginal zone and Retzius in 1893 described cells in the marginal zone with radial ascending processes; they are now all regarded as part of the CRC 'family' (Meyer et al., 1999). They are an

integral part of developing early cortical networks (Radnikow et al., 2002). Their importance in neurodevelopment has emerged following the discovery of the secretion of reelin protein by this family of cells (D'Arcangelo et al 1995) and they are likely to play key roles in cortical positioning and patterning of neurones. However, not all reelin immunopositive cells in layer I are CRC (Alacantra et al., 1998, Meyer and Goffinet 1998, Zecevic and Rakic 2001) as smaller layer I interneurones may also be reelin positive. CRC are therefore characterised on the basis of their cytology in addition to reelin expression.

CRC are horizontally orientated cells lying parallel to the pial layer with prominent processes emerging from the cell body also running parallel to the pial layer (Meyer and Goffinet 1998, Zecevic and Rakic 2001). The CRC also form a perpendicular lattice-type network in the later stages of development and they tend to be distributed non-uniformly over the cortical surface (Fairen et al., 2002). The nuclear diameter of the CRC is 10-13 microns, but other smaller neuronal types (6-11 microns) are also present in layer I during development. The paucity of mitotic figures in layer I at this stage suggests that additional CRC are recruited from elsewhere. It has been suggested that the earliest 'pioneer' cells to the marginal zone are reelin negative and calretinin or calbindin positive and that CRC subsequently arise from the subpial granule cell layer (SGL) (Meyer and Goffinet 1998). However, evidence in primate cortex suggest that CRC appear before the SGL develops (Zecevic and Rakic 2001). The SGL itself arises from the ganglionic eminence and may be the source of additional GABAergic cortical interneurones.

The numbers of CRC in primates peak at mid-gestation (Zecevic and Rakic 2001) and CRC in late gestation have been shown to undergo programmed cell death. In addition 'dilution' of CRC is likely to occur with the massive expansion of the cerebral cortex, which quadruples in size in the post-natal period. However, as equivalent dilution of smaller layer I neurones compared to CRC does not occur in the primate, selective loss of CRC is likely to occur with maturation (Zecevic and Rakic, 2001). Other studies of layer I in the developing human brain argue against a disappearance of CRC due to regressive changes such as programmed cell death (Spreafico et al., 1999). It is known that CRC persist in the mature mammalian cortex (Eriksson et al., 2001, Zecevic and Rakic 2001), but their function in adulthood is unknown. A possible role of CRC in brain repair after injury has been proposed (Super et al., 1997).

3.3.3 Molecules and genes controlling cortical development

3.3.3.1 The reelin signalling pathway

The identification of a mutation in reelin protein in the neurological mutant mouse, the reeler, has played a critical role in the evolution of our understanding of normal brain development (Rice and Curren, 2001). Reelin plays a key role in neuronal positioning and the formation of distinct cytoarchitectural regions in the brain as the cerebral cortex, hippocampus and cerebellum. Reelin is produced by discrete populations of cells in the brain (D'Arcangelo et a ., 1995). It binds to transmembrane receptors present on migrating neurones including VLDLR and ApoER2 (D'Arcangelo et al., 1999, Hiesberger et al., 1999). The cytoplasmic domains of these receptors in turn bind to Dab1 and trigger tyrosine phosphorylation of Dab1 (Trommsdorff et al., 1999). This induces an intracellular signalling cascade that instructs neurones to migrate to their proper location. Reelin is also considered to act as a detachment signal to migrating neurones (Hack et al., 2002) and is also required for the formation of the radial glia scaffold (Super et al., 2000, Forster et al., 2002). There are several other proteins identified as linked to the reelin signalling cascade, the study of which will provide further insight into brain development. These include BDNF, alpha3 integrin, pre-senelin, cdk5, p35, Emx2 and Tbr-1 (Rice and Curren, 2001).

The reeler mouse has long been recognised to exhibit widespread neuroanatomic deficits in the cerebral cortex, hippocampus and cerebellum. Birthdating analysis, using autoradiography of brain slices after administration of tritiated thymidine during development showed an absence of cortical layer I and an inverted cortical plate, with youngest neurones present in deeper locations (Caviness and Sidman, 1973). However, all morphological subclasses of neurones are present in the reeler cortex with normal physiological responses (Lemmon and Pearlman, 1981). Afferent projections, including from thalamo-cortical projections are also intact, although the trajectory of these fibres is altered with fibres ascending to the superficial layers first. Alterations in synaptic connectivity are also present in the reeler model. Analysis of the reeler mouse has shown that post-migratory neurones remain in close contact with radial glial fibres and therefore molecular interactions between radial glia and migrating neurones are affected. Reelin also affects

neuronal-neuronal interactions as abnormal neuronal aggregation also occurs. Reelin protein is also implicated in normal axonal branching, synaptogenesis and neurite outgrowth (Rice et al., 2001). Reelin protein persists during adulthood although its exact functions and role in the mature cortex are as yet uncertain.

The reelin protein is approximately 385kDa and several isoforms are present in the brain from cleavage of the full length protein (D'Arcangelo et al.,1999). The main body of the reelin protein consists of a series of eight internal repeats of 350 to 390 amino acids, each containing domains with epidermal growth factor-like motifs. Reelin protein is expressed by a relatively small proportion of cells with morphological appearances of Cajal-Retzius cells, and this has been demonstrated using immunohistochemical stains with reelin antibodies (CR-50) (D'Arcangelo et al., 1997). CR-50 acts as a blocking antibody to reelin in functional *in vitro* studies (de Bergeyck et al., 1998). Other antibodies have been generated against reelin, including clone 142 against an epitope at residues 164 to 189 whereas CR-50 is against amino acid residues 246-371. Clone 142 has been shown to be a specific marker for reelin, works in paraffin-embedded tissue (de Bergeyck et al., 1998) and is the one I used in this study. Reelin is important in the final stages of neuronal migration and provides positional information and instructing migrating neurones to detach from radial glia. Reelin secretion persists in the marginal zone throughout development and therefore each subsequent wave of migrating neurones receives similar instruction when they reach the top of the radial glial fibres. Reelin may therefore act as an attractant to migrating neurones.

Spontaneous mutations in the Dab1 gene in the Scrambler and Yotari mouse (Gleeson and Walsh, 2000) brain show neuroanatomical defects similar to the reeler mouse, which suggested that this protein acts in the same signalling pathway during migration. Dab1 is a cytoplasmic protein expressed at high levels in the developing CNS. Tyrosine phosphorylation of Dab1 promotes interaction with other proteins including Abl and Dab1 binds to transmembrane proteins, as APP and phosphoinositides. A dramatic increase in Dab 1 is present at the onset of cortical plate formation and migrating neurones express Dab1 when they invade the preplate (Rice and Curren, 2001) with higher levels of Dab1 present in the reeler mutant.

Disruptions in cdk5 or its regulatory subunit p35 produce migrational defects that differ from the reeler phenotype. Normal splitting of the preplate occurs and the

migration of the first cortical neurones proceeds normally (Kwon and Tsai, 1998) and there is formation of layer I. There is inversion of the cortical plate but there is failure of migration of later neurones (normal layers 2 and 3) and an increase in cellularity in the intermediate zone (between cortical plate and ventricular zone). The cortices in p35 and cdk5 engineered mutants are not identical; in the cdk5 mutant the subplate is in the middle of the cortical plate, whereas in the p35 mutant it is beneath the cortical plate (Gleeson and Walsh, 2000). In the cdk5 mutant model it is the cells destined for layers II-V that are found beneath the subplate. As such, p35 and cdk5 mutants reflect different capabilities in the ability of neurones to migrate through the cortical plate and subplate. It is possible that reelin or cdk5 have different effects on early and late migrating neurones or that distinct reelin/dab1 versus cdk5 pathways are activated. The exact link between reelin and cdk5 is as such uncertain (Park et al., 2002). Furthermore cdk5 is not considered to be involved in controlling the migration of GABAergic cells into the cortex (Gilmore and Herrup, 2001).

Cdk5 was originally identified as a member of the cyclin-dependent kinase family, which are involved in cell cycle regulation. However, no role in cell cycle regulation has been assigned to cdk5. Although it is expressed in many tissues, the kinase activity is detected only in the developing brain (Gleeson and Walsh, 2000). Cdk5, its activators p35 and p39 and their kinase substrates may mediate their effect on neuronal migration in several ways. They can induce actin reorganisation as Cdk5 modulates PAK kinases, which have a role in normal organisation of actin (Nikolic et al., 1996). They have an effect on neurite outgrowth, can phosphorylate neurofilaments, microtubule associated proteins tau and MAP1B and thus may exert their effects directly on the cyto-skeleton (Gleeson and Walsh, 2000). They also have an effect on neuronal morphology (Rashid et al., 2001). There is also evidence that links cdk5 activity to axon guidance, membrane transport and synaptic function (Dhavan and Tsai, 2001). Cdk5 dysfunction may also contribute to the pathology of neurodegenerative diseases such as Alzheimer's disease and ALS. Cdk5 itself is regulated by transcription factors Brn-1 and Brn-2 and nerve growth factor and can induce p35 expression; cdk5 is phosphorylated by c-Abl which controls its function (Dhavan and Tsai, 2001).

Double mutations in the reelin receptors ApoER2 and VLDLR genes produce cortical lamination defects very similar to the reeler mice with an indiscernible

layer I and inverted cortical architecture (Trommsdorff et al., 1999). This suggests that both of these receptors are capable of transmitting the reelin signal to migrating neurones. Mice deficient in either VLDLR or ApoER2 exhibit only subtle cortical defects. Both of these receptors are expressed in the developing cortical plate and in the intermediate zone (between the cortical plate and the ventricular zone) and binding of reelin protein to these receptors has been shown (D'Arcangelo et al., 1999). Reelin also binds to other transmembrane proteins, including cadherin-related neuronal receptors (CNRs), involved in homophilic or heterophilic cell-cell interactions (Kohmura et al., 1998) and to alpha-3 integrins (Dulabon et al., 2000).

3.3.3.2 Genes controlling cerebral patterning

The cerebral cortex is subdivided into areas that are distinguished from one another by differences in architecture, axonal connections and function. Based on cytoarchitectonic criteria, the human cerebral cortex was originally subdivided into more than 50 areas by Brodmann in1909. The conservation of areal divisions suggests a rigidly regulated regional specification program (Rubenstein and Rakic, 1999). Genetic studies in flies and nematodes have identified genes that regulate regional specification, segmentation, neurogenesis, programmed cell death, positional identity and commitment to a neuronal or glial fate (Schuurmans and Guillemot, 2002). In the last decades identification of the expression of *hox* genes (segmental organisation of the hindbrain), *Brn* and *Mash* 1 genes (regulators of forebrain development), *Dlx 1, Dlx2* (forebrain specific regulatory homeobox genes), *Emx* genes (expressed in a nested pattern in the forebrain), *caspase-3 and -9* (which regulate the number and survival of cells) have provided key insights into the regulation of regional cortical development in mammals, through the study of knockout models and single cell expression using advanced molecular biological techniques. The expression of such genes frequently exhibits transverse and longitudinal gradients (Rubenstein and Rakic, 1999) and in addition to this spatially distinct expression also shows temporal regulation (Hatanaka and Jones 1999). For example, distinct gradients have been shown for Emx2 and Pax6 but not Emx1 (Bishop et al., 2002). Genes encoding diffusible factors as *wnt* gene family and signalling pathway are also expressed in restricted regions (Cotter et al., 1999).

Cortical regionalisation is also dependent on the in-growth of specific thalamic afferents, which are in turn dependent on genes controlling axon pathfinding, which include the *ephrin* family (Mackerehtschian et al., 1999). The genetic differentiation of projection neurone versus interneuron appears to be distinct; *Dlx* genes regulate the latter GABAergic cells and *Pax6, Otx1. Emx 1* and *Tbr1* the former (Gorski et al., 2002). Emx1 expression for example appears restricted to glutamatergic pyramidal cells (Chan et al., 2001). Pax6 transcription factor is important in neuronal differentiation and migration and gliogenesis in the subventricular proliferative zone of the developing cortex (Warren et al., 1999).

Mutations in many of these genes result in cerebral malformations. Mutation of the Pax6 gene in mice results in small eye phenotype, which include cerebral cortical malformation (Warren et al., 1999) and in humans Pax6 mutations have been associated with, among other abnormalities, malformation of the anterior commissure (S.Sisodiya, personal communication). Heterozygous mutations in the Emx2 gene have been associated with schizencephaly (Brunelli et al., 1996, Guerrini and Carrozo, 2002)

3.3.4 Tangential migration and inhibitory neuronal populations in the normal cortex and epilepsy

3.3.4.1 Origins of cortical inhibitory interneurones

The importance of tangential neuronal migration during development has been more recently recognised than radial migration. This process does not require radial glial fibres and is the major mode of migration of interneurones which subsequently integrate into the cortex (Anderson et al., 2001, Lavdas et al., 1999, Wichterle et al., 1999, Marin and Rubenstein, 2001, Jiminez et al., 2002, Lavdas et al., 1999, Whicheterle et al., 1999). The cells migrate from the ganglionic eminences which are small masses of developing neurones that project into the walls of the lateral ventricles and include distinctive lateral, medial and caudal regions. Although in many animal species most, if not all, cortical GABAergic cells are considered to originate from the ganglionic eminence and migrate tangentially, recent human studies have reported that up to 65% of cortical GABAergic cells may in fact be derived from the ventricular zone (Letinic et al., 2002) . Furthermore some cortical GABAergic cells in primates may also be recruited from the subpial

granule cell layer (SGL) during development (Zecevic and Rakic 2001). An alternative mode of origin proposed is ventricle-directed migration from the ganglionic eminence (Nadarajah et al., 2002).

Neuronal precursors from each of the ganglionic eminences migrate into different regions of the brain. The migrating neurones travel to the neocortex within the marginal zone and intermediate zone (Polleux et al., 2002). The cellular signals and mechanisms controlling tangential cell migration are less well understood than for radial migration (Marin and Rubenstein, 2001). It has recently been shown that these tangentially migrating neurones are guided by molecular cues that are also involved in the projection of axons, e.g., Slits, Netrins, Semaphorins and Ephrins which act in a chemo-attractant or -repellent fashion (Park et al., 2002). In addition, during the process of migration and differentiation these neuronal precursors express distinctive transcription factors and respond to different molecular signals (Polleux et al., 2002), controlling their migrational pathway, destination and subsequent cell phenotype. For example, whereas cortical pyramidal neurones express Emx1 and migrate along radial glia (Chan et al., 2001, Marin and Rubenstein 2001), GABAergic neurones from the ganglionic eminence express Mash1 and Dlx1&2 (Anderson et al., 2001). Tangential migration is also stimulated by BDNF (Polleux et al., 2002). Evidence suggests that GABAergic interneurones do not follow an inside-out sequence of neurogenesis and they are generated in bursts from the lateral and medial ganglionic eminences (Hevner et al., 2002). The presence of Cajal-Retzius cells in layer I, pioneer neurones which play a key role in controlling radial migration and the formation of horizontal cortical layers, is also closely interrelated to normal migration of cells from the ganglionic eminence (Shinozaki et al., 2002, Meyer et al., 2002) and they may themselves also arise from tangential migration (Sarnat and Flores-Sarnat 2002).

3.3.4.2 Subtypes of inhibitory interneurones and their normal distribution in temporal neocortex

Interneurones in the cortex mostly contain GABA as a neurotransmitter, but they are highly diverse, their phenotype influenced by their local afferent connections in addition to developmental factors (Gonzalez-Albo et al., 2001). They make up to 15-25% of all cortical neurones. This heterogeneous group of neurones is further

sub-classified according to their neuropeptide content, receptor profiles, and calciu- binding protein content. These features show a close correlation with input-output relationships (Magloscky et al., 2000) and different interneurones have axon terminals that synapse on different regions of the targeted neurone leading to differences in the modulation and strength of their inhibitory effect. In the present study we focused on the distribution of calcium-binding protein containing interneurones and NPY expressing cells in the temporal neocortex in epilepsy, which are readily identified using immunohistochemistry.

The calcium-binding proteins parvalbumin (PV), calbindin (CB) and calretinin (CR) identify distinct subsets of interneurones (Gonzalez-Albo et al., 2001) present in all regions of the mammalian cortex (Conde et al., 1994). CB cells are the most numerous, followed by CR then PV and NPY positive cells (Gonzalez-Albo et al., 2001). No or very few PV- or NPY-positive neurones are found in layer I whereas CB- and CR- positive cells are found throughout layer I. Parvalbumin is expressed predominantly in the wide arbour (basket) cells and chandelier GABAergic interneurones in the cortex (Cotter et al., 2001). The distinguishing characteristic of the chandelier interneurone is the terminal portions of the axon which form short vertical strings of boutons resembling candlesticks (Foncesca et al ., 1993). PV- positive cell bodies are found in all layers of the temporal neocortex, except layer I, with a preponderance in layer IV and the lower part of layer III. All are non-pyramidal cells and include small neurones (10-20 microns diameter) in layer III-IV and large multipolar cells (25-30 microns) in layer III-V which are the basket cells. In addition, the cortical neuropil shows labelling with PV immunohistochemistry with a dense band in layer III-V of the temporal lobe and processes found throughout the other layers except layer I (Foncesca et al., 1993). The dense immunoreactive band in the middle cortical layers is made up partly from cortical processes and also from thalamo-cortical projections (Marco et al., 1996).

Calretinin is expressed in bipolar cells or double bouquet cells and Cajal-Retzius cells (CRC) which predominate in layer I and II (Conde et al., 1994). Calbindin labelled neurones are predominantly found in the upper cortical layers (I, II and III). They include double bouquet cells, or bitufted cells and horizontal cells in layer I. Co-localisation of CB and CR is seen in a proportion of bitufted cells or double bouquet neurones on immunostaining (DelRio and DeFelipe 1997). There is also weak labelling of pyramidal neurones observed with some antibodies to CB (Ferrer

et al., 1992a, Gonzalez-Albo et al., 2002). In addition to the cell soma, a CB-positive plexus is seen in the molecular layer and vertical bundles in layers III and V/VI. Calcium- binding protein expressing interneurones also show distinct expression profiles of glutamate receptors. For example, most PV- and CB-positive neurones express the GluR1 subunit whereas mainly PV neurones express NMDAR1 and no interneurones that are GluR2/3 positive express CB or CR (Gonzalez-Albo et al., 2001).

NPY immunoreactive neurones are a subset of local circuit interneurones. NPY acts as a local hormone or neuromodulator and is often co-expressed with other peptides (e.g., VIP, somatostatin); co-localisation with GABA has also been shown (Dellale et al., 1997). In the normal brain NPY immunoreactive neurones are highly concentrated in the cortex, the caudate and putamen, amygdala, hypothalamus and brainstem (Blinkenberg et al., 1990). Positive cells are multipolar, bitufted, triangular cells of 10-20 microns diameter and pyramidal cells are generally regarded as NPY negative (Chan-Palay et al., 1985, Blinkenberg et al., 1990). During development NPY neurones predominate in the subplate, where the largest NPY cells are found (Uylings and Delalle 1997). Thereafter degeneration of these subplate cells is seen and, after 1 year, increased NPY cells are identified within the cortex (Delalle et al., 1997). In normal cortex immunohistochemistry studies of NPY show a stereotypical laminar distribution of positive interneurones in all cortical regions (Blinkenberg e al., 1990, Brene et al., 1989, Gonzalez-Albo et al., 2001, Horung et al., 1992, Terenghi et al., 1987). Neurones are rarest in layer I and II and more numerous in deeper cortical layers and the white matter (Chan-Palay et al., 1985). It is thought that 60% of neurones are in the adult white matter compared to 40% within the cortex. A greater predominance of cortical over white matter neurones has been shown in frontal and temporal regions compared to parieto-occipital cortex (Hornung et al., 1992). Quantitative biochemical studies have also suggested a variation in NPY within regions of frontal cortex (Brene et al., 1989, Dawbarn et al., 1984). However, other studies report no difference in the distribution of NPY cells between frontal, parietal and temporal lobes (Chan-Palay et al., 1985).

The dendrites of these cells and axonal plexuses are well developed and strongly NPY positive. The axons have striking beaded varicosities (Chan-Palay et al, 1985) and complex terminal arbours (Horung et al., 1992), especially in older brains. The density of NPY fibres is highest in the superficial layer I (Blinkenberg et al., 1990,

Uylings and Dellale 1997), where they are arranged horizontally. The fibres in deeper layers are arranged in a random pattern (Hornung et al., 1992) with the highest density in layer IV. The fibres in layer I are considered to be of extra-cortical origin (Chan-Palay., 1985) although others suggest they are all of local origin (Horung et al., 1992).

NPY neurones have been considered to be involved in memory processes and loss of these cells has been noted in Alzheimer's disease. A possible role of NPY has been considered in constriction of cerebral blood vessels. The precise role of the extensive networks of NPY fibres in the cortex and white matter is unclear although an important role of NPY in seizure modulation has been proposed (Vezanni et al., 1999).

3.3.4.3 Alterations of inhibitory interneuronal cells in epilepsy including cortical lesions

Studies of non-principal neurones in epilepsy, the local circuit neurones, can provide valuable information regarding the vulnerability, adaptability and connectivity of these cells. Indeed, altered function and number of inhibitory interneurones, whether a primary or secondary event, is considered to be one cause of predisposition to seizures. In TLE, principal neurones are considered to be hyperexcitable and the three possible mechanisms for this are 1) altered membrane properties, 2) increased excitatory drive and 3) decreased inhibitory drive (Bernard et al., 1998, Schwarz et al., 2000). The loss of inhibitory drive could be a result of death of some interneurones, a functional disconnection from their excitatory afferents, down-regulation of their firing or other cause. For example the 'dormant basket cell' hypothesis was proposed to explain the contradictory observation of preservation of inhibitory cells but loss of inhibition in animal models of epilepsy (Sloviter, 1991b).

Alteration in inhibitory interneuronal populations has been shown in cortical tissue from patients with epilepsy although no distinct or single pattern has emerged (Ferrer et al., 1994). Loss of PV positive chandelier cells, considered to be the most powerful inhibitory neurones, has been proposed to be a key component in the aetiology of temporal lobe epilepsy (De Felipe 1999, Marco et al., 1996). Loss of PV-positive chandelier cells has been noted in lesional epilepsy as focal cortical

dysplasia together with an overall loss of GABAergic inhibitory interneurones, rearrangement of PV terminals and abnormal morphology of interneurones (Spreafico et al., 1998, 2000, Garbelli et al., 1999, Ferrer et al., 1992b). Loss of inhibitory interneurones, GAD and GABA transporter (GAT1) have also been shown in microdysgenesis like malformations (Spreafico et al., 2000). Depletion of inhibitory interneurones may explain the excitatory overbalance in these lesions. Such a local deficit of interneurones may represent a primary failure of tangential migration, survival or differentiation within the epileptogenic lesion. However, ongoing and selective secondary cell loss of interneurones is an alternative explanation and may explain the heterogeneity of interneuronal patterns observed between studies and cases (Marco et al., 1996, Ferrer et al., 1994).

Neuropeptide Y (NPY) is considered to be a powerful endogenous anti-convulsant (Furtinger et al., 2001, Vezzani et al., 1999a). Alteration in NPY systems has been shown in experimental epilepsy and human hippocampal sclerosis but not in cortical resections from patients with epilepsy. (See section (3.5.3) for inhibitory interneuronal populations in HS.) In animal models, NPY expression is enhanced in the hippocampus (Schwarzer et al., 1995), entorhinal cortex and temporal cortex following seizures, promoted by BDNF secretion (Vezzani et al., 1999b). In the hippocampus in HS, loss of hilar NPY interneurones (De Lanerolle et al., 1989, Mathern et al., 1995b, Sundstrom et al., 2001) but increased length of NPY fibres has been shown compared to autopsy controls (Furtinger et al., 2001), particularly involving the molecular layer of the dentate gyrus (DeLanerolle et al., 1989). This is considered to reflect adaptive NPY expression to counteract excess excitation from mossy fibres.

3.3.5 Hippocampal development.

The development of the hippocampus was studied by Arnold and Trojanowski (1996) in a series of brains ranging in age from 9 weeks of gestation to adulthood. Neurones destined for the hippocampal region are among the first born, but in terms of migration, and cytoarchitectural maturation the hippocampus is late compared to other regions. At the earliest stage in cortical development at 9 weeks the hippocampus is distinguished by the lack of a subventricular proliferative zone. By 15-19 weeks the hippocampus begins to flex over the parahippocampal gyrus

with the formation of the hippocampal fissure and by 25 weeks the volume of the hippocampus has expanded and the ventricular proliferative zone is virtually depleted. This is in contrast to the adjacent parahippocampal gyrus where cellularity in the ventricular and subventricular zone continues until 32 weeks. At 9 weeks the marginal zone in the hippocampus is densely populated with Cajal-Retzius cells. The number of these cells has reduced by 19 weeks, but they persist into adulthood. The subiculum matures at a faster rate than CA1 to CA4 subfields with maturation of neuroblasts into pyramidal neurones. The Cornu Ammonis forms a narrow band of cells compared to the adjacent subiculum and during development it is difficult to distinguish the boundaries of CA1- CA2 and CA2-CA3. Maturation of the neurones within the hippocampus proceeds in a gradient with the deeper neurones maturing before superficial neurones. This reflects the 'outside-in' course of neuronal migration. Maturation of CA2 and CA3 neurones at 25 to 32 weeks is likely to coincide with contact with mossy fibre afferents.

The three layers of the dentate gyrus are clearly evident by 19 weeks. The polymorphic layer is distinguished from CA4 by its increased cell density. The granule cell layer is sharply demarcated from the molecular layer. The granule cell layer continues to show an increase in cell density at 25 weeks in contrast to the rest of the hippocampus. The granule cells begin to mature and develop apical dendrites into the molecular layer by 34 weeks. Neurogenesis continues in the subgranular zone after all the post-mitotic neurones have migrated to the dentate plate. Mitotic figures, however, are not easily seen after 34 weeks. Interneurones migrate to the dentate gyrus and hippocampus via the ganglionic eminence (Polleux et al., 2002). By the end of gestation the architecture of the hippocampus is similar to that in adulthood. Myelination of tracts including the perforant pathway is visible at 9 months postnatal. There is likely to be some overlap in the molecular signals that control cortical and hippocampal development. For example, it has been shown that *doublecortin* has a role in the normal lamination of the hippocampus in mice (Corbo et al., 2002).

3.3.5.1 Normal anatomy of the adult hippocampus

The hippocampus is one of several related regions of archicortex which make up the *hippocampal formation*. The hippocampal formation comprises the *cornu ammonis* (CA1-4), dentate gyrus, the subiculum, pre and parasubiculum and the

Entorhinal cortex. The anatomical terminology used for the hippocampal formation in the present study is based on the original work of Lorente de No (1934), the atlas of the hippocampus by Duvernoy (1988) and the work of Amaral. The hippocampal subfields are defined by their position, shape and cytological characteristics. CA1 sector is medial to the subiculum and the pyramidal cells are smaller and less densely packed than in CA2. The transition between CA1 and 2 is characterised by a change in width of the pyramidal cell layer. This *stratum pyrimidale* is bounded on either side by the myelinated fibres in the stratum oriens and radiatum. The stratum oriens contains the basal dendrites of pyramidal cells and scattered interneurones. The boundaries between CA2 and CA3 are not clearly discernable and part of CA3 is enclosed by the blades of the dentate gyrus. 'Hilar neurones' therefore comprise CA3 pyramidal cells, CA4 pyramidal cells and interneurones of the polymorphic cell layer. In fact, there is ongoing controversy regarding the existence of distinct CA4 pyramidal cells in humans; however, both CA3 and CA4 cells have a unifying feature of being the recipients of mossy fibre axons, which distinguishes them from the CA1 pyramidal cells.

The granule cell layer of the dentate gyrus is easily defined. It is composed of a packed C-shaped ribbon of small neurones. The portion of the granule cell layer opposite CA1 is called the *suprapyramidal blade* and the other the *infrapyramidal blade*. The junction between the blades is referred to as *the crest*. The granule cell layer is 4-8 neurones deep and these are the only projection neurones of the dentate gyrus. Other neurones include interneurones such as basket cells and occasional chandelier cell-types (Freund and Buzsaki, 1996). The striking *mossy cells* identified in the polymorphic cell layer, which synapse on the apical dendrites of granule cells, are glutamatergic and therefore do not fit the classical description of interneurones.

The hippocampus has long been of interest to neuroanatomists because of its unidirectional synaptic circuit. Excitatory signals pass from the Entorhinal cortex pre-alpha cells via the perforant pathway, which crosses the subiculum, and then forms synapses with granule cells. The granule cells then send mossy fibre axons to synapse with CA4 and 3 pyramidal cells. These in turn send Schaeffer collateral fibres to CA1 (and CA2) pyramidal cells which then make synapses with cells in the subiculum and Entorhinal cortex (layer III), thus completing the loop. Mossy fibres can be identified in histological sections as they are zinc rich and in the Timm's method will react to produce a visible silver precipitate.

3.3.5.2 Animal models of hippocampal malformations

There are several animal models which show abnormal hippocampal development, useful in comparative studies. Hippocampal dysgenesis is seen in the *reeler* and *scrambler* mouse models. In the adult reeler mutant increased Cajal-Retzius cell numbers are found in the hippocampus although the number of principal neurones is normal (Coulin et al., 2001). In the p73 knockout mouse dysgenesis of the hippocampus is also seen. p73 is a member of the p53 family and implicated in cell survival and apoptosis and is expressed in the Cajal-Retzius cells (Yang et al., 2000, Meyer et al., 2002). In p73 deficient mice unusual arrangement of CA1-CA3 pyramidal cell layer is seen and the dentate gyrus lacks an infrapyramidal blade with the suprapyramidal blade appearing hypertrophied and extended. This suggests an abnormality of organisation rather than of granule cells migration. An absence of Cajal-Retzius cells and reelin secretion in the marginal zone was noted in these animals, but, in contrast to the *reeler* mouse, no neocortical laminar defects were seen (Yang et al., 2000). Genes controlling neocortical cerebral patterning may also be critical in the development of the hippocampus and archicortex. For example, the Emx2 mutant has a shrunken hippocampus and an absent dentate gyrus (Tole et al., 2000). Chemokines, which are involved in neuronal migration, may also influence hippocampal development and abnormalities in the dentate gyrus have been shown in mice lacking chemokine receptors (Lu et al 2002). Loss of neuronal determination genes e.g., NeuroD/BETA 2, leads to an absence of the dentate granule cell layer (Liu et al., 2000). Hippocampal malformation can be induced in animal models. Inter-uterine exposure to methylazoxymethanol (MAM) induces neuronal heterotopia in the hippocampus ; the heterotopic neurones display abnormal electrophysiological properties (Castro et al., 2002).

3.3.5.3 Granule cell neurogenesis

Ongoing neurogenesis in the adult is known to occur in the dentate gyrus. This was reported over 30 years ago in the adult rat. New cells are generated from progenitor cells in the subgranular proliferative zone of the dentate gyrus (Gould et al., 1998). Subsequent dendritic growth, synapse formation and axonal elongation

occur with integration of the new cell into local circuits. Neurogenesis in humans has more recently been confirmed (Eriksson et al., 1998, Singh-Roy et al., 2000). In the rat the number of new granule cells generated each month represents 6% of the total population suggesting they play an important role in hippocampal function e.g., memory processes (Cameron and Mckay, 2001). Granule cell neurogenesis in the rat is stimulated by BDNF (Katoh-Semba et al., 2002). Excitatory NMDA receptor activation, via perforant pathway stimulation, decreases neurogenesis. However, seizure activity appears to enhance neurogenesis (Parent et al., 1997), possibly stimulated by excessive cell death in the region.

3.3.5.4 Hippocampal dysgenesis in humans

Dysgenesis of the hippocampus has been described in human conditions other than epilepsy. In autism an increase in neuron density and a decrease in neuronal size has been described (Bauman, 1991). In schizophrenia a sizable literature on developmental abnormalities in the hippocampus has accumulated, some of which is controversial and has been refuted in other studies (Harrison, 1999). For example, smaller neuronal size has been noted in the hippocampus (Benes et al., 1991), hippocampal neuronal disarray, and increased number of neurones in the white matter (Conrad et al., 1991). Cytoarchitectural changes and 'tectonic' malformations have also been reported, involving CA1 and the subiculum in the hippocampus in temporal epilepsy in the absence of HS (Baulac et al., 1998, Thom et al., 2002a) (see Section 3.5).

3.4 Abnormalities of cortical development in epilepsy

Cortical malformations (previously referred to as neuronal migration disorders) have long been recognised as an underlying cause of epilepsy in a proportion of adults with early or late onset disease, as well as in the paediatric age group. They occur as a result of a disturbance in the normal migration and differentiation of nerve cells from the germinal matrix to the cortex at a critical time during development. These lesions were previously diagnosed and classified at post mortem examination, but, with the advances in neuroimaging they are more frequently diagnosed in life and surgical treatment may be appropriate in some cases (Sisodiya 2000). They represent a diverse group of pathologies (Crino et al.,

2002, Pilz et al., 2002) in which the normal laminar structure of the cortex is disrupted and abnormal neuronal morphologies may be present. They may either uniformly affect large regions of the cortex or be restricted to focal regions.

As immature neuroblasts generated in the periventricular matrix migrate centrifugally to the developing cortex along specialised radially orientated glial fibres and undergo a complex program of maturation, terminal differentiation, tangential migration or programmed cell death, interference with this complex process can occur at any step, involve both nerve cell or glia and may be genetically determined or caused by an external factor. In general, the type of insult causing the disturbance, its severity and time of occurrence, may influence the extent, location and type of malformation, and in turn affect the degree of clinical disability.

Structural malformations of this type now being recognised in adults with epilepsy are thus reducing the numbers of patients with the label of 'cryptogenic epilepsy'. It is estimated that 8-12% of cases of intractable epilepsy are associated with malformations of cortical development while 14-26% of paediatric surgically treated epilepsy patients have malformations (see Tassi et al., 2002). When lesions appear localised by imaging and electrophysiological studies, subsequent surgical excision may dramatically improve symptoms in some cases (Engel 1996, Sisodiya 2000). Surgical intervention may also be the appropriate therapeutic measure when seizures have become intractable. In other instances, surgical biopsy of a cortical malformation in the setting of epilepsy may be carried out as a diagnostic investigation where the differential diagnosis includes a neoplasm.

Much interest has recently focused on the molecules affecting neuronal migration which are defective in some brain syndromes. These may include molecules which affect cell motility in general; however, as in many syndromes only the brain is malformed, there must be specific factors that are uniquely expressed or regulated in the brain (Ross and Walsh, 2001). Firstly, migrating cells must receive signals to go, secondly to adhere to the glial fibres, thirdly to receive information on the direction of migration and finally a signal to stop migrating.

3.4.1 Classification of cortical malformations

Current classifications of these disorders are largely based on their structural appearances. As single gene mutations in selected malformations have been identified in recent years it is likely that a more precise molecular genetic classification will emerge (Crino et al., 2002) and the nomenclature of these lesions is likely to evolve (Kuzniecky and Barkovich, 2001). At present it is widely acknowledged that advances in the aetiology, diagnosis and pathogenesis of malformations of cortical development in epilepsy have not been paralleled by the evolution of a practical and universal nomenclature and that the present systems are unsatisfactory (Palmini and Luders, 2002, Tassi et al., 2002). Some classifications are predominantly based on neuroimaging appearances, others on histopathological features or embryological development. In addition, the type and timing of the environmental insult during development or the type of genetic mutation are likely to influence the pathological phenotype, as well as extent of the malformation observed.

It is generally agreed that a universally applied and practical classification system needs to be employed for both the clinical, radiological and histopathological diagnosis of these malformations in epilepsy. Such a classification may allow prognostic information to emerge; for example, it is suggested that some malformations termed 'focal cortical dysplasia' may have a better response to surgery than others (Sisodiya, 2000). The classification scheme should be based on scientifically valid observations and, as new information on the aetiology of these lesions emerges, it should be modified accordingly.

The term ' Malformations of cortical development' (MCD) is regarded as better than 'Neuronal migration disorders' to encompass these lesions as a whole as, although they are all developmental lesions induced during corticogenesis, they may not all represent defects in cell migration but also in cellular differentiation, maturation and defects in programmed cell death. In fact neuronal migration disorders are a subtype of MCD. The term 'Cortical dysplasia' has also been used as a generic term for all radiologically visible lesions, although their pathology may be diverse, including focal cortical dysplasia of Taylor type, polymicrogyria, hamartomas. 'Microdysgenesis' has been used by radiologists to indicate all 'occult' lesions on MRI whereas the pathology of these lesions may reveal distinct lesions

such as focal cortical dysplasia (see Tassi et al., 2002). Further discussion, on proposed and currently used classifications for MCD are reviewed in the following subsections of their neuropathology. Below is a table of the currently widely quoted classification system for MCD by Kuzniecky and Barkovich (2001) followed by the simplified system adopted in the following text and this study. The classification system I have adopted includes the same range of pathologies, based on radiological and pathological appearances, but without assuming pathogenesis where this is not established. For instance, in their classification (Kuzniecky and Barkovich, 2001) 'single ectopic white matter neurones' are categorised under 'neuronal migration failure', but in fact it is not yet excluded that these cells represent a failure of programmed cell death of subplate cells.

(For an outline of the further classification systems currently used in focal dysplasias See table 1 and text in Section 3.4.3.3.)

CLASSIFICATION SYSTEM FOR MALFORMATIONS OF CORTICAL DEVELOPMENT (Kuzniecky and Barkovich, 2001)

1) Malformations due to abnormal neuronal and glial proliferation
   a) Generalised
      i) Decreased proliferation (microlissencephaly)
         (1) Microcephaly with simplified gyral pattern of microlissencephaly with thin cortex
         (2) Microlissencephaly with thick cortex
      ii) Increased proliferation (none known)
      iii) Abnormal proliferation (none known)
   b) Focal or multifocal
      i) Decreased proliferation (none known)
      ii) Increased and abnormal proliferation (megalencephaly and hemimegalencephaly)
      iii) Abnormal proliferation
         (1) Non-neoplastic : Focal cortical dysplasia
         (2) Neoplastic (but associated with disordered cortex)
2) Malformations due to abnormal neuronal migration
   a) Generalised
      i) Classical lissencephaly (type I) and subcortical band heterotopia (agyria-pachygyria-band spectrum)
      ii) Cobblestone dysplasia (type 2 lissencephaly)
      iii) Lissencephaly : Other types
      iv) Heterotopia
   b) Focal or multifocal malformations of neuronal migration
      i) Focal or multifocal heterotopia
      ii) Focal or multifocal heterotopia with organisational abnormality of the cortex
      iii) Excessive or single ectopic white matter neurones
3) Malformations due to abnormal cortical organisation
   a) Generalised
      i) Bilateral diffuse polymicrogyria
   b) Focal or multifocal
      i) Bilateral partial polymicrogyria
      ii) Schizencephaly
      iii) Focal or multifocal cortical dysplasia (no balloon cells)
      iv) Microdysgenesis
4) Malformations of cortical development not otherwise classified

SIMPLIFIED CLASSIFICATION SYSTEM ADOPTED IN THIS STUDY

Diffuse or generalised malformations

Lissencephalies
Heterotopia
    Laminar, subcortical band (double cortex)
    Nodular, periventricular or subcortical
Polymicrogyria
Megalencephaly/hemimegalencephaly

Localised or focal malformations

Focal cortical dysplasia (FCD)
Microdysgenesis (MD)
Polymicrogyria
Schizencephaly
Heterotopia

3.4.2 Diffuse and generalised malformations

3.4.2.1 Lissencephaly

Lissencephaly describes a smooth brain lacking gyri. It is further subtyped according to its morphological appearances, the presence of associated abnormalities, and genetic characterisation. Two main histological patterns are identified, all with epilepsy as a predominant feature (Armstrong and Mizrahi, 1997):

Type 1 lissencephaly or classical lissencephaly, including Miller-Dieker syndrome and isolated lissencephaly sequence.

Type II lissencephaly or cobblestone lissencephaly – migration of neurones occurs beyond the marginal zones into the leptomeninges through gaps in the limiting basement membrane.

Lissencephaly, a condition with less than normal sulcation and thickening of the cortical grey matter, is characterised by a more or less four-layered cortex (the

majority of neurones being located in the fourth layer), which has no relation to the normal six-layered cortex, except that the marginal zone is preserved (Gleeson and Walsh, 2000). This large family of MCD overlaps with another condition, 'Double cortex syndrome' or subcortical band heterotopia. Lissencephalies are grouped together because of a shared common mechanism of incomplete neuronal migration to the cortex. Mutations in two genes, LIS1 and DCX, account for the majority of cases (75%) of classical lissencephaly (Pilz et al., 1998). Genotype and phenotype analysis reveal a gradient in the severity of the lissencephaly. There is evidence of differences in anterior-posterior gradients in MRI studies between XLIS and LIS1 cases, with more severe posterior involvement in LIS1 mutations and more severe anterior involvement in DCX families (Dobyns et al., 1999). Neuropathological findings in lissencephaly reveal microcephalic cerebral hemispheres with almost complete agyria, except for the temporal lobe and hippocampus. Callosal agenesis may be present. The claustrum and external capsule may be absent and the ventricles enlarged, with reduction in white matter volume (Armstrong and Mizrahi, 1997). The cortex is thick and four-layered: an outer cell-free layer (molecular layer), a superficial thin cell layer and cell sparse layer, and a deeper, thick disordered cell layer. Dysplasia of the olives and dentate nucleus may be present (Harding and Copp, 2002).

In isolated lissencephaly point mutations and deletions on LIS1 gene (17p13.3) are identified in 40% (Ross and Walsh, 2001). Mice with LIS1 mutations show abnormalities in the cerebral cortex, cerebellum and hippocampus, although, unlike the reeler cortex, there is no 'inversion' of the cortex (Ross and Walsh, 2001) and the cortex malformation is relatively milder compared to human phenotype. Birth-dating studies indicate poor layer specificity, with some neurones that should be destined for the superficial layers positioned in deeper layers (Gleeson and Walsh., 2000). LIS1 is expressed in the VZ and in Cajal-Retzius cells. LIS1 is an autosomal gene and individuals with lissencephaly have a mutation in one copy. Therefore individuals display haploinsufficiency, which suggests that a 50-fold decrease in LIS1 protein levels is sufficient for the lissencephaly phenotype (Gleeson and Walsh, 2000). LIS1 homozygous mice do not survive, suggesting two copies of the gene are required for other key events outside the CNS. The protein (Lis1) is the non-catalytic subunit of platelet activating factor (PAF)-acetylhydrolase (PAFAH) and one of its known functions is to regulate platelet-activating factor which is a potent pro-inflammatory phospholipid. High levels of

LIS1 are expressed in neurones and this may explain why the manifestations of mutation of this ubiquitously expressed protein are apparently confined to the brain. It is not clear if the influence of Lis1 on neuronal migration is through PAF. Lis1 also binds to tubulin and may stabilise the - microtubule cytoskeleton (Sapir et al., 1997, 1999, Reiner, 2000). Lis1 also binds to NudEL which regulates dynein function in migrating neurones (Hatten, 2002) and therefore it probably has an effect on nucleokinesis (Gleeson and Walsh, 2000).

The second type of classical lissencephaly called XLIS is seen in males of families with female members showing double cortex malformation. Mutations in doublecortin gene (DCX/XLIS) on chromosome Xq22 have been demonstrated. This phenotypic variation occurs because females inactivate one X-chromosome during development so that, on average, half of the neurones generated will reach the cortex. Double cortex patients have milder clinical manifestations than the males with lissencephaly, with 25% having normal intelligence and only mild to moderate epilepsy in contrast with patients with lissencephaly who display severe epilepsy and mental retardation (Dobyns et al., 1996). DCX is expressed in developing neurones and encodes a 40kDa soluble protein called doublecortin (Dbcn). This protein is expressed only in neurones (Gleeson et al., 1999), is tightly developmentally regulated and binds to tubulin structures to promote precipitation and stabilisation. Lis 1 and Dbcn may also be linked to molecules in the reelin pathway through cAbl and Dab1 (Ross and Walsh, 2001). There is no known interaction between Lis1 and Dbcn in cells and these proteins are unrelated to each other structurally (Gleeson and Walsh, 2000).

A new group of lissencephaly with cerebellar hypoplasia has been recognised (Ross et al., 2001) (LCH). Recently a mutation in the human reelin gene has been identified in one family (Hong et al., 2000). Mutations in astrotactin (ASTN1) in mice produce a similar malformation, this protein being required for glial-guided migration of neurones.

3.4.2.2 Grey matter heterotopia

Grey matter heterotopias are classified according to their anatomical location (periventricular, subependymal, subcortical, leptomeningeal) or by their morphological appearances (nodular, band, or laminar). They are becoming more

widely recognised in the adult population of epilepsy patients due to recent advances in neuroimaging (Raymond et al, 1994c, 1995). In some cases, 'mixed' rather than 'pure' types of heterotopias are encountered and in particular heterotopias may be seen as part of more complex malformations.

3.4.2.2.1.1 Laminar / band heterotopia (Double cortex syndrome)

Jakob in 1936 was among the first authors to describe laminar or band heterotopias of grey matter in the sub-cortical white matter (Jakob, 1936). This constitutes one of the generalised disorders of neuronal migration and is also referred to as the 'double cortex syndrome'. Most patients present with generalised or multifocal epilepsy and a variable degree of mental retardation, but in contrast to other related migrational disorders, such as agyria and pachygyria, it is more often compatible with survival into adult life. Recent advances in MRI imaging have improved recognition of this malformation in the setting of epilepsy during life (Barkovich et al., 1989, Palmini et al., 1991c). Surgical resection often yields inadequate results (Bernasconi et al., 2001).

Macroscopically, the lesion corresponds to bilateral, often symmetrical, bands of grey matter, separated from the overlying cortex by an intervening band of white matter. The heterotopia follows the contours of the overlying cortex, which is usually of normal thickness and has a normal gyral convolutional pattern. The thickness of the heterotopia can vary from case to case, ranging from 5 mm to 20 mm in one series (Raymond et al., 1995), and also from region to region in a single case. In one study there was a suggestion that thicker bands were associated with a less well-developed overlying cortex (Palmini et al., 1991c). The lobes affected are usually the fronto-central or parietal-occipital with less common involvement of the temporal, inferior frontal and cingulate cortices. The distance between the bands and the overlying cortex is also variable; in some cases it is only a thin strip of white matter, whilst in others the bands are located deeper within the centrum semi-ovale (Friede 1989, Raymond et al., 1995).

Histological examination of heterotopia reveals a composition of differentiated, randomly orientated and focally clustered cortical nerve cells of all types, including pyramidal cells. Fibrillary astrocytes and oligodendrocytes are also present. The overlying cortex displays a normal hexalaminar architecture in most cases. In other instances there may be poor delineation of cortical layers V and VI, which

merge with the underlying white matter and heterotopic tissue. In these instances the cortex may have abnormal gyral pattern and resemble a pachygyria. There are also cases where the overlying cortex is transitional in appearance between pachygyria and normal (Pinard et al., 1994). Quantitation studies have suggested an increased neuronal density in the cortex overlying band heterotopias suggesting a failure of programmed cell death.

A developmental link between agyria (lissencephaly) and laminar heterotopia was first reported in a study of two affected families in which females have laminar heterotopia and male offspring a more severe malformation (lissencephaly) (Pinard et al., 1994). Mutations in the doublecortin gene on the X chromosome have been identified in these families (Des Portes et al., 1998) and also some sporadic cases of double cortex (Allen and Walsh 1999). Mosaicisms in this gene have been associated with variable malformative phenotypes due to variable inactivation of the X chromosome (Gleeson and Walsh, 2000, Gleeson et al., 2000). Doublecortin is expressed in the brain during corticogenesis in the processes of migrating and differentiating neurones (Gleeson et al., 1999) and is a microtubule binding protein with roles in stabilising the neuronal cytoskeleton (Allen and Walsh 1999, Gleeson and Walsh, 2000).

3.4.2.2.1.2 Periventricular nodular heterotopia

Periventricular heterotopias are one of the more common forms of grey matter heterotopia encountered in adult epilepsy patients. A genetic predisposition for this lesion has been recognised with several members of the same family, usually females, affected by similar malformations (Eksioglu et al., 1996) with a susceptibility locus at Xq28; affected males appear not to survive gestation. A periventricular heterotopia gene (Filamin1) has been identified which codes for a 280kDa actin-cross linking phosphoprotein essential for normal cortical migration (Fox et al., 1998, Ross and Walsh, 2001). Depth electrode studies have shown that the source of the seizures is in the heterotopia (Kothare et al., 1998).

The malformation is characterised by nodules or bands of heterotopic grey matter beneath the ependymal lining of the outer margins of the lateral ventricles. The trigones and occipital horns of the lateral ventricles are the more commonly

affected sites, although the whole length of the lateral ventricles may be involved. In general, however, there is less often involvement of the frontal and temporal horns and the third and fourth ventricles are spared. In some cases isolated or scattered single nodules may be found along the body of the ventricle (Dubeau et al., 1995) giving a 'beads on a string' appearance on MRI. It has been noted that, when the temporal horn is involved, distortion of the hippocampal formation can be seen (Raymond et al., 1994c).

In one series of 13 cases the lesions were unilateral in five and bilateral in eight (Raymond et al., 1994c) and in a further series of 33 patients the lesions were unilateral in 58% of cases and bilateral in 42% (Dubeau et al., 1995). It was noted in the earlier series that when unilateral, the heterotopia was always right-sided, although such an observation was not confirmed by Dubeau. When bilateral, however, the heterotopias are often symmetrical. Associated dilatation of the lateral ventricles has been noted in some cases (Eskioglu et al., 1996, Dubeau et al., 1995).

Individual nodules range in diameter from 2–10 mm (Eskioglu et al., 1998), with one report arbitrarily dividing the nodules according to size – large nodules were greater than 5 mm and small nodules less than 5 mm (Raymond et al., 1994c). When the subependymal heterotopias appear 'band-like' or 'laminar' it has been noted that the outline is irregular and bumpy on MRI, suggesting a 'coalescing' of multiple nodules (Raymond et al., 1994c). The morphological type of heterotopia, i.e., single nodules or 'band like', does not appear to influence whether the malformation is bilateral or unilateral (Raymond et al., 1994c).

Histologically the heterotopias are composed of islands of mature nerve cells, resembling cortical neurones rather than those of deep grey nuclei. Multiple neuronal types have been recognised within the heterotopias, including medium to large pyramidal cells, small pyramidal cells and non-pyramidal cells. Nerve cells are orientated in multiple directions, but in some cases rudimentary laminae can be seen, reminiscent of cortical organisation including laminar arrangement of neurofilament positive neurones (personal observation). Intermingled GFAP positive fibrillary astrocytes and oligodendroglia may be present. The nodules are relatively sharply demarcated, separated by septa of myelinated fibres.

Calcifications are notably absent and no cytologically dysplastic nerve cell elements are observed. Cajal-Retzius cells have not been identified within nodules although small reelin-immunopositive neurones have been seen (personal observation). The lack of Cajal-Retzius cells may explain the relative lack of laminar organisation within these nodules compared to the adjacent cortex. Others have also noted networks of neurofilament positive fibres within the nodules and dense presynaptic terminals around the heterotopic cells with synaptophysin immunohistochemistry (Eskioglu et al., 1996). The origin of this synaptic input, whether local or distal, is not established. However, a recent study of nodular heterotopias in children using dye-tracing methods demonstrated limited connectivity of fibres into or out of the nodules (Hannan et al., 1999). In addition, abnormal immature calretinin-positive neurones were present within the nodules, which may be indicative of impaired inhibitory function although they were noted to be present in similar densities to the cortex. Inhibitory neuronal subsets have been identified in animal models of heterotopia (Sun et al., 2001). In a personal study of five cases of grey matter heterotopia calretinin, parvalbumin and GAD positive interneurones were present within the heterotopia but in significantly reduced numbers compared to cortex (Paper submitted). NPY positive interneurones have also been demonstrated in heterotopia (Hannan et al., 1999). I consider the source of interneurones in heterotopia is likely to be from the ventricular zone rather than tangential migration from the ganglionic eminence as for cortical GABAergic interneurones (Letinic et al., 2002).

As previously mentioned, periventricular heterotopia may be an isolated finding, either on neuroimaging or at autopsy, or complicated by other cerebral malformations. Associated abnormalities include microcephaly, agenesis of the corpus callosum, cerebellar hypoplasia, polymicrogyria, agyria, pachygyria and cortical dysplasia (Dubeau et al., 1995, Friede 1989). In Dubeau's study of 33 patients with periventricular heterotopias, 13 patients also had subcortical nodular heterotopias and this combination of heterotopias has also been documented in other series (Raymond et al., 1995). These cases with both periventricular and subcortical heterotopias were always unilateral malformations with heterotopias occupying the white matter of central-parietal or temporo-occipital regions forming 'mass'-like lesions of clustered nodules. In a considerable proportion of these cases, additional abnormalities of gyration of the overlying cortex, including polymicrogyria, thinning of the cortex, neuronal loss and abnormal sulcation, were

observed, whereas cortical malformations are less commonly observed with periventricular heterotopias alone. Occult abnormalities of the cortex, however, have been described in periventricular nodular heterotopia and this may be of relevance to the epileptogenesis (Sisodiya, 2000). In a proportion of cases hippocampal sclerosis has also been documented in association with subependymal heterotopias (Raymond et al., 1994a, Dubeau et al., 1995).

The likely pathogenesis of periventricular heterotopias proposed is that a fraction of the post-mitotic neurones are incapable of leaving the ventricular zone. In family pedigrees with these heterotopias, affected females show mutation in filamin 1 gene (FLN1). Filamin is an actin cross-linking protein expressed at high levels in cells in the lateral ventricle (Gleeson and Walsh, 2000) and may be important in the onset of cellular migration and is involved in the extension of the cell as it moves along the radial glial fibre. In addition to its effects on actin, Filamin1 also binds to other proteins that are possibly involved in neuronal migration including integrins and presenelin1 (Gleeson and Walsh, 2000). The identification of sporadic cases of periventricular heterotopia in females and males suggests that additional genes may be involved and in some cases FLN1 mutations are not identified (Spalice et al., 2002).

Although a genetic predisposition is suggested in many cases, exogenous factors such as focal subependymal haemorrhages or infarcts have also been implicated as a possible perinatal event. Intrauterine toxic, metabolic and infectious insults have also been proposed as causative factors. In some cases, maternal complications during pregnancy have been recorded, including pre-term deliveries, low birth-weights, twin pregnancies and pre-eclampsia, but no distinctive perinatal factors have emerged. In one report, occipital subcortical nodular heterotopias causing intractable epilepsy were associated with an ipsilateral hypoplastic left posterior cerebral artery, implying a prenatal ischaemic event as the cause of this localised malformation (Reutens et al., 1993).

Studies using invasive electrical recordings show there is evidence that periventricular nodular heterotopias are intrinsically epileptogenic (Dubeau et al., 1995, Kothare et al., 1998). The results of surgical treatment for these lesions have been uniformly disappointing.

3.4.2.3 Polymicrogyria

This is a condition in which there is excessive folding of an abnormally thin cortex which can be focal, e.g., perisylvian dysplasia (Armstrong and Mizrahi 1997) or generalised (Copp and Harding, 1999). Focal forms particularly affect the frontal, perisylvian, parieto-occipital or mesial occipital regions. The MRI may suggest pachygyria but high-resolution images will show classical polymicrogyria. Four-layered and unlayered types are recognised and likely to reflect timing of the causative insult during development. In the four-layered type the cortex comprises an outer molecular layer, cellular outer layer, a cell sparse layer and a disorganised inner layer. Associated cortical malformations may be present, including neuro-glial leptomeningeal heterotopias, FCD-like areas, abnormal cortical myelination, and calcification. Epilepsy and mental retardation are common findings. The aetiology is generally considered more related to environmental inter-uterine insults than genetic causes (Harding and Copp, 2002). One important cause is intrauterine CMV infection. In animal freeze-lesion models, a microgyric malformation similar to polymicrogyria is induced during development (Chevassus-au-Louis et al., 1999a) and the resultant hyper-excitability is caused by reorganisation of neuronal networks at the border of the malformation (Jacobs et al., 1999, Schwartz et al., 2000). Recent studies in human polymicrogyria have demonstrated an excess of reelin immunopositive Cajal-Retzius cells in the region of the malformation (Eriksson et al., 2000), which may represent part of the malformation or a cellular response to an area of cortical injury. However, the detection of polymicrogyria in several family members indicates that there are some inherited causes, for example in cases of peri-sylvian polymicrogyria. At least five distinct genetic syndromes with polymicrogyria have been observed. Diffuse and peri-sylvian polymicrogyria appears to be X-linked in families, although in other families an autosomal pattern of inheritance is demonstrated (Kuzniecky and Barkovich, 2001).

3.4.3 Focal malformations

3.4.3.1 Focal cortical dysplasia (FCD)

Focal cortical dysplasia is the most common malformation encountered in surgical series (Sisodiya, 2000) with characteristic neuropathological appearances. The term focal cortical dysplasia (FCD) has been used to describe a specific localised and well-recognised cortical malformation with a distinctive histological appearance. The features of FCD were first delineated by Taylor and colleagues (Taylor et al., 1971) at the Maudsley Hospital in their examination of lobectomy specimens removed from ten adults with intractable seizures. The detailed pathological features described in this first series define particular and common characteristics that have been verified subsequently by the histological findings in more recent series (Janota and Polkey, 1992, Palmini et al., 1991a, 1991b, Jay et al., 1993, Wyllie et al., 1994, Spreafico et al., 1998, Tassi et al., 2001, 2002, Urbach et al., 2002).

FCD may involve any lobe but it occurs more-often in the frontal than temporal lobes (Blumcke et al., 1999d, Kuzniecky and Barkovich, 2001) or around the central sulcus and it is usually unilateral. In more recent series extra-temporal FCD also predominate (Urbach et al., 2002). The clinical manifestations are variable; seizures usually begin in the first decade and may be partial simple motor, complex partial or secondary generalized. The localization of the lesion dictates the seizures (Kuzniecky and Barkovich, 2001). The abnormality is assessed and localised pre-surgically by electrophysiological and neuroimaging techniques and by intraoperative electrode recordings. In many cases FCD is sharply bound by normal appearing cortex on MRI. In cases where the extent of the surgical resection has been limited owing to the location of the lesion (e.g., involving motor cortex), the 'lesionectomy' may be partial and no normal cortical tissue is present at the surgical resection margins.

The macroscopic examination of the resected specimen is often unremarkable, although in some cases 'smooth cortex lacking sulci', 'thickening' of the cortical gyri or blurring of the cortical-white matter junctions has been observed (Taylor et al., 1971, Janota and Polkey, 1992). Detection by the pathologist of macroscopic abnormalities depends to some extent on the size of the resected tissue, this being less likely in small cortical biopsies carried out for diagnostic purposes than larger

therapeutic resection specimens as lobectomies and hemispherectomies. On slicing the specimens they may feel firmer or more 'rubbery' in consistency than normal brain tissue owing to the extensive gliosis often present. In some resections the region of FCD appears sharply bound by normal cortex whereas, in others, several foci of dysplasia are present within one specimen forming 'skip lesions'. This can in some cases make the assessment of the 'completeness of excision' difficult for a pathologist as further adjacent areas of dysplasia may have been left behind.

Histologically the predominant features are abnormalities of both the architecture and cellular composition of the cortex. Anarchic, hypercellular cortices with disruption of normal cortical lamination are the striking low-power features. In addition, impressions of persistent columnar alignment of cortical nerve cells in the vertical axis may be apparent. Heterotopic nerve cells may be present in the molecular layer of the cortex or found in increased numbers in the underlying juxtacortical white matter (Crino et al., 2002).

Cytologically, the cortical nerve cells show aberrant differentiation with bizarre, 'exotic' and cytomegalic nerve cells scattered in all cortical layers. These cells reach the size of Betz cells and many maintain an overall pyramidal shape. The Nissl material is often prominent, 'tigroid' in appearance and unevenly distributed, and nuclear membranes may show irregular 'elliptical' thickenings. In a proportion of these abnormal nerve cells, cytoplasmic vacuolation has also been reported (Duong et al., 1992, Yammanouchi et al., 1996). The orientation of these nerve cells often appears random within the cortex, with loss of the normal vertical polarity of the apical dendrites. Clustering of nerve cells may also be observed. Additionally silver impregnation techniques, such as the modified Bielschowsky stain, highlight increased numbers and abnormal branching and arborisation of dendritic processes (Janota and Polkey, 1992), although in a minority of cells a diminution in the number of processes may be seen (Wyllie et al., 1994).

Although most authors divide the cytological components of FCD into two predominant cell types, abnormal nerve cells and glia (Crino et al., 2002), many more recent papers (e.g., Palmini and Luders 2002, Tassi et al., 2002) distinguish the presence of 'Giant neurones' from 'dysplastic neurones' and 'immature neurones' and subdivide FCD according to the cell types present (See classifications in Table 1 and Section 3.4.3.3). Giant neurones are those that show an increase in size compared to normal layer V pyramidal cells and are found scattered in any

layer from II to VI. These cells display a preserved pyramidal morphology and do not show excessive staining with neurofilament antibody, although some have demonstrated increased staining with neurofilament and silver stains (Tassi et al., 2002). By contrast, dysplastic neurones show abnormal shape, orientation and cytoskeleton with silver and neurofilament stains and may be smaller than normal pyramidal cells. Immature neurones are round homogenous cells with a thin rim of cytoplasm and may also be referred to as 'oligodendroglial-like cells'.

In a large proportion of cases an additional feature in FCD is the presence of abnormal, enlarged astrocytic cells. These cells have been variably described as 'balloon cell glia', 'grotesque cells', 'dysplastic glial cells', or 'uncommitted cells' – the last term in recognition of their intermediate cytological appearance between nerve cell and astrocyte. They are histologically characterised by abundant glassy cytoplasm with large, sometimes multiple nuclei and relatively few processes, in contrast to the reactive-hyperplastic astrocytes. They are more often located in the deeper regions of the cortex, extending into the white matter in well-defined aggregates, although they may appear more superficially within the cortex (layer I). They are also morphologically reminiscent of similar cells seen in cortical tubers, subependymal giant cell astrocytoma and gangliogliomas. In two of the cases of FCD reported by Janota and Polkey (1992) the presence of these abnormal astrocytes was the dominant histological feature.

3.4.3.1.1 Immunohistochemistry in FCD

Cytoskeletal abnormalities have been documented in the abnormal nerve cells in areas of focal cortical dysplasia (Duong et al., 1992). In Duong's study, the coarse intracytoplasmic fibrillar inclusions showed positive immunolabelling with antibodies to high and medium-weight neurofilaments, phosphorylated neurofilaments, microtubule-associated protein (MAP), tau antibody and also weak staining with anti-ubiquitin labelling. Staining of the abnormal dendritic processes of these cells and absence of staining of the intracytoplasmic vacuoles was noted. These filamentous inclusions are reminiscent of the tangles observed in Alzheimer's disease; however, the nerve cells in cortical dysplasia do not label with paired helical filament antibody (abnormally phosphorylated tau), and ultrastructural studies do not confirm such structures. The positive staining of dysplastic nerve

cells with neurofilament epitopes was of strong intensity and visible in cells in all cortical laminae, as distinct from the weak staining patterns observed in nerve cells of laminae 2 and 6 in the normal cortex. The authors postulate progressive accumulation and phosphorylation of neurofilaments within the cytoplasm of these dysplastic cortical nerve cells, possibly due to a failure of normal axon transport, or increased transcription and production (Duong et al., 1992). Further studies have demonstrated increased expression of microtubule-associated protein MAP-2 (Yammanouchi et al., 1996) and early forms of MAP (MAP-1b and MAP-2c), (Yammanouchi et al., 1998, Crino et al., 1997) in the perikarya of dysplastic nerve cells in cortical dysplasia by immunohistochemistry and in-situ hybridisation techniques. These proteins play a role in normal growth and sprouting of neuronal processes. The increased expression observed in FCD may reflect an increased plasticity and remodelling of dendrites in these nerve cells or a failure in cell maturation.

The persistent staining of nerve cells with the embryonal form of N-CAM (E-NCAM) is also suggestive of a failure of cell maturation in FCD (Wolf et al., 1995a, Kerfoot et al., 1999). Biochemical analysis of samples of cortical tissue from epilepsy patients, including those with mild cortical dysplasia, however, showed a reduction in total N-CAM compared to control non-epileptic cortical tissue (Hamberger et al., 1993a and b). Expression of developmental neurofilament nestin and internexin in the cells of FCD also supports a developmental immaturity of these cells (Crino et al., 1997).

The balloon cell glia in FCD often show variable intensity of staining with antibodies to glial fibrillary acidic protein (GFAP) (Janota and Polkey, 1992, Jay et al., 1993) and this may correlate with the findings of variable amounts of intermediate cytoplasmic filaments on electron microscopy. Some of these cells label with vimentin (Palmini and Luders, 2002, Urbach et al., 2002). They may express markers of cytoskeletal immaturity including nestin and CD34 (Urbach et al., 2002). In addition, some cells label with neuronal markers and may even show dual labelling with neuronal (synaptophysin, neurofilament, PGP9.5) and glial markers (Vinters et al., 1993, Vital et al., 1994), thus reflecting an intermediate glial-neuronal differentiation. This may be reflected in their morphology on H&E, with cells having a nucleolated nucleus characteristic of a neurone and glassy hyaline cytoplasm, a feature more suggestive of astrocytic differentiation (Crino et al., 2002). Reactive astrocytes are often prominent both in the cortex and the white

matter and can be distinguished from balloon cells in the region of dysplasia as, although they express GFAP, they do not express nestin and often have prominent cellular processes. Due to the vague resemblance of these balloon cell glia to neoplastic gemistocytic astrocytes, an assessment of the proliferation of these cells was carried out (De Rosa et al., 1992). De Rosa found virtual absence of labelling with cell proliferation marker (PCNA) and considered increased AgNOR numbers observed in these cells as a reflection of increased cell ploidy rather than cell turnover. In addition, Wolf et al. (1995a) showed only occasional positive labelling of glial and neuronal cells in cortical dysplasias with Ki67 antibody, in keeping with the malformative nature of the lesion. A further study using MIB1 (Ki67) to label cells in cycle showed low labelling in regions of dysplasia particularly in multinucleated balloon cells; this may be indicative of amitotic division of balloon cells in FCD (Crino et al., 2002). Recent immunohistochemistry studies have demonstrated doublecortin immunoreactivity in giant, dysplastic cells of FCD and TS which may be relevant to the pathogenesis of these lesions (Masashi et al., 2002). No doublecortin gene mutations were, however, identified in the cytologically similar dysplastic cell elements in ganglioglioma (Becker et al., 2002b), suggesting this gene not to be involved in the latter.

3.4.3.1.2 Sub-classification of FCD and radiological appearances

Terminological issues abound in the classification of FCD. Some groups (Tassi et al., 2002, Garbelli et al., 2001) have proposed adopting subcategories of 'Taylor-type focal cortical dysplasia' for the typical lesion with balloon cell glia and dysplastic neurones as opposed to other 'non-specific cortical dysplasias' lacking these cell types. Palmini and Luders also propose subdividing FCD in two types (types I and II) depending on whether or not dysplastic neurones or balloon cell glia are present (see Table 1). There is evidence from structural imaging that cortical dysplasia with dysplastic cell elements are more readily visualised on MRI (Palmini and Luders, 2002); however, it is well recognised that 'Taylor type' FCD can be normal on MRI (Desbiens et al., 1993) and in one series of 15 cases of Taylor type FCD the MRI was normal in 33% (Tassi et al., 2002). Typical MRI appearances of FCD include an increase in cortical thickness, blurring of the cortical-subcortical transition zone and an increased signal on T2-weighted, proton density or FLAIR sequences as confirmed in our recent study at Queen Square (Gomez-Anson et al., 2000). In many cases a tail like extension of the cortical signal tapers into the underlying white matter towards the lateral ventricle (Urbach et al., 2002, Tassi et al., 2002),

which is probably identical to the focal 'transmantle' cortical dysplasia described by Kuzniecky (Kuzniecky and Barkovich, 2001). There is some evidence that 'transmantle' FCD cases are associated with more widespread cortical abnormalities on quantitative MRI (Sisodiya and Mitchell, personal communication) and probably correlate with greater numbers of balloon cells and hypomyelination of the underlying white matter (Urbach et al., 2002). In many cases the histopathological changes of FCD extend outside the radiologically defined lesion and, similarly, the epileptogenic zone extends out of the imaging boundaries of the lesion (Tassi et al., 2002).

3.4.3.1.3 Pathological lesions identified in association with FCD

Additional histological features reported in FCD include subpial layers of myelinated axons, a reactive cellular astrocytosis, subpial fibrillary (Chaslin's) gliosis, loss of axons, and increased numbers of corpora amylacea (Janota and Polkey, 1992).

Tumours have been reported to occur adjacent to cortical malformations and include pilocytic astrocytomas, fibrillary astrocytomas, gangliogliomas, dysembryoplastic neuroepithelial tumours (DNT) and meningioangiomatosis (Wyllie et al., 1995, Prayson et al., 1993, Daumas-Duport et al., 1988, Prayson and Estes, 1995). The presence of slow-growing, mixed glial-neuronal 'hamartomatous' tumours such as ganglioglioma and DNT, which are the most common tumours found occurring near to areas of cortical dysplasia, raises the possibility of a common origin for these lesions. Indeed the cytological elements of ganglioglioma (atypical neurones and glial cells) are similar to cortical dysplasia and, in cases of small biopsies, it is difficult to distinguish between the two (Prayson and Estes., 1993). Alternatively these low-grade tumours may be arising secondarily on a background of a malformed cortex.

Less commonly reported features associated with cortical dysplasias are pathologies of an inflammatory, degenerative or destructive nature. Chronic meningeal inflammation, small infarcts (Desbiens et al., 1993), contusions of the cortex and dystrophic calcifications were observed in a small proportion of cases in one series (Prayson and Estes, 1995b). Emphasis is placed on the need to exclude an iatrogenic cause for any secondary pathology, such as insertion of depth electrodes prior to surgery. In a study of cortical dysplasia in paediatric epilepsy, cystic

encephalomalacia, cystic infarcts, calcification, non-specific inflammation including microglial nodules, and Rasmussen's encephalitis were seen in addition to the malformative abnormalities (Mischel et al., 1995). The combination of Rasmussen's encephalitis with cortical dysplasia has been documented by others (Robitaille 1991, Rosenberg et al., 1996). It has been reported in combination with other lesional pathologies, as cavernomas, in patients with epilepsy. Rasmussen's encephalitis is a chronic encephalitis of unknown aetiology characterised by neurono-glial nodules and chronic inflammation with neuronal loss and gliosis and it is associated with intractable seizures in childhood. It has been speculated that the co-existence of Rasmussen's encephalitis with a second pathology throws a light on the pathogenesis and that it results from a disruption in the blood-brain barrier allowing an 'auto-immune' process to occur (Hart et al., 1998). However, it is commonly recognised that excessive neuronophagia may occur in the setting of cortical dysplasia as a result of ongoing death of neurones (Crino et al., 2002).

Hippocampal sclerosis may also occur in combination with cortical dysplasia (temporal or extra-temporal) giving a 'dual pathology' for the origin of seizures. The exact incidence of hippocampal sclerosis in the setting of malformations is unknown, as in many cases the hippocampus is not available for pathological examination and post-mortem studies are lacking. In one series, using volumetric MRI, but without pathological confirmation, malformations were present in 15 out of 100 patients with hippocampal sclerosis, and in one of these cases the malformation was a cortical dysplasia (Raymond et al., 1994a). In another study, hippocampal sclerosis was documented in 43% of cases of architectural dysplasia and in all cases the dysplasia was ipsilateral to the hippocampal sclerosis (Tassi et al., 2002). In other studies (Ho et al., 1998) hippocampal dual pathology was seen in 87% of cortical dysplasia cases. This same study demonstrated a high incidence of bilateral hippocampal sclerosis in association with FCD on MRI. A histo-pathological study of glial-neuronal cortical malformations showed the additional presence of hippocampal sclerosis in three of 24 cases (Wolf et al., 1995a) and in another study it was observed in 9% of cases when mesial temporal lobe structures were included in the resections (Prayson and Estes, 1995). The possible relationships between these two lesions are either that the hippocampal sclerosis is secondary to or 'kindled' by prolonged seizures induced by the malformation or (a currently favoured hypothesis) that both share a common malformative-embryonic origin (Blumcke et al., 2002, Tassi et al., 2002). Another report has documented increased hippocampal neuronal loss when the 'second' lesion, e.g., cortical

dysplasia, is adjacent to the hippocampus compared to similar lesions in more anterior or posterior locations (Mathern et al., 1995a). Another study of cortical dysgenesis in epilepsy, however, demonstrated that hippocampal sclerosis, if present, may occur contralateral to the malformation (Raymond et al., 1995). From this it can be concluded that any patho-physiological link between cortical dysplasia and hippocampal sclerosis needs further study. However, in view of an association between FCD and hippocampal sclerosis, it is vital in pre-surgical evaluation that hippocampal volumetric MRI studies are carried out; the possibility of dual pathology will guide the appropriate surgical approach as removal of both lesions may be necessary.

3.4.3.1.4 Developmental links between tuberous sclerosis and FCD

According to National Institutes of Health consensus criteria (Hyman and Withmore, 2000), the major features of tuberous sclerosis (TS) in the CNS are subependymal nodules and cortical tubers. Subependymal giant cell astrocytomas (SEGA) are benign tumours, usually diagnosed in the first two decades of life, and their association with TS is well established (Thom and Scaravilli, 1997). They are typically located in the lateral ventricle wall. Histologically they are composed of cells arranged in broad sheets, many with abundant eosinophilic cytoplasm and eccentric nuclei. GFAP expression can be weak in these cells, but expression of neurofilament and class III tubulin has been shown (Hirose et al., 1995). Subependymal nodules are smaller hamartomatous lesions with similar cellular components. Cortical tubers are histologically characterised by the presence of dysplastic neurones and abnormal balloon cell glia and have histological similarities to FCD lesions. It is controversial as to whether FCD represents a 'forme fruste' of TS as initially suggested in Taylor's monograph (Taylor et al., 1971). FCD is more often a solitary lesion, lacks clinical features of TS and the radiological and macroscopic features of tubers are less subtle compared to FCD (Taylor et al., 1971, Crino et al., 2002). However, when a single cortical lesion is present at post mortem or in surgical resection, in the absence of clinical, radiological or genetic information regarding TS, the distinction of TS from FCD on histological grounds alone may not be possible (Crino et al., 2002). In the initial pathological descriptions of FCD by Taylor et al. (1971) the histological similarities to tubers were described. It was acknowledged that no single feature was present as pathognomonic of a tuber, but histological features considered more suggestive

included a paucicellular cortex with relatively more atypical glial cells and a peculiar 'wheat-sheaf' arrangement of astrocytes in the subpial region. Tubers are often extensively gliotic (Crino et al., 2002). In one study it has been suggested the severity of cyto-architectural abnormalities is more marked in TS (Palmini et al., 1991a,b) although others have suggested the opposite (Jay et al., 1993) and others have demonstrated a continuous spectrum of cyto-architectural disorganization in both lesions (Wolf et al., 1995a). Neurones with extreme cytomegaly and abnormal somato-dendritic morphologies are considered to characterize tubers (Crino et al., 2002).

Tuberous sclerosis is an autosomal dominant disorder which results from mutations in one of two non-homologous genes *TSC1* and *TSC2*. The *TSC1* gene encodes a protein hamartin and *TSC2* codes a protein tuberin that are structurally distinct. Both proteins are widely expressed in normal tissues including brain, liver, kidney, skin, muscle and heart. Hamartin interacts with the ERM family of actin binding proteins and may contribute to cell adhesion and migration. Tuberin may have a role in the regulation of DNA synthesis and the cell cycle (Catania et al., 2001). Furthermore tuberin and hamartin interact through the Akt pathway to regulate cell growth and cell size. It has been suggested that enhanced cell size may compromise normal neuronal migration (Crino et al., 2002).

In hamartomas in other organs in TSC1 or 2 it has been shown that a second hit somatic mutation in the unaffected allele has occurred. Reduction of gene products hamartin and tuberin has been demonstrated in the cells of tubers and SEGA (Mizuguchi et al., 2000, Arai et al., 1999), although robust expression was noted in another study using western blotting (Johnson et al., 1999). Therefore in the case of cortical tubers it is likely that these lesions are a result of haplo-insufficiency. Alternatively a second hit mutation in TS genes may have occurred in one cell type, e.g., glial cells, which subsequently resulted in the cortical malformation (Crino et al., 2002). Studies of differential gene expression by single cell populations and dysplastic neurones in cortical tubers (White et al., 2001) may shed light on the pathogenesis of these lesions. Similar studies in typical FCD lesions may allow distinction of FCD from tubers. Loss of TS genes (TSC1 and 2) were not demonstrated in one study of FCD-like lesions (Wolf et al., 1997b); however, in a more recent study by Blumcke and co-workers, loss of heterozygosity on the TSC1 gene and polymorphisms was noted in the micro-dissected balloon cells of FCD lesions (Becker et al., 2002a). One hypothesis is that FCD arises

following a somatic mutation in a neural progenitor cell, but it remains to be seen whether altered hamartin function in these balloon cells can be demonstrated (Crino et al., 2002)

3.4.3.1.5 Aetiology and mechanisms of epileptogenesis in FCD

It is widely held that FCD is a developmental disorder. Familial cases of FCD have been recorded suggesting a genetic predisposition in some cases (Rorke, 1994, Kuzniecky, 1994), although no candidate gene has been identified and in many studies a genetic predisposition is lacking (Montenegro et al., 2002). The search for a candidate gene in FCD is an area of intense research (Crino et al., 2002) and the possibilities are wide. Crino suggests that there are two competing hypotheses regarding the formation of FCD. It may be the result of a somatic mutation in a precursor cell that results in a clonal progeny. The mutation may involve one of the identified MCD genes. The cellular changes observed in FCD indicate that such a gene could affect cortical development in time frames spanning early-middle and late development (Mischel et al., 1995, Cotter et al., 1999). Alternatively an external event may affect the development of multiple precursor cells in a region. Such pathogenic factors may act either during migration or in post-migrational maturation, differentiation and/or proliferation, or may interfere with programmed cell death. Environmental assaults, e.g., radiation, ischaemia, or toxins, are possible factors in some cases and in one study of neuronal migration disorders a possible prenatal aetiological factor, such as exposure to X-rays, second twin dying in-utero, etc., was identified in 63% of cases (Palmini et al., 1991 a,b) There is also evidence of discordant incidence of FCD in monozygotic twins suggesting an environmental insult (Briellmann et al., 2001). Such an insult may act pre- or post-natally, but the cytoskeletal changes in FCD suggest an underlying maturational failure in the cells. Finally, there is also a theory that FCD is a result of a defect in radial glial cells.

Possible candidate genes involved in FCD include those in the reelin pathway. In a recent study at our institute we demonstrated abnormal aggregates of cdk5 in balloon cells and dysplastic neurones using immunohistochemistry (Sisodiya et al., 2002, see figure 4e-g). Interestingly another group did not demonstrate any mutations in the cdk5 gene in their analysis of gangliogliomas (Becker et al., 2002b), a cytopathologically related lesion. Cotter et al. (1999) demonstrated

abnormal expression of proteins in the *wnt* signalling pathway in the cells of FCD. The w*nt* pathway influences gene transcription and developmental patterning and, in turn, cell fate, shape and mobility.

FCD is considered to be intrinsically epileptogenic (Sisodiya, 2000). Intracranial recordings provide evidence that the FCD lesion harbours the ictal onset zone. Resected human FCD maintained *in vitro* has been shown in electrophysiological studies to have epileptiform activity (Mattia et al., 1999). *In vitro* recordings in human FCD slices have shown that a proportion of dysplastic neurones generate repetitive ictal discharges (Avoli et al., 1999). Seizure genesis in focal cortical dysplasia is likely to be the result of abnormal neuronal circuitry, altered inhibitory-excitatory nerve cell populations, aberrant synaptogenesis or neurotransmitter imbalances. Altered connectivity between FCD and the adjacent cortex may predispose to synchronous paroxysmal discharges, but it is well documented that in some cases of FCD this does not occur. An alternative hypothesis raised by Crino et al. (2002) is that genes responsible for the malformation may also predispose to susceptibility to epilepsy.

In a typical case of FCD containing dysplastic nerve cells, analysis of subpopulations of local circuit neurones using calbindin and parvalbumin immunoreactive studies showed abnormalities in the distribution and morphology of inhibitory interneurones (Ferrer et al., 1992b) with mainly a reduction in these cell types in the regions of dysplasia (Spreafico et al., 1998, Ferrer et al., 1994). A reduction in GABAergic cell types in FCD and tubers may suggest a failure of these cells to migrate or their premature loss (Crino et al., 2002). In addition, a reduction in $GABA_a$ receptors has been shown (Crino et al., 2001). Neuropathologically confirmed cases of FCD, localised by abnormal stereoelectroencephalogram, showed altered patterns of catecholamine neuronal circuitry within the dysplastic foci and in the surrounding areas using anti-sera to tyrosine- hydroxylase and dopamine-beta-hydroxylase enzymes, thus suggesting the hypothesis that catecholamines may be relevant in seizure propagation and limitation (Trottier et al., 1994). In addition, abnormally high concentrations of amino acids (ethanolamine and glycine) have been observed in tissues recovered from epileptic foci showing mild cortical dysplasia (Hamberger et al., 1993a). More recently, abnormalities in the distribution of glutamate receptors on pyramidal cells have been demonstrated in FCD (Spreafico et al., 1998, Garbelli et al., 1999, Najm et al., 2000) as evidence for intrinsic hyper-excitability of FCD and increased expression

of NR2B mRNA has been shown in FCD. From these findings, an increase in lesional excitatory neurotransmission and a decrease in inhibitory drive may result in the high degree of intrinsic epileptogenicity. Over-expression of drug resistance proteins in FCD, as well as in other malformations associated with epilepsy, may also contribute to the development of refractory seizures (Sisodiya et al., 1999, 2001).

3.4.3.1.6 Clinical outcome following surgery

In a recent review of surgical outcome for patients with malformations of cortical development it was shown that a seizure-free outcome in approximately 40% of cases was achieved, which is similar to the outcome to cortical malformations as a whole (Sisodiya, 2000). Many factors may influence the outcome reported in FCD surgical series. The length of follow-up is critical in the analysis and comparison of these studies. In many reports the post-surgical follow-up is less than 2 years, which may under-report late recurrences. For example, Urbach et al. (2002) reported good outcome (Engel class I) in 100% of patients following resection of FCD with balloon cell, although the follow-up in this study was limited to one year. Another recent series also reported a better post surgical outcome for FCD than mild cortical dysplasia, with 75% Engel Class Ia following surgery, although again the follow-up in most cases was less than 2 years (Tassi et al., 2002).

Differences in surgical approaches may influence the outcome achieved with FCD. For the best outcome it is considered that the whole 'epileptogenic zone' should be resected and completeness of excision is likely to be important in FCD, although in many reports the surgical resection margins are not commented on (see Sisodiya, 2000). The epileptogenic zone is defined as the volume of brain necessary for the generation of seizures. This region is delineated by a combination of functional imaging, EEG and structural imaging studies pre-operatively or invasive monitoring, as in many cases the epileptogenic zone extends beyond the boundaries of the area of dysplasia (Sisodiya et al., 2000, Tassi et al., 2001). The extent of resection is also governed by the proximity or encroachment of FCD on eloquent cortex. Therefore in the analysis of benefits of surgery in FCD many factors are to be considered. Although in many centres FCD surgery is more likely to be considered in the paediatric age group, it was observed that there was no difference in outcome, in terms of seizure control, between children and adults with FCD (Sisodiya et al., 2000).

3.4.3.2 Microdysgenesis

Meencke and Janz (1984) outlined the pathological appearances of microdysgenesis, a microscopic malformation, in post mortem tissue from patients with generalised 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. It has been documented that the histological features of microdysgenesis (also known as 'neuronal heterotopia' or microscopic malformation) account for up to 37% of the malformative lesions found in the brains of patients with epilepsy (Meencke and Veith, 1992), being more often encountered in patients with an older age of onset of epilepsy. By definition, microdysgenesis is not visualised either by neuroimaging studies or on naked eye examination of the cortical tissue and its presence requires histological confirmation. However, it is still regarded by many as a controversial entity and its significance and functional relevance in epileptogenesis has been debated and treated with some degree of scepticism (Lyon and Gastaut, 1985). This controversy has arisen as a result of:

- Similar pathological changes having being identified in neurologically normal people (Kaufman and Galaburda, 1989), dyslexia (Humpreys et al., 1990), autism, and psychosis (see Harrison, 1999), and in mentally retarded patients – all without a history of epilepsy.
- Other studies of patients with psychosis (Beasley et al., 2002) or idiopathic generalised epilepsy (Opeskin et al., 2000) have failed to identify significant microdysgenetic features.
- 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, show large nodular aggregates of mal-positioned 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.

- Confusion has arisen in the classification of this lesion as distinct from other cortical malformations, e.g., the terms microdysgenesis and focal cortical dysplasia have often been used synonymously and interchangeably (Prayson and Estes 1993, Mischel et al., 1995). Other terms used include architectural dysplasias (AD) (Garbelli et al., 2001, Tassi et al., 2002), microdysgenetic nodules or ' hamartias' (Blumcke et al., 1999d), 'mild cortical dysplasia' or 'non-balloon cell dysplasia' (Honavar and Meldrum, 1997).

- The features of microdysgenesis are often reported in association with other pathologies, e.g., hippocampal sclerosis, making determination of its significance in isolation difficult (Sisodiya, 2000).

- There is a lack of standardised criteria for the histopathological diagnosis of microdysgenesis in specimens from patients with epilepsy. In addition, there is lack of clarity regarding which microscopic abnormality is the most clinically significant, the defining feature of microdysgenesis, and whether only some or all features need to be present in order to make a diagnosis. 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.

One of the original descriptions of microdysgenesis (Meencke and Janz, 1984) was based on the study of eight autopsy specimens from patients with primary generalised epilepsy. Seven of these eight cases showed a variety of 'disturbances of the brain architecture'. These included:

- Excess of nerve cells in the subpial region (uni- and bi-polar)
- Increased nerve cells in the molecular layer of the cortex (either singly or in nodules)
- An indistinct boundary between cortical laminae 1 and 2
- Protrusions of nervous tissue into the leptomeninges
- Persistent columnar alignment of cortical nerve cells
- Increased numbers of single heterotopic nerve cells in the white matter
- Ectopic nerve cells in the hippocampus and Purkinje cell dystopia in the cerebellar cortex

However, this descriptive study was not supported by quantitative or morphometric analysis of these heteromorphic malformations. The authors also speculated on the functional significance of these changes, stressing the 'qualitative' nature of the analysis and noting that ectopic nerve cells were recorded predominantly in areas where they were more easily observed by the pathologist, such as the molecular layer of the cortex, cerebellar cortex and the subcortical white matter. They also acknowledged that cellular disturbances are probably not limited to these observed structures. A high incidence of cerebral microdysgenesis, supported by quantitative data, was reported in the brains of children with West's syndrome, Lennox-Gastaut syndrome, childhood absence seizures and juvenile myoclonic epilepsy (Meencke 1985b). In more recent years, additional abnormalities have been described, including minute grey matter heterotopias, neuronal satellitosis and perivascular aggregations of glial cells (Armstrong, 1993). In the hippocampus dentate granule cell dispersion is considered by some to represent microdysgenesis (Cotter et al., 1999).

3.4.3.2.1 Aetiology and cellular mechanisms of epilepsy

Microdysgenesis is thought to arise as a result of a later disturbance in cortical development occurring after the seventh embryonic month (Meencke and Veith, 1992). Interestingly, increased numbers of heterotopic nerve cells in the frontal white matter have been demonstrated in patients with post-traumatic epilepsy (Meencke, 1983) indicating that disturbed nerve cell migration may be a predisposing factor even where there is another precipitant for the epilepsy. The heterotopic neurones within white matter are believed to represent the arrested migration of nerve cells destined for the cortex. More recently it has been suggested that they may be remnants of the deeper embryonic subplate, representing some of the earliest neurones in corticogenesis that have failed to undergo programmed cell death (Rojiani et al., 1996). Additionally, a number of studies have demonstrated the presence of neurotransmitters and neuropeptides within these heterotopic white matter nerve cells, implying a possible functional role of these cells in seizure genesis (Rojiani et al., 1996). The heterotopic nerve cells in the molecular layer may represent either residual Cajal-Retzius nerve cells or additional or misplaced pyramidal cells that would normally lie in adjacent laminae (Meencke 1985a). A malformation similar to microdysgenesis, with ectopic collections of neurones and glia in lamina 1 and underlying cortical dysplasia, has been observed in a proportion of New Zealand black mice (Sherman et al., 1985). This malformation

arises spontaneously in these mice, a strain that also develops severe autoimmune disease and learning deficits. An increase in the absolute cortical nerve cell numbers has been demonstrated in the regions of these malformations in these mice suggesting a primary defect in the regulation of nerve cell numbers, in addition to migrational failure. In addition, experiments involving freezing regions of rat brain early in life have induced lesions similar to microdysgenesis with ectopic neuronal nests in the molecular layer (Humphreys et al., 1989) and malformations similar to microdysgenesis occur in the Ihara rat, which develops spontaneous seizures (Chevassus-au-Louis et al., 1999a). These animal studies suggest that malformations similar to microdysgenesis can occur both spontaneously or after an episode of cortical damage.

3.4.3.2.2 Quantification methods and morphometric studies in MD

In an aim to refine diagnostic criteria several quantitative studies have been carried out in temporal lobectomy specimens from patients with epilepsy. The quantitative analysis studies have centred on the white matter neuronal component of microdysgenesis, which is more amenable to such studies. However the majority of these have not used stereological methods. (Hardiman et al., 1988, Emery et al., 1997, Kasper et al., 1999). The findings of these studies can be summarised as follows :

1. In an evaluation of 49 temporal lobes from patients with intractable seizures, neuronal ectopia was observed in the subcortical white matter in 96% of the epilepsy patients as compared to 72% of autopsy age-matched controls (Hardiman et al., 1988). Cell densities of up to 8 cells/2 mm^2 were seen in 43% of the epilepsy cases, but in none of the control material which represented a significant finding. In some cases cell densities as high as 15 cells/2 mm^2 were observed. 'Bare areas' in the cortex and focal neuronal clusters were also seen in association with these white matter changes. These patients with microdysgenesis (greater than 8 cells/2 mm^2) had a favourable outcome following surgery, even without hippocampal resection, and the authors comment that this implies a functional importance of the ectopic nerve cells in the propagation of seizures.

2. Meencke confirmed that neuronal densities in the molecular layer of the frontal cortex are significantly increased in the brains of epilepsy patients compared to

age matched controls (Meencke, 1985a). In a further study it was noted that the density of nerve cells in the molecular layer in epilepsy patients approached that observed in the newborn. There was also an age-dependent reduction in these dystopic cells in the epilepsy group (Meencke and Janz, 1984). It was postulated that this loss in the epilepsy patients may be due to their greater vulnerability or a delayed post-maturational effect. In the same study, an increase in neuronal densities in the white matter of the frontal lobe of epilepsy patients was confirmed when compared to normal controls (Meencke, 1985a).

3. Emery (Emery et al., 1997) demonstrated increased temporal lobe white matter neuronal densities in patients with temporal lobe epilepsy compared to controls (4.08 neurones/mm^2 compared to 1.68/mm^2 in controls), but with considerable overlap between the two groups.

4. In a more recent study of temporal lobe microdysgenesis, clustering of cortical neurones in layers II–IV and glioneuronal hamartias predominated in patients with epilepsy compared to normal controls and the presence of more than ten neurones per high-power field of white matter was associated with a worse post-operative outcome (Kasper et al., 1999).

5. The only stereological study of temporal neocortical abnormality in temporal lobe epilepsy was published recently by Bothwell et al., (2001). In a study of 8 patients with TLE , they demonstrated no loss of neurones in the cortex and no significant increase of neurones in the white matter compared to controls but evidence of neuronal hypertrophy in epilepsy. They showed estimates of 1.2 million white matter neurones in Brodman's area 38 in controls compared to 1.5 million in temporal lobe epilepsy with mean neurone density measurements of 750/mm^3 in controls compared to 1160/mm^3 in the epilepsy patients.

6. In a study of layer I cellularity in microdysgenesis-like architectural dysplasias and focal cortical dysplasia Cajal-Retzius cell densities of 7.5/mm^2 were present in FCD compared to 13.73/m^2 in the architectural dysplasia (Garbelli et al., 2001).

3.4.3.2.3 Surgical outcome in MD

The previous quantitative studies of temporal lobe surgical resections summarised above (section 3.4.3.2.2) have provided conflicting results in terms of clinico-pathological correlation; in one, 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). In remaining studies, correlation with outcome is not provided. In a recent study by Tassi et al. (2002), patients with minor 'microdysgenetic' malformations had a poorer outcome post-operatively when compared to more severe dysplasias, as Taylor-type FCD, although in this study the quantitative methods used to estimate the white matter neurones are not clearly defined.

There are several possible explanations for these inconsistent findings. Different methodologies have been employed, mainly using biased or assumption based cell-counting techniques. It has been suggested that neuronal density 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 neuronal density within temporal lobe white matter is unknown and therefore the area selected for quantification in these studies may influence this measurement. The presence or not of a second pathology, as hippocampal sclerosis which may influence the surgical outcome, is variable in these studies (Kasper et al., 1999). Finally, in all previous studies on this topic, only the density of large 'gangliod' or pyramidal neurones (larger than 10 or 12 microns diameter) were 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. It is certainly acknowledged that the clinical relevance of microdysgenetic malformations or mild architectural dysplasias are disputed, particularly where they involve the temporal lobe, compared to the more severe cortical dysplasias (Palmini and Luders, 2002). In a recent text book of surgical pathology it is quoted 'How often these dysplastic (microdysgenetic) lesions are encountered depends on the experience and persistence of the observer and their threshold for accepting minor variations in cytoarchitecture as bona-fide abnormalities' (Burger et al., 2002).

3.4.3.2.4 Neuroimaging in lateral temporal lobe and microdysgenesis

Microdysgenesis is not detected with conventional MRI and a preoperative diagnosis of this abnormality in the lateral temporal lobe is not as yet possible in patents with HS undergoing surgery. Quantitative MRI studies in HS studies have suggested more widespread neocortical abnormalities in some cases, but this does not distinguish between dysgenetic or acquired pathologies (Sisodiya et al., 1997). More recent volumetric MRI studies measuring regions of temporal lobe in patients with AHS have shown volume loss in proportion to hippocampal atrophy consistent with a common process involving both temporal lobe structures (Moran et al., 2001). More subtle white matter abnormalities in the temporal lobe reported on MRI adjacent to HS include poor demarcation between grey and white matter and increased signal intensity on T2 weighted images (Choi et al., 1999, Mitchell et al., 1999). The increased T2 signal is in proportion to the white matter atrophy in these studies, which suggest they are measuring a common pathological process. Possible neuropathological correlates of these features include gliosis, myelin loss, perivascular-atrophy and corpora amylacea deposition as well as microdysgenesis (Choi et al., 1999, Mitchell et al.,1999, Meiners et al., 1999). Proton magnetic resonance spectroscopy of the temporal lobe adjacent to HS have also shown abnormal spectra interpreted as correlating with myelin loss rather than gliosis in one study (Meiners et al., 2000) and with microdysgenetic features in another (Stefan et al 2001). PET studies of temporal lobe white matter in HS may prove a more sensitive investigation for microdysgenesis showing hypometabolism (Choi et al., 1999) and increased Flumazenil binding in cases with higher white matter neuronal densities (Hammers et al., 2001).

3.4.3.3 Proposed revisions of classification for focal malformations

The terminology for focal dysplasias in epilepsy is not clearly defined. Various terms have been used based on a 'hotchpotch of genetic, clinical, imaging, histological and embryological criteria' (Tassi et al., 2002, see also Kuzniecky et al., 1999, Palmini et al., 1991a, Mischel et al., 1995, Barkovich et al., 1996, Cotter et al., 1999, Sisodiya 2000). Microdysgenesis has been used as a term to describe any type of MCD not seen radiologically, whereas it is acknowledged that in a proportion of these cases severe histopathological abnormality may be seen, including the presence of balloon cells and dysplastic neurones (Palmini and

Luders, 2002, Tassi et al., 2002, Ferrer et al., 1992b). Others have used the term for subtle derangements in cortical architecture including cortical laminar disorganisation, single or small aggregates of white matter neurones, a persistent subpial granule cell layer and marginal glioneuronal heterotopia (Mischel et al., 1995, Palmini and Luders, 2002). Armstrong draws a distinction between the terms microdysgenesis and cortical dysplasia according to the extent of cortical involvement: in cortical dysplasia the whole thickness of the cortex is involved whereas in microdysgenesis patchy cortical involvement is present (Armstrong and Mizrahi, 1997).

It has been suggested that the term microdysgenesis be abandoned and that the abnormalities observed are referred to as 'a mild form of cortical dysplasia' (Palmini and Luders, 2002). Tassi et al., 2002) state that the term microdysgenesis has become 'seriously misleading' as a result of it being applied to a wide variety of cortical abnormalities and they avoid using it in their classification (see table below).

In the scheme proposed by Palmini and Luders cortical resections are assessed for the following: the presence of dysmorphic (dysplastic) neurones, balloon cells, giant neurones (neurones of greater size than those found in the pyramidal cell layers and found scattered in any layer) and immature neurone (either single or in small clusters). Although the authors acknowledge that a 'definitive understanding of the relevance of each cell type or architectural abnormality' or their relationship to clinical and imaging findings are unknown, they propose the following classification as set out in Table 1.

Table 1. Classification Schemes for Focal Malformations in Epilepsy

<table>
<tr><th colspan="2">Palmini and Luders 2002</th><th>Tassi et al., 2002</th><th>Kuzniecky and Barkovich, 2001</th><th>Previously commonly used terminology</th><th>Terminology used in this study</th></tr>
<tr><td colspan="2">Mild Malformations :</td><td rowspan="4">Architectural dysplasia (AD):
Abnormalities of cortical layering
+/- immature neurones
+/- layer I and white matter neuronal heterotopias</td><td>Type III Malformations due to abnormal cortical organisation
3. Focal cortical dysplasia (no balloon cells)
4. Microdysgenesis</td><td>Microdysgenesis

Mild cortical dysplasia</td><td rowspan="3">Micro-dysgenesis:

White Matter Heterotopia

Glio-neuronal Hamartias

Layer I Hypercellularity

Cortical layering disorders including neuronal clustering</td></tr>
<tr><td>Type I
Layer I abnormalities</td><td>Type II
White matter heterotopia</td><td>Type II Malformations due to abnormal migration
B3. Excessive white matter neurones</td></tr>
<tr><td colspan="2">Focal Cortical Dysplasias:</td><td rowspan="2">Type I Malformations due to abnormal neuronal and glial proliferation.
B. Focal type 3a Focal cortical dysplasia with balloon cells</td><td rowspan="2">Glio-neuronal hamartias

Focal cortical dysplasia (Taylor)</td></tr>
<tr><td colspan="2">Type Ia
Architectural abnormalities: Dyslamination only</td><td>Cyto-architectural dysplasia (CD):

Cortical dyslamination and giant pyramidal cells</td><td rowspan="3">Focal Cortical Dysplasia

Dislamination, Balloon cells, Giant neurones, Dysplastic Neurones.</td></tr>
<tr><td colspan="2">Type Ib
Architectural abnormalities: Dyslamination plus immature

or giant neurones</td><td rowspan="2">Taylor-type Focal Cortical Dysplasia (TFCD):

Cortical dyslamination plus dysplastic neurones +/- balloon cells</td><td>Focal cortical dysplasia (Taylor)</td></tr>
<tr><td colspan="2">Type IIa
Architectural abnormalities: Dyslamination plus dysplastic neurones but without balloon cells</td><td>Focal cortical dysplasia (Taylor)</td></tr>
<tr><td colspan="2">Type IIb
Architectural abnormalities: Dyslamination plus dysplastic neurones plus balloon cells</td><td></td><td></td><td></td></tr>
</table>

Furthermore, Palmini and Luders subdivide mild malformations into type I where the abnormalities involve layer I (neurones in the molecular layer, a persistent subpial granule cells layer or marginal glio-neuronal heterotopia) and type II lesions with abnormalities outside layer I (heterotopic white matter neurones or dysgenesis of the hippocampus).

The system suggested by Palmini and Luders is compared to other classifications. In the more simplified classification of focal malformations proposed by Tassi et al. (2002) they define three subgroups of malformations: Architectural dysplasias (AD), Cytoarchitectural dysplasia (CD) and Focal Cortical Dysplasia Taylor type (TFCD). AD broadly correlates with Meencke's description of microdysgenesis. The most prominent histopathological features were disorganisation of the cortical layering. Layer I and II were clearly defined; however, they reported that in some cases layer II was thinner with evidence of a reduction in the number of neurones. Increased numbers of neurones were seen in layer I including Cajal-Retzius cells. Abnormal grouping of neurones in layer IV was seen and the border between layer V and VI incomplete. In this group no giant or dysplastic cells are seen but in 25% of cases isolated or clustered immature neurones were present (identified by small, uniform size, large nuclei and thin rim of neurofilament positive cytoplasm). Ectopic neurones were present in the white matter, although in this study they were not quantified. In CD, in addition to laminar disruption, giant pyramidal neurones were found in the cortex, particularly in the upper layers. In TFCD additional features included the presence of dysmorphic neurones plus or minus balloon cells. The term "$TFCD_{BC}$" has been also adopted to refer to Taylor FCD with balloon cells to distinguish this from that without (Urback et al., 2002). In TFCD, Tassi et al. (2002) also comment that although the molecular layer is thicker than normal the overall cellularity is reduced. Overall the system proposed by Tassi et al. (2002) seems a more practical and 'user friendly system' which may in time be more widely adopted (see Section 8.7: Conclusions).

Previously used nomenclatures are also listed in the table 1. 'Hamartia' (or microdysgenetic nodule) is a term that has been used for microscopic (0.2-1.0 mm) nodular intracortical aggregates of mature and immature nerve cells, glia and oligodendroglial-like cells (Blumcke et al., 1999d, Wolf et al., 1993 and 1995a). Hamartias have been documented adjacent to DNT and gangliogliomas

and also described in the context of microdysgenesis (Armstrong 1993, Armstrong and Mizrahi, 1997). The cells within hamartias may show positive immuno-labelling with immature cell markers including embryonal NCAM and stem cell marker CD34 (Blumcke et al., 1999d). Hamartias are of course distinct from glio-neuronal *hamartomas*, which describes macroscopically larger lesions. A hamartoma is essentially a localised disorganised mass of tissue composed of mature cellular elements indigenous to that site. In patients with epilepsy they include those associated with neurocutaneous syndromes and vascular malformations. They are not regarded as low-grade tumours as they show little capacity for growth or cellular proliferation. Unlike other cortical malformations, they form better-defined gross lesions. In more long-standing cases, dystrophic features such as calcification may be present.

The table also includes the equivalent classification for focal malformations within the broad scheme for all cortical malformations proposed by Kuzniecky and Barkovich (2001) as detailed in the previous section (Section 3.4.1). Although a thorough and detailed system based on mechanistic processes, this is considered to be a less practical classification scheme for the histopathologist to adopt when diagnosing focal lesions. For instance 'microdysgenesis' and 'excessive single white matter neurones' are grouped as distinct entities in different classes. In their scheme they also distinguish focal cortical dysplasia type I (without balloon cells) from focal cortical dysplasia type II (with balloon cells) (Kuzniecky and Barkovich, 2001).

For the purposes of our present study we have adopted a simple classification scheme that is used at Queen Square. 'FCD' is used for all cortical dysplasias with either giant neurones, balloon cells or dysplastic neurones whereas the term 'microdysgenesis' is used for other focal cortical malformations lacking these cellular elements but demonstrating either or all of the following; white matter heterotopia, layer I abnormalities and cortical dyslamination.

3.5 Hippocampal sclerosis (HS)

3.5.1 Epidemiological and clinical aspects related to HS

Large studies of patients with pharmacoresistant TLE and surgical therapy regimen of the epileptogenic area have undoubtedly confirmed hippocampal

sclerosis (HS) (syn. Ammon's horn sclerosis - AHS; mesial temporal sclerosis-MTS) as the major pathological finding. Although the pathogenesis of HS remains controversial, the clinical history in most patients follows a characteristic schedule. In a series of (725 + 384) patients obtained from two large European epilepsy centers, including the University of Bonn Medical Center and Institute of Neurology, London, three periods can be identified. Most patients presented with an initial precipitating injury before the age of 4 years. In the Bonn series, 53% of patients (as reviewed from the clinical charts) and 47.5% of patients in London (as reviewed between the period 1996-1998) experienced early insults. The majority of those patients in the Bonn series (70%) had a record of complex febrile seizures before and in the London series complex or complicated febrile convulsions were noted in 34%. This observation closely parallels data consistently reported in the literature. Birth trauma, head injury or meningeal infections were other early childhood lesions observed in TLE patients. The mean age of onset of spontaneous complex partial seizures is then between 9-11 years. As a matter of fact, neither structural, molecular or functional analysis can be sufficiently obtained during this silent period. Retrospective analysis of psychological development in these patients, however, may indicate already substantial differences compared with close relatives in the same families (see Blumcke et al., 2002). Nevertheless, the diagnosis of HS following surgical resection is obtained only after a long period of ineffective antiepileptic medication in most patients. The mean age at the time of operation is typically between 31-34 years with a duration of epileptic seizure history of 23 years. In most other series reported so far, both genders were equally distributed in our cohort and a familiar history of TLE was very rare, rendering a genetic substrate and/or inheritance unlikely, and almost confined to specific epilepsy syndromes.

Neurosurgical resection either by 2/3 temporal lobe resection or selective amygdalohippocampectomy, result in complete seizure relief in more than 77.5% (Engel class I), whereas a further 12% largely benefit from significant reduction of seizure frequencies (Engel class II).

3.5.2 Patterns of neuronal loss in HS

HS is typically a unilateral process, affecting either hemisphere equally (Briellmann et al., 1999), with involvement of the whole length of the hippocampus (Quigg et al., 1997). In some cases more focal damage may be observed (Bronnen et al., 1995, Babb et al., 1984) and, in others, bilateral sclerosis (Mathern et al., 1996a, Free et al., 1996, Barr et al., 1997, Van Paesschen and Revesz 1997). In so called 'classical HS' selective loss of pyramidal cells is seen in the CA1 subfield and in the hilar region, including CA4 pyramidal cells, with accompanying astrocytic gliosis. Pyramidal cells of CA2 and dentate granule cells appear more resistant (Bruton, 1988). In 'severe HS' almost total neuronal loss is seen in all hippocampal subfields and may be accompanied by marked deposition of corpora amylacea (Chung and Horoupian, 1996). In the pattern of HS termed 'end folium gliosis', encountered in 3-4% of surgical cases (Bruton, 1988), the neuronal cell loss appears confined to the hilus and includes loss of both principal cells and interneurones. This pattern of hippocampal atrophy is less easily detected on pre-operative MRI and is regarded to be associated with a later onset of epilepsy than classical HS and a worse post-operative seizure outcome (Armstrong 1993, Engel 1996, Van Paesschen and Revesz 1997).

Quantitative histological studies have been carried out in HS series mostly using two dimensional or semi-quantitative techniques. Pathological grading systems have been proposed to categorise the severity of neuronal loss in HS (Wyler et al., 1992, Davies et al., 1996a, 1996b, Watson et al., 1996), for example, grade I HS : less than 10% of neuronal dropout in CA1 up to grade IV HS : more than 50% neuronal loss in all sub-fields (Wyler et al., 1992). This is based on a semi-quantitative assessment of neuronal loss in histological sections and such analyses have proved useful in allowing pathological correlation with clinical parameters, such as the age of onset of epilepsy and duration of seizures (Davies et al., 1996), and with neuroimaging features (Watson et al., 1996). These grades may also reflect a progressive evolution of HS from grade I through to IV, mirroring ongoing hippocampal atrophy that has been occasionally reported in sequential neuroimaging studies (Van Paesschen et al., 1998, Nohria et al., 1994, VanLandingham et al., 1998, Jackson et al., 1999). More rigorous stereological methods of quantifying neuronal loss and gliosis in HS have also been employed (Van Paesschen et al.,

1997b, Billington et al., 2001) which provides more reproducible and reliable quantitative data, albeit more time consuming, and these methods have provided a good correlation with quantitative MRI analysis.

Marked cytological alterations have been observed in surviving neurones in HS using immunohistochemistry, electron microscopy and confocal imaging techniques (Blumcke et al., 1999b). These include enlargement and accumulation of neurofilaments in end-folial cells (Thom et al., 1999a, Blumcke et al., 1999b) and abnormal dendritic nodular swellings and ramifications of these neurones . These features are considered to more likely represent secondary or adaptive cellular changes due to the altered connectivity in the reorganised hippocampus than a primary cellular abnormality.

Neuronal loss and gliosis may also be present in adjacent limbic structures including the amygdala (Yilmazer-Hanke et al., 2000) and parahippocampal gyrus. This is referred to as mesial temporal sclerosis (MTS). Neuronal loss in layer III of the entorhinal cortex was also shown in some cases adjacent to HS (Du et al., 1993). The layer III entorhinal neurones receive and send projections (together with pre-alpha cells of layer II) to the hippocampus and subiculum. Whether neuronal loss in layer III represents a primary or secondary effect of the seizures is not clear. Damage to the entorhinal cortex has also been observed in volumetric MRI studies considered likely to be related to the privileged electrical dialogue between these two regions (Salmenpera et al., 2000, Bernasconi et al., 2003). The extent of any temporal neocortex neuronal loss seems to correlate with the severity of hippocampal damage (Bruton 1988, Moran et al., 2002). Neocortical neuronal loss again appears to be layer specific, with cortical layers II and III more affected (Cavanagh and Meyer1956, Thom et al., 2000).

3.5.3 Specific neuronal vulnerability

Loss of the principal pyramidal cells in HS is established, but recent studies have focused on the vulnerability or resistance of specific subsets of interneurones within the hippocampal formation which may influence the

intrinsic circuitry of the hippocampus and seizure propagation. Most interneurones contain the neurotransmitter GABA, but can be further subdivided according to their connectivity, calcium binding protein content and neurotransmitter receptor status (Freund and Buzsaki, 1996).

Neuropeptide Y (NPY) and somatostatin expressing inhibitory interneurones are normally numerous in the hilum and form a dense plexus of fibres in the outer molecular layer of the dentate gyrus which co-localises with GAD (Amaral and Campbell, 1986). Using immunohistochemistry, selective loss of NPY and somatostatin cells in the hilum was noted in HS (Mathern et al.,1995b, de Lanerolle et al., 1989). NPY containing axons also appeared to be reorganised in the dentate molecular layer in HS and in models (Patrylo et al., 1999, de Lanerolle et al., 1989, Bouilleret et al., 2000) and ectopic expression of NPY in granule cells has been observed following seizures (Vezzani et al., 1999b). This is likely to represent plasticity in NPY inhibitory mechanisms in the epileptogenic hippocampus. A more recent quantitative study using *in-situ* hybridisation, however, has suggested that NPY and somatostatin cells are lost in proportion to the overall cell loss and not specifically 'targeted' in the disease process (Sunderström et al., 2001).

The calcium binding proteins (CBP) calbindin(CB), parvalbumin (PV) and calretinin (CR) label different and non-overlapping subsets of inhibitory hippocampal interneurones and the resistance or susceptibility of these cells in HS may directly effect hippocampal epileptogenesis. CB positive cells are mainly involved in the inhibition in the dendritic region of principal cells whereas CR positive interneurones probably selectively innervate other interneurones (Magloczky et al., 2000). These cells can be readily identified using immunohistochemical techniques. An early study had suggested preferential survival of CB and PV immunoreactive neurones in HS (Sloviter et al., 1991a). More recent quantitative studies have shown a selective loss of PV immunoreactive neurones in CA4 subfield, disproportional to the overall cell loss (Zhu et al., 1997). It has been suggested that delayed maturation of CA4 interneurones renders them more susceptible to insults early in life. Loss of hilar parvalbumin and somatostatin interneurones was also noted and associated with the development of chronic seizures after status epilepticus in animal models (Gorter et al., 2001).

CBPs are not restricted to inhibitory cells and the glutamatergic granule cells of the dentate gyrus are also normally immunoreactive for CB. In HS, however, loss of CB expression by granule cells has been reported (Maglocsky et al., 1997 and 2000). Granule cells are typically more resistant to damage in HS than other principal neurones and it has controversially been proposed that the loss of calbindin actually protects these cells from Ca2+ mediated neuronal damage (Nagrl et al., 2000). The distribution of CB positive interneurones in the dentate gyrus in HS was shown not to differ from controls in one study, but striking enlargement of their cell bodies with enhanced expression of CB and modification of the dendritic trees and synapses of these cells was noted (Magloczky et al., 2000). These cytological changes may suggest an enhanced metabolic rate of CB cells in HS. CR cells do not appear to show abnormal distribution in HS (Blumcke et al., 1999a), but increased numbers of a subset of CR positive neurones, the Cajal-Retzius cells, were shown in some HS patients (Blumcke et al., 1996a). In another study, however, a marked reduction in CR positive cells in the dentate gyrus in HS was noted in the majority of cases which correlated with the severity of sclerosis (Magloczky et al., 2000). All studies however have confirmed an expansion of CR positive axonal networks in the molecular layer of the dentate gyrus in HS (Blumcke et al., 1996a and 1999, Magloczky et al., 2000). These fibres represent the excitatory supramammillary pathway terminating on granule cells and this observation may represent enhanced excitation (Magloczky et al., 2000). Paradoxically, however, it has recently been demonstrated in studies of CBP knockout mice that the absence of these proteins does not appear to affect the numbers of interneurones or excitotoxic mediated cell loss in epilepsy (Bouilleret et al., 2000). Therefore the precise role of calcium binding containing interneurones in the pathogenesis of TLE is uncertain.

Loss of hilar mossy cells, an excitatory interneurone with distinctive dendritic arborisations, was shown in HS cases compared to patients with generalised seizures (Blumcke et al., 1999b and 2000b). These cells can be demonstrated with immunohistochemistry for metabotropic receptor (mGluR7b). These excitatory interneurones normally project to inhibitory basket cells and their loss may result in a reduction in feed-forward granule cell inhibition, supporting the experimental 'dormant basket cell hypothesis' proposed by Sloviter (Sloviter, 1991b). However, it is recognised in animal models that

basket cells also receive direct excitatory input from the granule cells and perforant pathway fibres, thus bypassing the mossy cells (Kneisler et al., 1995).

3.5.4 Granule cell dispersion

The observation of disorganisation or dispersion of granule cells (GCD) into the molecular layer of the dentate gyrus in HS was first described in detail by Houser (Houser 1990, Houser et al., 1992). Dispersed granule cells appear separated from the normally compact cell layer, which gives an impression of an undulated irregular border with the molecular layer. In some cases the deep hilar-border of the granule cell layer is also ill defined. As a result in HS the cell layer appears broadened with a mean width of 180 microns in TLE patients compared to 100 microns in control subjects (Houser 1990). The dispersed cells often appear elongated or fusiform in shape, reminiscent of migrating neurones. Less often, a bi-laminar arrangement of granule cells is observed (Houser et al., 1992, Lurton et al., 1997) or nests of GC are present in the hilum (Houser 1990). The incidence of GCD in HS surgical series varies from 34-45% (Lurton et al., 1997 , Houser 1990).

It has been suggested that GCD represents a primary abnormality of neuronal migration or an underlying hippocampal malformation (Houser et al., 1992). There are occasional reports of GCD in association with cortical malformations in the absence of a history of seizures and with bilateral hippocampal involvement (Harding and Thom, 2001). Disorganisation of the granule cell layer and ectopic localisation has also been noted in several animal models with cortical malformations, such as the reeler and p35 mutant mice (Wenzel et al., 2001). The presence of GCD in human HS has also been correlated with epileptic events occurring early in life including febrile seizures (Lurton et al., 1998, Houser 1990) suggesting a vulnerability of these neurones at this time period. It has further been shown that the presence of GCD correlates well with the severity of hippocampal neuronal loss (El Bahh et al., 1999). This would suggest that GCD may represent an epiphenomenon of HS, rather than a primary abnormality, the migration of granule cells perhaps being influenced by neurotrophin secretion during seizures or other cellular signals (Lurton et al., 1998).

In animal models of epilepsy, such as the pilocarpine model, there is evidence to suggest that abnormally migrated granule cells are newly generated cells, neurogenesis being stimulated by the seizures (Parent et al., 1997). Rapid dispersion of granule cells has been demonstrated following injury (Omar et al., 1999) and it has been shown that newly generated cells can migrate as far as CA3 and integrate into CA3 neuronal network (Scharfman et al., 2000). Abnormal connections formed by new cells may contribute to seizure development (Parent et al., 1997) although experimental inhibition of neurogenesis does not prevent mossy fibre reorganisation in epilepsy models (Parent et al., 1999). Recent studies have confirmed that regeneration also occurs in human adult granule cells (Eriksson et al., 1998) and neuronal progenitor cells have been isolated from the dentate gyrus (Singh-Roy et al., 2000). This pool of precursor cells may have important physiological roles, but it is conceivable that in human epilepsy, stimulated by seizures, an increase rate of granule cell neurogenesis occurs leading to the abnormal cell localisation and reorganisation observed in HS. It is possible that cells, in the subgranular cell layer in the dentate gyrus, which express GFAP, function as one group of transient granule cell precursors (Seri et al., 2001). This is in parallel with the recent observations that radial glial cells in the developing cortex divide to form new neurones (Fishell and Kriegstein, 2003). Interestingly, in human studies of HS the presence of GCD has been associated with immature glial cells (Crespel et al., 2002) although no co-localisation of GFAP and nestin was noted in another study of dentate gyrus neural precursors in TLE (Blumcke et al., 2001).

As evidence of neurogenesis in human HS, this later study has confirmed the stem-cell intermediate filament protein nestin in granule cell neuronal precursors in young patients with MTLE, before the age of two years (Blumcke et al., 2001). Similar cells were not found in adult HS cases and whether the nestin positive cells represent newly generated cells or a delay in hippocampal development in these younger patients is not clear. Studies of cell cycle proteins, including Ki67, showed low expression in dentate gyrus subgranular layer in adult hippocampi from patients with epilepsy (Del Bigio, 1999). Although this is an insensitive technique for measuring cells with a low turnover rate, it suggests that neurogensis in HS is a rare event and likely to be dependent on age.

If migrated granule cells do represent newly generated cells, any differences in the physiological properties of these less mature cells remain to be investigated. Electrophysiological studies in human HS have already demonstrated the existence of distinct populations of granule cells, one group showing abnormal excitability (Dietrich et al., 1999). We also know from animal studies that there is considerable potential for adaptability and plasticity of GC. For example, induction of inhibitory cellular mechanisms by increasing basal expression of GAD (Sloviter et al., 1996b), NPY induction (Vezzani et al., 1999), loss (Maglocksy et al., 1997) or gain (Scharfmann et al., 2002) of calbindin expression and, in human surgical HS tissue, altered ionotropic and metabotropic glutamate and GABA neurotransmitter receptor profiles (Loup et al 2000, Mathern et al., 1999a) has been shown. It is plausible that such plasticity could be enhanced in newly generated GC, which could contribute to the seizure propensity.

3.5.5 Aberrant axonal reorganisation : Mossy fibre sprouting

In 1974, using Golgi techniques, Scheibel and colleagues identified aberrant axons from granule cell neurones ascending into the molecular layer of the dentate gyrus in hippocampal specimens from patients with epilepsy (Scheibel et al., 1974). It has long been considered that reorganisation of the excitatory glutamatergic mossy fibre pathway is a key event in the development of chronic seizures (Sutula et al., 1989). More recent experimental findings, however, in which mossy fibre sprouting is prevented, suggest that it is not an essential process to the generation of spontaneous recurrent seizures (Longo and Mello 1999) and that it is not the only factor important in hippocampal seizures (Longo and Mello 1997, Gorter et al., 2001). Mossy fibre sprouting in human HS specimens results in aberrant innervations of other granule cells, synapsing at the apical dendrites and also with CA1 pyramidal neurones resulting in both feedback and feed-forward excitation (Babb et al., 1991, 1992, Mathern et al., 1994, 1995a, 1995c, 1995d). In addition, aberrant mossy fibres also innervate interneurones, suggesting that new inhibitory circuits are established (Kotti et al., 1997).

Mossy fibre sprouting in the supragranular layer of the dentate gyrus can be demonstrated using the Timms histochemical method which highlights the zinc rich mossy fibre synaptic terminals (Babb et al., 1991, Babb 1991) or with

dynorphin immunohistochemistry (Houser et al., 1990). Increased expression of growth associated protein GAP-43 in the supragranular layer is thought to indicate active mossy fibre sprouting in HS specimens (Proper et al., 2000). Similarly increased syanaptogenesis in this region has been demonstrated by studying the distribution of 5'nucleotidase activity, which localises in regions with more active synaptic turnover (Lie et al., 1999). Overall reorganisation of synaptic terminals in HS has also been demonstrated in human specimens using immnohistochemistry for synaptic antigens such as synaptophysin, which shows a loss in CA4 and increased labelling in the dentate gyrus molecular layer (Honer et al., 1994, Davies et al., 1998, Proper et al., 2000). Similarly prominent immunolabelling for chromogranin in the inner molecular layer of the dentate gyrus is shown to correspond with reorganised mossy fibres in patients with epilepsy (Kandlhofer et al., 2000). In parallel with increased synaptogenesis, elaboration and increased complexity of granule cell dendrites in the internal molecular layer has been demonstrated in HS patients (Von Campe et al., 1997).

Sprouting of MF is considered to result from epilepsy-induced loss of target cells. However, in animal models it may be an early event, occurring within 4 weeks following kindling (Elmer et al., 1996) and independent of hippocampal cell loss, possibly regulated by neurotrophic factors (Adams et al., 1997). Preliminary studies also suggest that mossy fibre sprouting is likely to be independent of any granule cell neurogenesis (Parent et al., 1997, Covolan et al., 2000).

3.5.6 Adaptive reorganisation in HS – neurotransmitter systems

Alteration in the distribution of neurotransmitter receptors has been extensively investigated as a pathogenic mechanism in the hyperexcitability of the hippocampus in TLE . The 'GABA' hypothesis proposes that a deficit in inhibitory GABAergic transmission is implicated in seizures. $GABA_A$ and to a lesser extent $GABA_B$ receptor subtype expression (Barnard et al., 1998) and reuptake mechanisms have been studied in human HS tissues. Many alterations in GABA transmission may represent an adaptive mechanism in the brain in response to repetitive seizures and increased expression of $GABA_A$ receptors has been documented in animal models of epilepsy as a compensatory

mechanism (Fritschy et al., 1999). In human tissues any additional contribution of AED therapies to alteration in receptor number and function should be considered. In human HS loss of $GABA_A$ receptors using immunohistochemical methods was considered to be a result of the overall neuronal loss (Wolf et al., 1994a). Autoradiographic studies of $GABA_A$ receptor binding, however, have suggested that the absolute levels of the receptor are reduced in hippocampal neurones, which may represent a primary fault (Hand et al., 1997). This is supported to some extent by more recent work using autoradiography for benzodiazepine receptors showing reduction of this receptor in excess of overall loss (Sata et al., 2002). Selective upregulation of particularly the $GABA_A\alpha 2$ subunit (and to a lesser extent $\alpha 1$, $\beta 2/3$ and $\gamma 2$ subunits) in remaining granule cells in HS has been observed using immuohistochemistry with selective subunit antibodies, highlighting the plasticity of these neurotransmitter systems in HS (Loup et al., 2000). Increase in $GABA_A$ receptors has been suggested to occur in both HS and non-HS TLE with increasing age. $GABA_A$ receptors normally comprise 2α and 2β subunits and either a γ, δ or ε unit. It has yet to be demonstrated if increased expression of subunits in granule cells in human epilepsy correlates with assembly of functioning units. A co-ordinated expression of receptor subunits in granule cells however has been shown in both normal and epilepsy tissue, which may suggest a functional impact on these cells (Brooks-Kayal et al., 1999). Furthermore in animal models, such as the kindling model of TLE, increased expression of GABA subunits in GC is observed at synapses by EM and associated with increased GABA mediated currents (Nusser et al., 1998).

$GABA_B$ receptor changes have also been demonstrated in human HS tissue. $GABA_B$ receptors inhibit neurotransmitter release from pre-synaptic terminals and cause late inhibitory synaptic potentials (Barnard et al., 1998). Increased expression of $GABA_B$ 1 receptor has been shown in the subiculum of HS cases and in surviving CA1 neurones and granule cells with augmented receptor binding using *in situ* hybridiation and autoradiography techniques (Billington et al., 2001). More recent study has suggested a reduction in $GABA_B$ in HS granule cells although other areas maintain expression (Munoz et al., 2002). Further *in vitro* work, labelling $GABA_B$ receptors with agonist 3H-CGP62349,

suggested a reduction in receptor in CA3 and the hilum in HS patients (Princivalle et al., 2002).

Upregulation of excitatory metabotropic glutamate receptors (mGluR 1) has been observed in the dentate gyrus in both human and animal models of HS, which may contribute to the development of chronic seizures (Blumcke et al., 2000a). In addition, upregulation of the inhibitory metabotropic receptor mGluR4 in the dentate gyrus and granule cells in hippocampal specimens was also observed, which, may contribute to the dampening of seizure activity (Lie et al., 2000). In an immunohistochemical study of excitatory ionotropic glutamate receptors in human HS, a reduction of labelling for NMDAR1 and AMPA (GluR2/4) receptors in CA1, 3&4 was noted, but, when corrected for the reduced neuronal densities in these subfields, no differences from the control group were seen (Blumcke et al., 1996b). In other studies, employing *in situ* hybridisation techniques, an increase in pyramidal and granule cell AMPA receptor mRNA was shown, however (Mathern et al., 1997b,d), and an increase in granule cell NMDAR1 and 2 receptor mRNA (Mathern et al., 1999b), which is also supported by autoradiographic studies (Brines et al., 1997).

Alterations in astrocytic function in the gliotic hippocampus have also been shown. Astrocytes show physiological changes of immature astrocytes, including prolonged depolarisation, that may contribute to seizure generation (Hinterkeuser et al., 2000, Schroder et al., 2000). In a further study of rat and human hippocampi in temporal lobe epilepsy, glial cells in area of neuronal loss were associated with alterations in extracellular potassium that also may affect conduction of seizure activity (Heinemann et al., 2000).

3.5.7 The pathogenesis of HS and developmental aspects

There appear to be predictable patterns of cell loss and alterations to the intrinsic circuitry of HS. However, the factors critical to the initiation of the cell loss and hippocampal reorganisation are still debated and the precise aetiology of HS still remains elusive.

A significant cerebral insult (or initial precipitating injury - 'IPI') occurring early in life, such as a febrile or prolonged seizure, is often reported in a third to a half of cases in retrospective studies of patients with HS (Falconer et al., 1964, Cavanagh and Meyer 1956, Davies et al., 1996, Bruton 1988, Mathern et al., 1995c, Annegers et al., 1987). The 'injury' hypothesis implies that this insult irreversibly damages or alters the hippocampus and acts as a template for the progression to HS following a 'latent' interval. There appears to be an age-specific sensitivity for this injury, with more severe neuronal loss demonstrated when the IPI occurs before age 7 (Mathern et al., 1995c, Davies et al., 1996). In a recent study, hippocampal oedema was observed within 5 days following a febrile convulsion, but not following status epilepticus (Scott et al., 2002). The injury hypothesis is supported by experimental studies, which demonstrate HS-like patterns following prolonged seizures and status epilepticus (Roch et al., 2002). However, it fails to explain why in half of HS patients a history of an IPI is absent and why HS is predominantly a unilateral disease process following such a 'global' cerebral insult (Berkovic and Jackson, 2000). The fact that only 2-7% of children with a history of febrile seizure go on to develop epilepsy later in life (Maher and McLachlan, 1995, Cendes et al., 1995) is an indication that there are likely to be other factors at play in the aetiology of HS. It is also of interest to note that a pattern of cell loss identical to that observed in HS is reported to occur sporadically in elderly patients as a rare cause of dementia (Leverenz et al., 2002).

The other important question is whether progressive loss of neurones occurs in the hippocampus as a result of ongoing seizures and accumulative damage. In a study of patients with newly diagnosed epilepsy using serial imaging, progressive hippocampal damage was not evident (Liu et al., 2002). Another longitudinal MRI study, however, suggested incurred hippocampal damage is directly related to the number of generalised seizures the patients had (Briellmann et al., 2002). There is experimental evidence that repeated brief seizures produce progressive hippocampal neuron loss (Kotloski et al., 2002) which may also be the case in humans (Kalvianinem and Salmenpera 2002, Mathern et al., 2002). The patterns of neuronal loss, however, may differ according to the age of the individual and following prolonged seizures (or status) versus recurrent seizures; the relative contribution of repetitive seizures to the hippocampal neuronal loss therefore remains to be clarified (Holmes, 2002, Pitkanen et al., 2002).

Temporal lobe epilepsy is generally regarded as an acquired disorder with only a small genetic contribution. Genetic predisposition to some forms of temporal lobe epilepsy and febrile convulsions has been identified (Berkovic et al., 1996, Ottman et al., 1995, Poza et al., 1999] and recently, in a family with both febrile convulsions and TLE but without HS (Baulac et al., 2001). Gene polymorphisms in interleukin –1 receptor antagonists have been demonstrated in a group of patients with HS; as these are major pro-inflammatory cytokines this may indicate increased susceptibility of these patients to neurotoxic damage (Kanemoto et al., 2000). This observation was not repeated in a different study population, however (Heils et al., 2000), and a genetic predisposition to HS as well as the role of inflammatory cytokines in the aetiology of HS still remains to be confirmed. For example, it is uncertain whether increased expression of inflammatory cytokines in TLE represents a protective or deleterious mechanism (Crespel et al., 2002a).

More recent attention has focused on an underlying mal-development of the hippocampus as a primary abnormality predisposing to HS and also to febrile seizures. In an MRI study of families with familial febrile convulsions a subtle pre-existing hippocampal abnormality was detected (Fernandez et al., 1998) and HS has also been reported in patients in association with isolated malformations of the hippocampus (Baulac et al., 1998). In addition, an abnormal persistence of calretinin positive Cajal-Retzius cells in the hippocampus has been reported in HS specimens (Blumcke et al., 1996a, Blumcke et al 1999a). Cajal-Retzius cells, through the secretion of Reelin protein, play a critical role in neuronal organisation in the developing brain (D'Arcangelo et al., 1995). Higher numbers of Cajal-Retzius cells were particularly observed in patients with HS with histories of febrile seizures. It is plausible that such an injury occurring early in life disrupts normal hippocampal development and maturation (one manifestation of which is an excess of Cajal-Retzius cells), which in turn predisposes to HS. As it has recently been suggested that reelin in the adult cortex has a role in plasticity and axonal remodelling these cells may also be important in the reorganisation of circuitry occurring in HS.

The final argument supporting a mal-developmental basis for HS comes from the observation that HS is often observed in association with subtle

cytoarchitectural malformations in the neocortex, also called microdysgenesis (Meencke and Janz 1984, Armstrong and Mizrahi 1997, Hardiman et al., 1989). This may be indicative of a more widespread mal-developmental process involving both mesial and lateral temporal lobe structures. One cytoarchitectural feature observed in microdysgenesis is also an excess of Cajal-Retzius cells in the molecular layer (Garbelli et al., 2001) which parallels findings in HS.

HS is also well recognised to occur in association with more severe or obvious cortical malformations, vascular malformations and low grade glio-neuronal tumours (Raymond et al., 1994a, Cendes et al., 1995, Li et al., 1999). In these so-called 'dual pathology' cases there is evidence of less severe hippocampal principal cell loss than in HS cases without a second lesion (Levesque et al., 1991, Mathern et al., 1995d, 1996b, 1997a). It is possible that in these cases the epileptogenic extra-hippocampal lesion 'kindles' the hippocampal neuronal loss, i.e., the hippocampal sclerosis in these cases is a secondary event. It has been shown, however, that in patients with dual pathologies, removal of both the lesion and the abnormal hippocampus has the best outcome in terms of seizure control (Li et al., 1999), emphasising the role of the hippocampus in temporal lobe seizures even where there is a second pathology.

3.6 Other common lesions identified in epilepsy surgical series

3.6.1 Tumours

Supratentorial neoplasms are frequently accompanied by epilepsy; although any type of tumour may cause seizures, the most frequent association is seen with low-grade gliomas, including astrocytomas (e.g., pilocytic astrocytoma, pleomorphic xanthoastrocytoma, subependymal giant cell astrocytoma) and mixed glioneuronal tumours (ganglioglioma and dysembryoplastic neuroepithelial tumours – DNT) (Burger et al., 2002).

3.6.1.1 Dysembryoplastic neuroepithelial tumours

Cavanagh in 1958 identified 'certain small tumours' in temporal lobe specimens removed for the treatment of epilepsy which bore a similarity to

oligodendrogliomas but which he considered to be hamartomatous in nature (Cavanagh, 1958). Thirty years later, the characteristics of the DNT were outlined by Daumas-Duport et al. (1988) in a retrospective analysis of 39 cases from young patients with intractable partial seizures. These tumours shared common features, including a cortical location, multinodular architecture, and a heterogeneous cellular composition. Additional studies over recent years have confirmed this typical pattern of clinical presentation, natural history and pathological features associated with these neoplasms. In the current WHO classification of brain tumours, DNT is grouped under 'neuronal and mixed neuronal-glial tumours.

3.6.1.1.1 Clinical features.

DNTs are typically associated with medically intractable seizures of the partial complex type. Rare cases without a history of preceding epilepsy have been documented (Thom et al., 1999). The type of epilepsy is usually concordant with the site of the tumour, e.g., complex partial seizures of temporal lobe type occurring with temporal lobe based tumours. Secondary generalisation of seizures is also present in a proportion of patients. The onset of epilepsy is usually in childhood, adolescence or early adulthood and there is often a further interval of several years before surgery is undertaken. There have been occasional reports of DNTs diagnosed in older patients in 7th decade.

There is usually no family history of seizures in patients with DNTs and, apart from occasional documentation of these tumours arising in patients with neurofibromatosis (Lellouch-Tubiana et al., 1995), they are not associated with other congenital malformations or neurocutaneous syndromes. Developmental milestones, intelligence and neurological examination are usually normal in patients with DNT, although mild impairment of memory has been observed with some temporal lobe lesions.

3.6.1.1.2 Pathological features

DNTs occur predominantly in an intracortical and supratentorial location. They favour the temporal lobe – this being the site of involvement in 94% of cases in

a series from the National Hospital, London (Raymond et al., 1994b), in 62% of the original series (Daumas-Duport et al., 1988), and in 59 of 74 cases in the most recent large study (Honavar et al., 1999). In some instances these tumours arise in mesial temporal structures, involving the hippocampus and amygdala. The frontal lobe, including the cingulate gyrus, is the next most common site of involvement with relatively fewer cases encountered in the parietal and occipital lobes. There have also been rare reports of these tumours occurring in deep grey nuclei, midline structures and cerebellum (Kuchelmeister et al., 1995). The tumours are nearly always solitary, but cases with multifocal deposits of tumour, including involvement of the temporal lobe, have been noted (Leung et al., 1994).

The gross appearance of these tumours in resected specimens is somewhat variable; some appear soft, others firm; some are well demarcated and nodular; others have ill-defined boundaries. Macroscopic evidence of cystic change may be apparent in a proportion, but calcification is usually not a gross finding. There is also considerable variation in the size of DNTs, which may vary from a few millimetres to near lobar dimensions. However, the majority of tumours measure a few centimetres across.

Histologically these tumours have a complex nodular or multinodular architecture with additional smaller satellite nodules found adjacent to the main tumour mass. They can expand and focally replace the cortex, and they are often observed to extend into the leptomeninges and the underlying white matter. A cellular infiltrative margin, however, is not a feature of these tumours. The cytological composition of the DNT is typically heterogeneous with a complex mixture of nerve cells, astrocytes and oligodendroglial-like cells.

In the initial description of DNT, Daumas-Duport et al. describe the 'specific glio-neuronal element' which forms a large component of the tumour, replacing areas of cortex with a relatively monomorphous appearance. It is composed of nerve cells, randomly placed, suspended within a basophilic mucoid matrix in which they appear to float. Atypicality of these nerve cells is not readily apparent and they appear generally mature, although occasional bi-nucleated forms may be seen. The intimately related glial component of this 'element' is mainly oligodendroglial in appearance with astrocytic cells being

less well represented. The glial cells are often arranged in a columnar fashion alongside perpendicularly orientated parallel arrays of thin-walled capillaries and axons. Such an orientation of oligodendroglia around vessels can form pseudo rosette-like structures or even a more loosely structured alveolar pattern, but perineuronal satellitosis by glial cells is not typically observed. This specific glio-neuronal element may also be readily apparent on cytological smear preparations of fresh tissue stained with toluidine blue and thus suggest an intra-operative diagnosis of DNT.

The nodular part of the DNT either comprises well defined cellular nodules with mixed cell populations or a single cell type may predominate. This forms the most heterogeneous component of the tumour, showing considerable morphological variation both within and between cases. Nodules composed of rounded oligodendroglial-like cells (OLCs) with perinuclear haloes are commonly encountered in DNT. Within these nodules delicate branching networks of thin-walled capillaries are often seen which are reminiscent of those observed in oligodendrogliomas. In addition to oligodendroglial nodules, diffuse oligodendroglial hypercellularity may be present in the cortex adjacent to DNTs, together with mucin accumulation but without the perineuronal satellitosis typically observed in oligodendrogliomas showing cortical infiltration.

The astrocytic component of DNTs may be minor and only apparent after immunohistochemical analysis with GFAP antibodies. In other instances it may have the appearance of diffuse sheets or nodular areas of well-differentiated fibrillary astrocytes with areas of microcystic change. Growth patterns similar to the pilocytic astrocytoma have also been seen within DNTs with additional features as glomeruloid capillary proliferation, Rosenthal fibres and eosinophilic granular bodies in these regions. Even more unusual astrocytic patterns such as 'polar spongioblastoma' have been documented in the setting of a DNT (Daumas-Duport et al., 1988). However, ependymal differentiation has not been noted.

Randomly orientated, mature ganglion cells are often observed scattered throughout DNTs, within the nodules as well as the specific glio-neuronal element. A laminar pattern of organisation or vertical orientation of these neurones is not apparent, which suggests they are not entrapped cortical cells.

Occasional bi-nucleated nerve cells have been reported (Daumas-Duport et al., 1988, Raymond et al., 1994b) but dysplastic or atypical nerve cells are rarely seen within the lesion itself and may be more readily apparent at the edges of, or between, nodules. The mature ganglion cells are highlighted with synaptophysin immunohistochemical staining. Scattered small collections of neuroblasts were also observed in one series of DNTs (Raymond et al., 1994b). Daumas-Duport et al. (1988) describe neuroepithelial components in DNT that 'defy characterisation' by histology or immunohistochemistry, but the presence of a primitive neuroepithelial element was not noted in this initial series.

Histological evidence of dystrophic calcification may be encountered in these tumours, either as calcospherites or mineralisation of vessel walls (Raymond et al., 1994b, Hirose et al., 1994). Other series have failed to confirm calcifications within a DNT. The vasculature of these tumours generally comprises thin-walled capillary-sized vessels. Hyalinisation and thickening of the vessel walls has been reported and, in addition, areas of glomeruloid capillary proliferation have been noted. Malignant neovascular proliferation as seen in glioblastoma, however, is not a feature of DNT. We have also demonstrated these tumours may undergo spontaneous haemorrhage (Thom et al., 1999). Deposition of pigment including iron and neuromelanin have been reported in DNT, and may be focal or widespread (Bhaskara et al., 2000).

3.6.1.1.3 Histogenesis and progression of DNT

There has been some speculation as to the exact nature of the OLCs and whether they represent glial, neuronal cells or more primitive precursor cells. Positive staining of a proportion of these round cells and the intervening fibrillary matrix has been demonstrated immunohistochemically with antibodies for synaptophysin, raising the possibility they are of a neuronal cell lineage. In addition, positive staining of a small number of these OLCs was also demonstrated using anti-neurofilament antibodies in one study. Electron microscopy studies of the OLC component have shown evidence of advanced neuronal differentiation with neuritic processes and synapse formation and occasional dense core granules (Leung et al., 1994, Biernat et al., 2001) and mature neuronal markers as NeuN (Wolf et al., 1997a) in a proportion of these

round cells. In contrast to this, occasional OLCs have also shown positive staining with GFAP antibodies. These findings suggest that the OLC are a heterogeneous population of cells, some showing glial differentiation. At least a proportion may indeed be small neurocytic cells. No cells have been observed showing transitional or intermediate cytological features between the OLCs and the larger mature ganglion cells of DNT by light microscopy. Interestingly, studies of typical oligodendrogliomas have also suggested features of neuronal differentiation in a proportion of cases, with immunohistochemical and ultrastructural investigations (Ng et al., 1994). Thus the histogenesis of the OLC in DNT and its relationship to neoplastic oligodendroglial cells is still uncertain (Wolf et al., 1997a).

Increased cellularity, pleomorphism and reniform and fleurette-like multinucleated cells may be observed in the glial element of DNT and occasional mitosis may also be seen (Hirose et al., 1994). Immunohistochemical analysis of cell proliferation in DNTs using antibodies such as PCNA (Raymond et al., 1994b, Taratuto et al., 1995) and MIB1 (Ki67) (Prayson et al., 1996, Wolf et al., 1995a) have shown in general a low cell turnover of the glial component of the tumour with only sporadic staining of nuclei (usually less than 1%). However, higher labelling indices (up to 5%) have occasionally been noted in these tumours. Further analysis using cell cytometry in one study showed the majority of DNT to be composed of diploid cell populations. Necrosis is not a feature of DNTs.

There is considerable debate as to the nature of DNTs; these lesions were regarded as hamartomatous by Cavanagh (1958), but favoured as benign neoplasms by Daumas-Duport et al (1988); in the current WHO classification they are regarded as grade I neoplasms. In support of a benign neoplasm, the biological behaviour of DNT is that of a slow-growing intracortical lesion with deformity of the overlying calvarium, suggesting a long-standing process. They are histologically non-infiltrative lesions and lack features of malignancy, including excessive mitotic figures, necrosis and malignant vascular proliferation. There have been no reports of recurrent behaviour and well-documented cases of transformation to glioblastoma are lacking. Although they may form large lesions, symptoms of mass effect or raised intracranial pressure

are not commonly observed, but there is evidence from cell proliferation studies and serial MRI to support their slow expansion and growth.

In favour of a hamartomatous or malformative nature for the DNT is the participation of many distinct neuroepithelial cell lines within the lesion, its common association with dysplastic/malformative changes in the adjacent cortex, and its occurrence mainly in children and young adults with these patients often having symptoms of long-standing epilepsy. These features all indicate that DNT arises on a background of cortical malformation.

Thus there is both evidence that DNT has a 'dysembryoplastic origin' but also that in contrast to true hamartomas they are not static lesions, showing evidence of slow expansion over the years and thus behave as benign neoplasms.

It has been suggested that DNTs arise from the secondary germinal layers of the cortex or the subpial granular layer. This appears at around the eighth week of gestation and can persist for a few months after birth. These germinal cells are mitotically active and multipotential. The round 'oligodendroglial-like cells' within DNT have been demonstrated to show both glial and neuronal differentiation by immunohistochemical and ultrastructural means and it has been suggested that these cells may be derived from progenitor cells of the secondary germinal layers capable of bi-divergent differentiation (Hirose et al., 1994).

Identification of DNT is paramount as these tumours represent surgically curable cortical neoplasms with an excellent prognosis and alleviation of epilepsy. Despite marked histological heterogeneity, the overall indolent biological behaviour is well established from larger studies with longer follow up (Honovar et al., 1999). Adjuvant therapy such as radiation treatment or chemotherapy is not advocated (Kirkpatrick et al., 1993).

3.6.1.1.4 Adjacent focal dysplasia associated with DNT

Dysplastic features in the cortex adjacent to DNTs is a common observation, especially in larger, well-orientated resection specimens with sufficient

marginal tissue. In the initial report by Daumas-Duport et al. (1988), it was present in most cases, and resembled either 'focal cortical dysplasia', as described by Taylor (1971) or milder forms of cortical malformation. In some cases the 'specific glio-neuronal element' of the DNT merged with the cortical dysplasia. Dysplasia was also noted in 12 of 16 cases in the National Hospital series (Raymond et al., 1994b) and took the form of disturbed cortical lamination, focal aggregates of nerve cells and, in one case, dysplasia of the dentate gyrus, which was adjacent to the DNT. Architectural disorganisation of the cortex was observed in nine of ten DNT (Prayson et al., 1996), in 22 of 74 cases (Honavar et al., 1999) and 'frequently' in larger series (Pasquier et al., 1999). The presence of Typical FCD with balloon cells and dysplastic neurones adjacent to DNT is not commonly encountered in personal experience. Obviously the extent of the tumour resection of DNT will influence how often adjacent cortical malformations are identified. In some cases it may be difficult to distinguish cortical infiltration adjacent to a tumour from true architectural dysplasia.

The histopathological variations encountered in DNTs may be bewildering, particularly to the novice pathologist, due to their complex and heterogeneous architecture. The diagnosis is easier to establish in a large resection than in a small biopsy specimen. The differential diagnosis in a small biopsy specimen can include oligodendroglioma, astrocytoma and oligo-astrocytoma. Daumas-Duport supports their division in to 'simple forms', where only the specific glio-neuronal element is present, and 'complex forms', where this element is combined with cellular nodules and/or focal cortical dysplasia (Daumas-Duport, 1993). Recent reports have identified tumours with a transitional or mixed appearance between DNT and gangliogliomas (Hirose and Scheithauer, 1998) and we have seen several such cases in our department; this may suggest a common histogenesis of these tumours. A small biopsy composed of only the specific element should therefore in theory be sufficient for confirmation and diagnosis of a DNT. However, reports of otherwise 'typical' DNT lacking the glioneuronal element (Iwanega et al., 1995), or 'non-specific DNT forms (Daumas-Duport et al., 1999) have been reported potentially broadening the spectrum of DNT. Where the typical clinical and MRI picture is present, but the specimen is too small to definitely confirm a DNT, a cautious diagnosis is a more pragmatic approach, impressing to the clinician that other neuro-glial tumours cannot be excluded with certainty; closer follow-up is warranted in

such cases. In small biopsy specimens cytogenetic analysis may prove a useful test to allow distinction of DNT from astrocytic and oligodendroglial tumours. In early studies DNT do not appear to show the 1p, 19q deletions that characterise oligodendrogliomas (Perry et al., 2003, Prayson et al., 2002, Fujisawa et al., 2002).

3.6.1.1.5 Intrinsic epileptogenicity of DNT

DNTs are strongly associated with epilepsy although the interictal spiking and ictal seizure onset may arise from regions adjacent to the DNT. This may indicate that adjacent cortical dysplasia is the cause of the seizures. This hypothesis raises the question of why seizure outcome is better following the treatment of DNT than cortical dysplasia alone. The neuronal component within the DNT may also be the epileptogenic source. High levels of glutamate receptor types have been shown with high expression of NMDAR1 and 2 on mature neurones and a smaller proportion of oligo-like cells expressing both these and GluR1, 2 and 5-7 (Aronica et al., 2001). Stronger labelling for glutamate receptors has been shown at the margins of a DNT compared to normal adjacent cortex (Adamek et al., 2001).

3.6.2 Cerebral cortical changes as a result of Epilepsy

Neuronal loss and gliosis as a consequence of seizure-mediated damage has been long recognised (Spielmeyer, 1927). The most affected areas are the hippocampus, neocortex, with laminar nerve cell loss predominantly involving layers II, III, the thalamus and amygdala (Honavar and Meldrum 2002, Du et al., 1993, Yilmazer-Hanke et al., 2000, Tassi et al., 2002). It appears that similar patterns of damage may be observed in patients with chronic repetitive seizures as following status epilepticus (Honavar and Meldrum, 2002). In some cases asymmetrical damage may result in cerebral hemiatrophy, often with ipsilateral hippocampal sclerosis. A pattern of subpial fibrillary gliosis (Chaslin's gliosis) is a common feature in the brains of patients with chronic epilepsy, although not specific for epilepsy. Corpora amylacea are frequently present in excessive numbers in regions of neuronal loss (Chung and Horoupian, 1996).

Cerebellar atrophy is also a common finding. A variety of patterns are described in patients with chronic epilepsy including post-ictal Purkinje cell loss, atrophy secondary to phenytoin administration, perinatal damage, ischaemic injury occurring during seizures, in addition to crossed cerebellar atrophy associated with cerebral hemiatrophy (Gessaga and Urich, 1985). We have also observed a pattern of cerebellar damage, predominating in the posterior lobe, which may result from chronic cerebral trauma (Crooks et al., 1999).

The presence of neocortical traumatic lesions and epilepsy are often linked. In some cases epilepsy follows an episode of head injury. In many cases, however, cortical injuries are a sequel of seizures. The risk of early or late seizures developing following head injury is increased by the presence of an open head injury, skull fracture, dural laceration, and intracerebral haematoma, as well as the localisation of the injury. Fronto-temporal contusions are the commonest pattern of head injury observed following a seizure. Repetitive head injuries following seizures may also result in neurofibrillary tangle formation in neurones similar to those observed in neurodegenerative diseases as Alzheimer's and dementia pugilistica (Thom and Scaravilli, 1997).

3.6.3 Rasmussen's encephalitis

Rasmussen's encephalitis is a rare and devastating seizure disorder resulting from an inflammatory process of unknown aetiology. Typically it begins at a young age with onset of severe chronic unilateral motor seizures with progressive neurological deficit and cortical atrophy (Rasmussen, 1958). Early treatments, such as immunosuppressive therapies and surgical treatments may be more effective in slowing the disease progression (Granata et al., 2003). The pathology is that of a chronic polio-encephalitis, typically unilateral with secondary cortical scarring. The inflammatory process is also unilateral but occasional bilateral pathology has been demonstrated at post mortem (Tobias et al., 2003). It is characterised by neuronophagia, perivascular cuffs of lymphocytes, and diffuse microglial hypercellularity. Most infiltrating lymphocytes are T cell type with CD8+ cells predominating (Prayson and Frater, 2002). The severity and activity may vary and Robitaille (1991)

classified cases according to their histological features: Active disease – with ongoing inflammation ; Active and remote disease – inflammation with necrosis; Remote disease – neuronal loss and gliosis with few microglial nodules ; Non-specific changes – neuronal loss and gliosis with few inflammatory cells. Serial MRI shows a good correlation with intensity of the inflammatory disease on biopsy (Bien et al., 2002a) and it is considered that most of the brain damage occurs in the first 12 months of the illness (Bien et al., 2002c).

Investigations have failed to demonstrate unequivocally an infectious agent. An autoimmune process against glutamate receptor protein GluR3 has been implicated in experimental models (Rogers et al., 1994, He et al., 1998). Recent studies suggest that glutamate receptor antibodies are not a specific marker for Rasmussen's encephalitis (Wiendl et al., 2001) being found in other focal epilepsies. A cytotoxic T-cell mediated response to MHC class I expressing neurones in Rasmussen's has recently been shown (Bien et al., 2002b). The presence of Rasmussen's encephalitis has also been demonstrated to occur in association with FCD, vascular malformations and other mass lesions, which may provide a window on aetiology, in terms of breakdown of the blood-brain barrier and exposure to potential new antigens (Hart et al., 1998).

3.7 Background to stereological methods applied to neuropathology in present study

Stereology is a mathematical method that enables data to be obtained regarding the estimation of the number, size and area of objects in a three dimensional structure by sampling in two dimensions (Howard and Reed, 1998) allowing a quantitative estimate of the measured feature. Key features of this method involve the unbiased random sampling of the specimen being examined and the application of unbiased geometrical and mathematically valid geometric probes. Unbiased stereological sampling protocols are referred to as ' design-based' techniques. The largest contribution to modern design-based stereological counting techniques in the last decade has been made by Hans Jorgen Gundersen (Howard and Reed, 1998). The main techniques used in neurosciences concern the estimation of tissue volume, cell number and size,

and length and area measurements. In the present study, estimation of cell number and axonal lengths were the measurements made and the following section focuses mainly on the science behind these techniques.

3.7.1 Cell number estimation

Estimation of cell number (neurones and glia) is one of the most important areas of stereological applications in neurosciences. The total number of neurones can be estimated by measuring their number per unit volume and multiplying by the reference volume. Alternatively neuronal densities (ND) can be calculated. There are four categories of cell counting methodologies : 1) Simple profile counts in histological sections, 2) Serial section reconstructions, 3) assumption based methods which are profile counts with geometric correction factors, and 4) stereological methods (Coggeshall and Lekan, 1996). Evaluation of the number of neurones is an important parameter in studies of the central nervous system as alteration in their numbers may occur as a result of a developmental cause or pathological or aging processes and may allow characterisation of the disease. When considering the neuronal density, it must be considered that any alteration in this measured value may be the consequence of alteration in neuronal number, alteration in the tissue volume or both (West 1999a). The main stereological approaches used in cell enumeration are the fractionator and the disector methods.

3.7.2 Sampling methods

For accurate measurements, random sampling of the specimen should be applied at all levels of examination, including selection of blocks, sections, fields and individual objects such that every element of an object has an equal probability of being sampled. Uniform or systematic random sampling is a method widely employed in stereology whereby the starting point is randomised and thereafter the tissue sampled at equal intervals. This method is easy to apply and gives estimates that have a lower variability (Gundersen and Jensen, 1987); the only inherent problem which should be considered is if there is a natural periodicity to the structure being sampled.

3.7.3 The disector principle

This is based on a 'scanning' approach to quantitate the number of cells in an object. A 3-D object is ' scanned' through a moving 2-D plane and each object is counted the first moment it hits this plane ensuring that it is included only once. In practice, such 3-D scanning is achieved in MRI studies, confocal microscopy and in conventional transmission light microscopy using a high numerical aperture objective lens (Howard and Reed, 1998). An alternative approach in conventional microscopy is to serially section an object and reconstruct it to count the objects; this method is, however, considered too labour intensive to be practical. The disector principle (Sterio, 1984) consists of a more simplified approach of examining a pair of sections a known distance apart; using this method if an object is seen in the first section but not the second ('look-up' section), it is counted. This method is referred to as the *physical disector*. The sections should be placed not so far apart that small objects lying between sections are missed. Also, it is critical that the two sections are perfectly aligned (or registered), which is often the most time consuming aspect of this technique. Practical approaches to register sections together include tandem projection microscopes where sections are aligned using a fixed anatomical feature as a guide, e.g., the subpial boundary or a blood vessel, or by using digitised images. Once aligned, fields can be randomly selected across the whole section and objects counted. The numerical density (N_v) of cells can then be calculated with the equation:

$$N_v = \frac{1}{A.h} \cdot \frac{\Sigma Q}{\Sigma P}$$

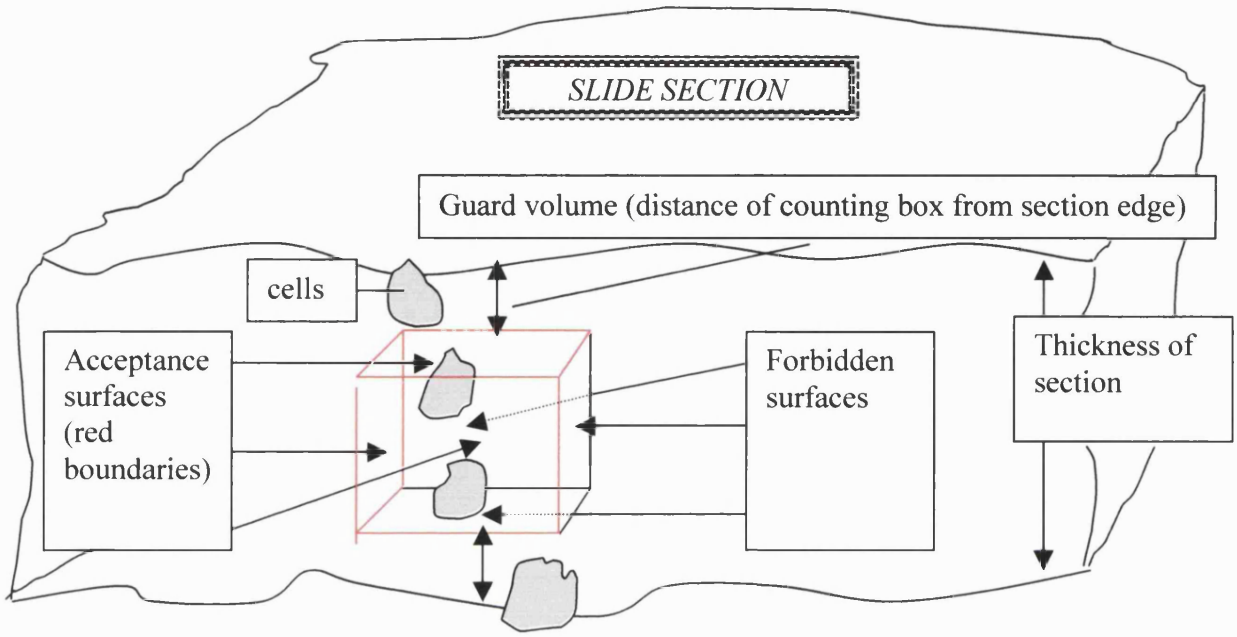
where A = the area of the counting field, h= the distance between the sections, Q is the sum of particles counted and P is the number of fields that are counted within the reference space. If the volume (V) of the specimen is known (see Cavalieri principle below) the total number of objects/cells (N) can therefore be calculated from the following :

$$N = V \cdot N_v$$

3.7.4 Optical Disector

The *optical disector* method is based on the principle of continuous scanning through a thick histological section. In a conventional microscope the depth of field (the point of the section that is in sharp focus) increases as the numerical aperture (NA) of the lens decreases. Therefore to obtain a thin and sharply focused optical plane a lens of high numerical aperture should be used. The highest NA lenses are high magnification (> x60) oil immersion lenses. If thick histological sections are used (>25 microns) objects will come in and out of focus and can be counted as the slide is scanned through. The main benefit of this method is that the need to align objects in separate sections becomes unnecessary. Cells are counted in fields using an unbiased counting frame superimposed on the image by use of a drawing tube, a graticule or a by using a video monitor. Another piece of apparatus required is a microactor which can accurately measure the distance travelled through the depth of the section, in the 'z' direction.

In essence the cells are counted within 3-D 'boxes' or 'bricks' of known dimension using an unbiased counting frame (Howard and Reed, 1998). All cells within the box or touching three acceptance surfaces are counted, as in the diagram below (two sides of the 2D frame and either the upper or deeper surface of the counting box). Cells touching the three 'forbidden' planes or outside the box are excluded. In practice a thick section (> 8 microns at least, but usually around 20-30 microns) is used (Williams and Rakic 1988) and the optical section set at random within the section at a distance away from the surface of the section (guard volumes). The microactor is zeroed and the slide is scanned through a known thickness (z) which is the height of the counting box, say 10-15 microns. As each cell is viewed it is considered whether it is within the 2D grid or frame and also whether it comes into focus within the depth of the box. For practical purposes it is better to set the box size so that there are 2 to 5 cells or 'counts' per box so that the count may be kept in the head.



This counting method requires that particles are essentially convex in profile. The same equation can then be used to calculate neuronal (or cell) density:

$$N_v = \frac{1}{A.z} \cdot \frac{\Sigma Q}{\Sigma P}$$

where z is the height of the counting box in this instance. The application of the optical dissector method to histological sections of brain specimens is now widely used and is also referred to as ' a direct 3-D cell counting method' (Williams and Rakic, 1988).

The fractionator method is an alternative method to estimate cell number. Described by Gunderson (1988), it is a method that is not influenced by tissue shrinkage. Similar to the disector method, 'physical' and 'optical' fractionator methods are used. The basic principle is that a known fraction of the whole object is analysed and every cell within this fraction is counted. The fraction analysed is obtained by uniform random sampling. The overall cell number can then be calculated.

3.7.5 Strategies for the number of cells to count

In any experiment the question of the number of cells that need to be counted and the number of cases to be examined in order to achieve a valid estimate is an important one. It is dependent on the variation of distribution of the cells within the structure, the variability between cases as well as the extent of the differences between the cases and the controls and the level of accuracy that a particular experiment requires. A sampling scheme of 100-200 counts in ten sections is generally considered to provide adequate precision. Pilot studies are often advisable and carried out on a small number of cases to assess precision and whether more sampling in individual cases or more cases are required (West, 1999a).

3.7.6 Are stereological methods (3-D cell counting) superior to 2-D cell counting?

No method of counting cells is guaranteed to be free of error and all counts are 'estimates' of the true value. When counting cells is to be undertaken in any neuropathological study it should be considered what level of accuracy is required and the margin of error that is acceptable by carrying out a pilot study in cases and control groups as above. It has been strongly argued by many neuroanatomists that 3-D approaches should always be used when estimating cell numbers in neurobiological systems as they offer unbiased estimates of cell numbers. A survey of scientific papers carrying out quantitative analysis in neurobiology showed that only 5% used stereological methods (Coggeshall and Lekan, 1996). In 1996 the Journal of Comparative Neurology adopted a unprecedented policy that only papers using unbiased stereologically-based estimates would be considered acceptable (Coggeshall and Lekan 1996, Saper 1996).

However, some workers continue to propose that simple 2-D cell counting methods have merits, being more practical to apply and even in providing more accurate estimates of cell number (Benes and Lange, 2001). These alternative '2-D' methods largely comprise simple profile counts of cells in thin sections. However, in a single section, whether or not a cell is counted is directly related

to its size and length, normal to the plane of section. As such, counting the profile number per unit area on single slides will never give a meaningful estimate of cell number (Howard and Reed, 1998) as large cells will be 'over-included'. Therefore the use of correction factors, as Abercrombie's, (Abercrombie, 1946) have be used to compensate for variations in cell size and section thickness :

$$\text{Number} = \text{count} \times \frac{\text{section thickness (H)}}{\text{Section thickness (H)} + \text{cell height (h)}}$$

The main flaws with Abercrombie's method are that fragments of cells cut by the blade on the section surface are included as whole cells therefore leading to overestimation of cell number (split cell error). In addition, with small cells it is often difficult to distinguish fragments of neurones from glia or other cells. Unless serial sections are used to measure the cell height, the cell height (h) is equated with the diameter in a single section (based on the assumption that cells are spherical, which they are clearly not, and that they are all orientated in the same direction). In addition, marked variations in section thickness (H) can occur in adjacent paraffin sections (e.g., in our experience '20 micron' sections cut on a microtome can vary in reality from 10-30 microns). So estimation of section thickness from the microtome calibration can also lead to errors, either over or underestimations. Therefore such cell counting methods using correction factors as Abercrombie's are inherently 'assumption based'.

One of the main arguments against using stereological methods is that they are perceived as more difficult to apply in practice, for example, acquisition of expertise in the preparation of thick sections and the setting up and extra expense of equipment. In addition, they are regarded as more time-consuming than simple cell counting methods (Benes and Lange, 2001), although this point is refuted by others (West and Slomanka, 2001, Geuna 2001). In reality the equipment to carry out stereological cell counts such as using the optical dissector method is not expensive, requiring only an adapted microscope with a 'z' stage-measuring device (West 1999b, Williams and Rakic 1988). The technical workload is also similar to that for 2-D assumption based methods, as both methods may require the cutting of multiple sections (for the 2-D methods in order to carry out estimations of nuclear size). A problem may arise in the application of stereological probes to valuable archival sections, which are not

randomly selected or where serial sections or thick sections are not available and 2-D methods may then be the only option. Furthermore, as semi-automated design-based image analysis systems become widely available (e.g., the Olympus CAST-grid system, Denmark or Histometrix system, Kinetic-Imaging, Liverpool, UK), the application of stereological probes to neurobiological studies should become more practical and efficient tools to use.

It has been argued by Benes and Lange (2001) that stereological counting methods are not appropriate methods in the analysis of the cerebral cortex where neurones are not distributed randomly but in clusters and layers. Therefore, because of the small size of the sampling boxes typically used with the optical disector, this may lead to sampling error. They argue that the larger window size that can be used with 2-D cell counts allows a more 'accurate assessment of the spatial distribution of the cells to be obtained'. However, supporters of the optical dissector method claim that this method categorically does not assume a uniform or homogenous distribution of particles within a tissue (West and Slomanka, 2001, Baddeley, 2001). Furthermore, it has been shown that non-stereological counts give more precise, accurate and repeatable measurements, even if these are inherently biased compared to stereological methods. However, proponents of 3-D counting methods argue that in order to achieve high accuracy in experimental results both random error (variability) and systematic error (bias) should be reduced. Bias is considered the most serious error as it is a consistent discrepancy between the truth and the results and cannot be eliminated by taking more data and cannot be detected (Baddeley, 2001). Although stereological methods may be less 'precise' (reproducible) they are regarded as fundamentally superior because of the lack of systematic 'bias' (West, 1999b).

Tissue shrinkage has been another argument considered to affect counts made using 3-D methods more so than 2-D methods (Benes and Lange, 2001). When a brain is fixed in 10% formalin an average increase in volume by 8.5% occurs (Aherne and Dunhill). However, when a paraffin section is de-waxed, 60% of the height on the section may be lost and up to 80% in a frozen section (Coggeshall, 2001). Whether this leads to distortion of the neurones is uncertain, but it is argued that it may lead to an overestimate in cell density when using 3-D counting methods. The extent of tissue shrinkage is often difficult to measure (Howard and Reed, 1998). Howeve,r when counting cells

using 2-D methods in thin histological sections, as all the cells are counted, any tissue collapse will also result in overestimation. In 2-D methods the problems of 'lost caps' of cells, which are split by the microtome, are overcome with 3-D counting in thick sections. By use of the guard volume (see diagram above) between the counting box and the section edge, these cell artefacts are avoided (West, 1999b). Coggeshall concludes that although 2-D counting methods may be appropriate in some cases, methodological requirements must still be met, for example, estimation of cell size (in Abercrombie's method), which may be more time-consuming than 3-D methods in the long run (Coggeshall, 2001).

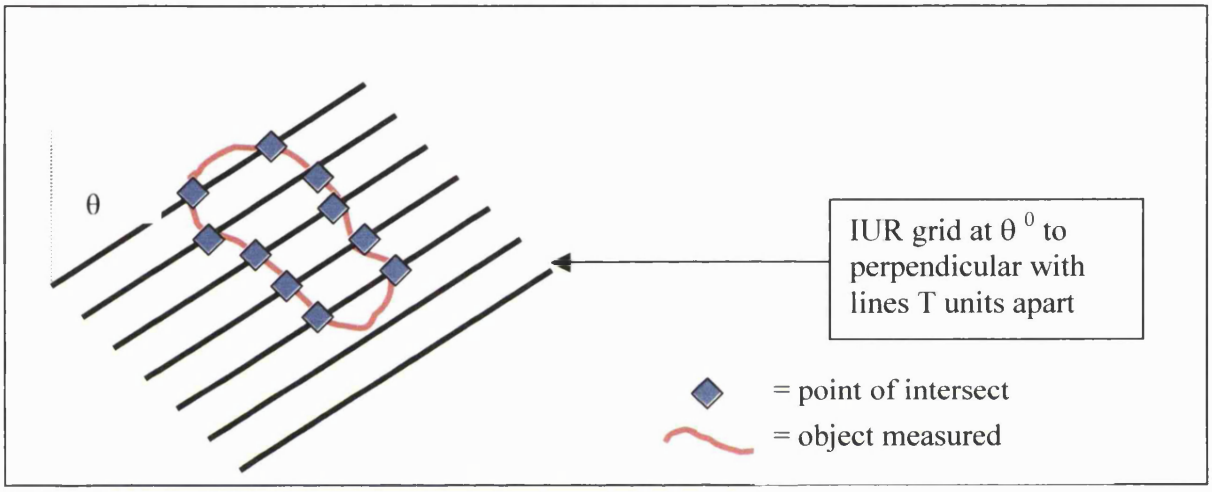
An alternative method for the estimation of cell number on tissue sections is to measure their separation: the interneuronal distance or the spatial distribution of cell. Such methods include the Voronoi tessellation (Duyckaerts and Godefroy, 2000) which are employed in 2-D rather than in 3-D and 'nearest – neighbour' formulae which can be applied to cortical (Schmitz et al., 2002) and white matter neurones (Beasley et al., 2002).

3.7.7 Estimation of length

Length estimations in neurobiology are required in the analysis of axons, microtubules, capillaries, etc., where such structures are embedded in the tissue blocks and then sectioned, transecting each structure to produce truncated profiles.
An estimation of length can be made from 2-D Dimensional sections using Buffon's needle principle (Aherne and Dunhill, 1982). In 1777 George Buffon correctly proposed the probability that a needle length l, if thrown at a series of parallel lines T distance apart, would intersect with one of them is :

$$P = \frac{2 \times l}{\pi T}$$

This law became the foundation of geometric probability and by inverting this equation, i.e., by measuring the probability (or actual number of intersections), the lengths of needles (or the structure of interest in the section) can be measured. In practice the structure of interest, for example, as in our study fibre length in the tissue sections, is superimposed with a parallel grid and the number of intersections with this grid counted.
For this purpose an isotropic uniform random (IUR) grid can be used. This is a 2-D grid with randomised orientation of parallel lines in a 0-180^0 axis separated by an equal interval (T).



Using this geometric probe, superimposed upon a two-dimensional object, the length of an object can be calculated by means of the following equation:

$$\text{Buffon's needle relationship : Length} = \frac{\pi \cdot T \cdot N}{2}$$

where N is the number of intersections and T is the distance between grid lines. The application of IUR grids to tissue sections can also be used to measure the length density of a structure in tissue sections (the length per unit volume). This involves the analysis of uniform random sections of tissue and profiles using an unbiased counting rule (Howard and Reed, 1998).

3.7.8 Estimation of volume

Methods for estimating volume of tissue include weighing, water immersion and the Cavalieri method. The Cavalieri method is widely used and a sound mathematically validated stereological principle. This method employs a series

of parallel sections through an object, which are a fixed distance apart (T). The first section should be at a uniform random interval (between 0 and T) and thereafter sections taken at equal intervals (T) from this. The area of each section is measured using point counting methods and the areas are summed and multiplied by the slice thickness using the following equation:

$$V = T \times a/p \times \Sigma P_i$$

where a/p = the area associated with each point, T is the distance apart of the sections and P is the number of points landing on the section to the *ith* section. This method can be used in practice for measuring the volume of objects ranging from a single cell to a whole organ.

3.8 CONCLUSION TO INTRODUCTION

The pathological criteria for microscopic malformations in temporal lobe epilepsy, including microdysgenesis, remain poorly defined. A stereological approach in the analysis of these malformations may provide objective, unbiased numerical criteria so that these cases may be better identified and allow more accurate clinico-pathological correlates. Identification of pathognomonic features of microdysgenesis, particularly features not present in normal cortex, would also aid their recognition. Hippocampal sclerosis is the commonest pathology in TLE, but the significance of granule cell dispersion in relation to hippocampal mal-development is not clear. Furthermore, the Cajal-Retzius cells have a critical role in normal cortical and hippocampal development and study of these cell populations in microdysgenesis and hippocampal sclerosis may provide evidence if these cells are involved in the pathogenesis of these lesions. Studies of the distribution of interneuronal populations within microscopic malformations may provide information of their developmental origins and the intrinsic epileptogenicity of these lesions.

4. AIMS OF STUDY

The main objective of this study was a quantitative and stereological analysis of microscopic malformations in patients with temporal lobe epilepsy and hippocampal sclerosis undergoing temporal lobectomy to clarify the diagnosis of these lesions. As outlined in the introduction, the terminology and diagnostic criteria for focal malformations in epilepsy are far from clear (see Section 3.4.3.3) and, as a result, good clinical correlative data on the functional significance of these lesions are lacking.

The main components to this study were:

I. REVIEW OF ALL TEMPORAL LOBECTOMIES: I reviewed the pathological diagnosis in all patients undergoing epilepsy surgery at the National Hospital for Neurology and Neurosurgery between 1993-2000 to identify the types and frequency of main pathologies including hippocampal sclerosis. This first section was necessary in order to select cases for the following studies.

II. WHITE MATTER NEURONE STUDY: In selected patients with hippocampal sclerosis I aimed to quantify microdysgenetic features. I aimed to apply stereological principles to quantitative estimates of white matter neuronal densities and layer I neuronal densities. I aimed to quantify the densities of different neuronal subtypes in different anatomical regions of the temporal lobe to investigate any variation in their distribution. I also aimed to quantify the degree of white matter microscopic gliosis to investigate if this influenced neuronal numbers and to correlate white matter pathology with the presence of cortical microdysgenetic features. Finally, I correlated all these measurements with the clinical outcome.

III. CORTICAL MYELINATION IN MICRODYSGENESIS: I investigated any abnormal patterns of cortical myeloarchitecture in patients with microdysgenesis in comparison to those described in more severe

malformations of cortical development and in relation to clinical outcome.

IV. CYTOARCHITECTURAL ABNORMALITIES IN HIPPOCAMPAL SCLEROSIS: In patients with hippocampal sclerosis I investigated the incidence of potentially developmental cytoarchitectural abnormalities in relation to patterns of hippocampal neuronal loss and clinical outcome.

V. CAJAL-RETZIUS CELLS: In view of the critical role of Cajal-Retzius cells in cortical and hippocampal development I aimed to quantify these cell populations in focal cortical malformations, including microdysgenesis and focal cortical dysplasia, and in hippocampal sclerosis with cytoarchitectural abnormalities. This would highlight any potential role of these cells in the pathogenesis of these lesions.

VI. CORTICAL INHIBITORY INTERNEURONES IN FOCAL MALFORMATIONS: I aimed to study inhibitory interneuronal populations in focal malformations to investigate their involvement in the dysgenetic process and potential contribution to epileptogenesis.

5 Materials and Methods

5.1 Handling of specimens from patients with epilepsy – general principles

Material from patients who have undergone epilepsy surgery is a valuable asset for neuropathological studies. It provides the unique opportunity to investigate the mechanisms of epileptogenesis in optimally preserved tissues and to correlate the pathological findings with recent neuroimaging and other pre-operative investigations. Surgical material has several advantages over post mortem neuropathological material as there is no delay in fixation so cellular and antigen preservation are favourable. There is usually no confounding pathology, such as pre-mortem cerebral hypoxia, which is often present in PM material and patients undergoing epilepsy surgery are on average younger in age than those identified at PM, so that, although the epilepsy is well established, the disease process itself is not as advanced. Currently, the patient undergoing surgery gives consent pre-operatively for tissues surplus to diagnostic requirements to be used for research purposes. The tissue removed during the surgical procedure can be handled in such a way as to maximise its value for research without compromising the histological diagnosis.

A standard protocol and guidelines for handling of epilepsy surgical material in the Division of Neuropathology at the National Hospital was established in 1992. On arrival in the laboratory the fresh specimen is dealt with by a member of the medical staff, under my supervision, or usually myself. The dimensions are measured in three planes (Anterior-posterior axis (A-P), superior to inferior axis (S-I) and the depth of the cortical resection from the pial surface to the underlying white matter (D)). The macroscopic appearances are described and any focal lesional pathology recorded. Photography of the specimen is also carried out at this stage (see Figure 1, page 112). The tissue specimen is orientated and margins inked where necessary. For example, in our laboratory the inferior temporal gyrus of temporal lobectomy specimens is marked with India Ink to allow identification in stained sections. The tissue is sliced fresh at 3-5mm intervals. At this point fresh tissue can be supplied for neurophysiological studies. Representative blocks may be snap frozen for subsequent neurochemical, receptor or molecular studies, others are fixed in

glutaraldehyde for electron microscopic studies (including grey and white matter) and the majority of the remaining tissue is fixed in formalin for routine neuropathology and fixed in paraformaldehyde and cryo-preserved for immunohistochemistry. Specimens of hippocampus are received fresh, as separate specimens to the cortical resections and are orientated and measured in three dimensions including the longitudinal axis. The fresh specimen is sliced coronally at 3-5mm intervals and in general 3-5 slices are made. The specimen is then photographed (see Figure 1). The hippocampal structure is identified (CA1 and the hilum). A slice including the hilum is reserved for Timms staining by fixation in sodium sulphide solution. A second slice is snap frozen for receptor and molecular studies and the remaining tissue fixed in 10% formalin. The formalin fixed tissue is then routinely processed on a long processing cycle (one week) and paraffin wax embedded. Standard stains used in the examination of tissue from a temporal lobectomy include H&E, luxol fast blue with cresyl violet (LFB/N) to assess the cyto- and myelo-architecture, Bielschowsky silver technique to assess cytoskeletal abnormalities and GFAP to assess the presence of gliosis.

5.2 Ethical permission for study

Ethical approval for the following immunohistochemical and morphological studies has been granted for archival (pre-2000) surgical tissue and for that acquired since 2000 by the Joint Research Ethics Committee for the National Hospital for Neurology and Neurosurgery and the Institute of Neurology. The data base held conforms with University College guidelines in accordance with the Data Protection Act.

5.3 Case selection and controls

5.3.1 Review of all temporal lobectomies 1993-2000 (Study I)

All surgical resections from adult patients who had undergone epilepsy surgery for the treatment of refractive epilepsy between the years 1993-2000 at the National Hospital for Neurology and Neurosurgery were reviewed. The cases were ascertained from the records in the Division of Neuropathology and entered into a database; 413 cases were identified. The original slides were retrieved from file and reviewed together with the surgical reports held in

pathology files. In a small number of cases further stains, including immunostains, were requested following review. The pathological diagnosis was also entered into the database. Pre- and post- surgical clinical information wherever available, including post-operative seizure control, was also entered in the database. This clinical information was available from clinical neurologists reviewing patients in clinics and from review of patient records ; only patients with a follow-up period of at least 2 years were included in the present analysis.

5.3.2 Case selection for study of white matter neuronal densities, layer I neuronal densities and cortical microdysgenetic features (Study II)

Thirty-one temporal lobe specimens were initially selected for detailed morphometric analysis of microdysgenetic features including white matter neuronal densities. For this study cases operated between 1994 and 1999 were randomly selected from the pathology archive. They included 17 right and 14 left sided temporal lobectomies. They were selected without knowledge of the clinical outcome. In all cases the adult patients had suffered from medically intractable temporal lobe seizures, with a mean age at surgery of 36 years. Standard pre-operative investigations, including MRI, were compatible with unilateral hippocampal sclerosis and no other lesion could be detected on pre-operative MRI. In each case the original 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 similarly processed and hippocampal sclerosis of the classical type, with cell loss in CA1 and hilar subfields, was confirmed in all cases. Control cases for this study included fifteen temporal lobe specimens, which were similarly analysed. The mean age of the control group was 52 years. Nine of the controls were from the right and 6 from the 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.

Table 2. Details of controls used in study II and in Study VI

Case control number	Age /sex	Cause of Resection / Death	Post mortem delay	Fixation Time
1.	27 M	Surgical resection ; Temporal lobectomy to treat brain swelling following acute cerebral trauma. No significant pathology	NA	1 day
2.	47 M	Surgical resection : DNT in the hippocampus	NA	5 days
3.	42 F	Surgical resection : Cavernous haemangioma in the hippocampus	NA	5 days
4.	38 F	Surgical resection : Cavernous haemangioma in the hippocampus	NA	5 days
5.	85 F	PE	3 days	7 days
6.	61 M	MI	6 days	8 days
7.	43 M	MI	1 days	4 days
8.	49 M	MI	1 day	4 days
9.	63 M	MI	3 day	7 days
10.	45 F	Carcinomatosis	1 day	7 days
11	56 F	NR	NR	7 days
12.	65 F	NR	NR	7 days
13.	NR	NR	NR	14 days
14.	45 F	MI	2 days	7 days
15.	67F	MI	NR	14 days

NR=not recorded, MI=myocardial infarct, PE=pulmonary embolus

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 remaining controls are presented in Table 2.

Following this initial study of white matter neurones in the 31 cases, a second phase of the study was carried out. A further 50 consecutive temporal lobectomy specimens for epilepsy carried out between the period 1996-1998 were selected from the pathology files. In all these cases white matter neurones in the temporal lobe were identified on initial routine sections and hippocampal sclerosis was the main pathology with no second cortical lesion.

5.3.3 Case selection for study of cortical myelination in microdysgenesis in epilepsy (Study III)

All temporal lobectomy specimens from 1993-2000 were reviewed and the myelin stained sections (LFB) examined for the analysis of patterns of cortical myelination in the superficial layers. In normal temporal neocortex thin myelinated fibres are observed running horizontally in the molecular layer. Surgical lobe cases with abnormal cortical myelination patterns were identified from the files. Control cases for this study included eight surgical temporal lobectomy specimens from patients with temporal lobe epilepsy (age range 24-50 years) without abnormal cortical myelination, six with hippocampal sclerosis and two with mass lesions in the hippocampus (one DNT and one cavernoma), none of which showed features of temporal lobe microdysgenesis. Identical tissue processing, staining and quantitation were carried out on both groups as detailed below.

5.3.4 Case selection for study of cytoarchitectural abnormalities in hippocampal sclerosis (Study IV)

For this study I examined 206 anterior temporal lobectomy and hippocampectomy specimens. All the patients were adults at the time of surgery with mean age 31.6 years (range 15-58) and suffered medically refractory temporal lobe epilepsy. In all cases standard pre-operative investigative protocols, including MRI measurements and EEG studiedswere carried out. In

183 cases, of a possible 251 in which a pathological diagnosis of HS was made , were selected where blocks and slides were all available. In these cases a second well-defined extra-hippocampal pathology was not identified. A second control group of 23 cases were also selected in which an extra-hippocampal, presumed epileptogenic pathology was identified in the temporal lobe but the hippocampus was also surgically removed. These dual pathology cases (often referred to as 'mass lesion' temporal lobe cases) were included to provide a comparison group to cases with HS alone. The extra-hippocampal pathologies in these cases included DNT (6), ganglioglioma (2), meningioangiomatosis (1), cavernoma (4), old infarct (5), Rasmussen's encephalitis (2), old traumatic lesions (4), with one patient having both chronic encephalitis and a cavernoma. In addition, post mortem hippocampal tissue from six age-matched neurologically normal patients was used as control tissue for the stereological estimation of granule cell number.

5.3.5 Case selection for study of Cajal-Retzius cell populations (Study V)

The cases of FCD and MD and controls selected in part VI of the study (see section 5.3.6) were used for the study of Cajal-Retzius cell populations. Cases and controls used in study II (see Section 5.3.2) were also analysed. In addition, 23 cases of HS from study IV (see Section 5.3.4) were included and 13 control specimens without HS. The HS cases were seleted according to the severity of granule cell dispersion (see Methods Section 5.5.3.2).

5.3.6 Case selection for study of inhibitory interneurones in focai corticai malformations (Study VI)

The cases for this part of the study were selected from the neuropathology records at the Institute of Neurology/National Hospital for Neurology and Neurosurgery and the Institute of Child Health/Great Ormond Street Hospital for Children, London. (This work was carried out with the collaboration of Dr. B. Harding, Department of Neuropathology, Great Ormond Street Hospital.) All of the patients had undergone surgery for intractable epilepsy. Twelve patients with Focal cortical dysplasia (FCD) were selected from files. All had a single lesion visible on MRI, were operated between 1995-2000 and in all cases adjacent cortex without dysplasia was also present in the resection

specimen. Nine FCD cases were paediatric (8 frontal, 1 hemispherectomy) with an age range 1-15 years (mean 6.7 years) and three were adult (2 temporal, 1 frontal) with an age range 26-35 years (mean 31 years). Twelve cases of MD involving the temporal lobe were selected, all operated between 1995-2000 and all adults (age range 28-53 years, mean 37 years). In all MD cases hippocampal sclerosis was also present. The diagnosis of FCD was based on the typical MRI and histological features showing an abnormal disorganised region of cortex with dysplastic neurones and, in most cases, additional balloon cells. The diagnosis of MD was based on the findings from study II and based on the presence of high white matter neuronal densities on NeuN immunostained sections (>2000 neurones /mm^3) or distinctive cortical cyto-architectural abnormalities of MD (see Section 6.2.1 and 6.3). The control tissues included normal post mortem tissue from five paediatric cases (age 1-5 years, including frontal and temporal cortex) and normal surgical and post mortem tissue from eight adult cases (age 28-88; mean 59 years, all temporal cortex). The fixation time for all the paediatric post mortem cases was less than a week and for adult cases ranged from 1 to 14 days (mean 7 days). The post mortem interval for the adult cases ranged from 1 to 6 days (mean 2.4 days). In all FCD cases, adjacent cortex with no neuropathological features of dysplasia was also available for comparison.

5.4 Specimen preparation and immunohistochemistry techniques.

For study I the tissue was processed and stained as outlined in Section 5.1 above and the original slides, including immunostained sections for GFAP, were reviewed.

In study II, the archival tissue blocks from the selected temporal lobe specimens were retrieved and further serial sections cut at a thickness of 20-25 microns and immunostained with the following primary antibodies: GFAP, NeuN and Calbindin. The GFAP sections were counterstained with Cresyl violet and the NeuN and Calbindin counterstained weakly with haematoxylin. Thick sections were required in order to use the optical disector technique. Between 4-9 blocks were present in each case (mean 6).

In study III further sections were also cut at 20-25 microns and immunohistochemistry for GFAP, neurofilament, phosphorylated neurofilament, NeuN and Calbindin D-28-K was carried out. In addition, double immunolabelling for GFAP and Calbindin was carried out using ethylcarbazole and nickel enhanced diaminobenzidine as the respective chromogens.

In study IV the hippocampal specimens were formalin fixed for 2 to 5 days, sliced coronally at 0.4 cm intervals, routinely processed (mean 4.5 blocks per case) and serial sections stained with H&E and Luxol fast blue with cresyl violet. After initial review further sections were selected for Bielschowsky silver staining and GFAP, neurofilament and phosphorylated neurofilament immunostaining. In 75 cases Timms staining for mossy fibres was carried out. For this staining, a fresh hippocampal slice was immersed in buffered 1.2% sodium sulphide solution, lightly fixed in paraformaldehyde, processed with silver developer, counterstained with haematoxylin and mounted.

In study V the selected sections from the cases were sectioned at 7 microns and immunostained with reelin, calretinin and calbindin primary antibodies.

For study VI in each case the fresh tissue was rapidly formalin-fixed and paraffin-embedded ; 1-3 representative blocks selected were further sectioned

at 7 microns thickness and immunostained for calretinin, calbindin, parvalbumin and NPY antibodies.

A standard immunohistochemistry technique was employed as follows and details of the antibodies used in these studies, including source, clone, dilution, pretreatments and incubation, are shown in Table 3. Sections were de-waxed and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked in 0.6% hydrogen peroxide and methanol for 15 minutes. Sections were then pre-treated, as appropriate with the primary antibody (see Table 3). For immunohistochemical stains requiring microwave pre-treatment sections were microwaved for 20 minutes in 0.01M citrate buffer pH6 followed by protein blocking with 10% normal swine serum for 20 minutes. Sections were incubated either overnight at 4 °C or for 1 hour at room temperature. The antibodies were diluted in 1% BSA and 0.05% tween. Detection systems used included avidin-biotin techniques or the LSAB kit from DAKO Corporation, USA. Antibody staining was visualised with chromogen DAB. In addition, double immunolabelling for GFAP and Calbindin was carried out using ethylcarbazole and nickel enhanced diaminobenzidine as the chromogens and the different colours were distinguishable with light microscopy (see Figure 5g). Counterstains used were either cresyl violet (for GFAP) or haematoxylin (all other antibodies). The slides were mounted.

Table 3. Details of antibodies used in studies

Primary Antibody	**Clone Monoclonal Polyclonal**	**Source**	**Dilution**	**Pretreatment protocol**	**Other information**
GFAP	Polyclonal	Dako, Cambridge	1:400	Microwave	
NeuN	A60	Chemicon, Harrow	1:500	Microwave	
Calbindin	D-28-K Monoclonal	Sigma, Poole	1 : 200	Microwave	
Calretinin	Polyclonal	Swant, Switzerland	1:1000	Trypsin (0.1%)	
Parvalbumin	Monoclonal	Swant, Switzerland	1:8000	Microwave	
NPY	Polyclonal	Sigma, UK	1:500	Microwave	
Neurofilament 70-kDa 200-kDa non phosphorylated	2202MF	Euro-diagnostica	1 : 10	No pretreatment	
Phosphorylated neurofilament	2F11	Dako,UK	1:100	Microwave	
Reelin	Cone 142 Monoclonal	Gift from Prof A Goffinet, Belgium	1:1000	Microwave	

5.5 Quantitative methods

5.5.1 Study II : Counting rules for cell densities

Cell densities were estimated using a direct three-dimensional cell counting technique and the optical disector method (Williams and Rakic, 1988 – see Section 3.7). For this purpose 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 (the z axis). An oil immersion lens (magnification x 100, aperture 1.4) was used to provide a narrow depth of field together with an eyepiece graticule for the counting box (dimensions 100 μm square and depth 10μm). The thickness of the section was measured first by zeroing the gauge at one edge of the section (where the first cell in any field came into focus), focusing through the section the other edge (where the last cell in the field just moved out of focus). Although sections were cut on the microtome at a 20 micron setting for 3-D cell counting, after de-waxing and staining the actual measured thickness varied from 10-30 microns. Any section less than 14 microns was deemed unsuitable for cell counting and a further section was cut and stained. When the section thickness was measured (T microns) the plane of focus was moved to a distance (T-10)/2 microns away from the top of the section using the digital gauge. The gauge was then reset at zero and this became the top of the counting box. This ensured an equal 'guard volume' on either side of the counting box to avoid counting cells damaged by the microtome. Cells were then counted from this plane, focusing through this section to a depth of 10 microns.

Cell counting rules : 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. Cells outside the counting box or touching one of the three forbidden planes, either within or outside the box, were not included. In the NeuN stained sections all immunopositive cells were counted. The antibody stained both the nucleus and the cytoplasm but the nuclear membrane was more clearly defined compared to the cytoplasmic boundary. Therefore for cell counting purposes the nuclear border was considered as the cell boundary when intersections with the counting grid occurred. The eyepiece graticule was further divided by a grid into 100 boxes (10 x 10 microns each), to allow approximation of neuronal diameter during counting. Neurones were

categorised as larger or smaller than 10 microns diameter. Similar counting procedures and rules were carried out in all following studies employing 3-D cell counting techniques. The maximum number of neurones counted in each counting box in the white matter was 4 and in many counting boxes no neurones were seen. When the analysis of one counting box was complete the score was recorded on a result sheet and the stage moved manually to the next counting box taking care to avoid overlapping fields. The process was then repeated. When the counting box reached the edge of a section, if the centre of the grid in this field was overlying tissue, the neurones in this box were counted ; if not overlying tissue, this box was not included. The total number of counting boxes per case was also recorded on results sheets in order to calculate the neuronal density.

5.5.1.1 Pilot study to determine the number of counting boxes required in the white matter

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 LFB stained section as a reference. The boundaries of the lower claustrum in the white matter of the superior temporal gyrus were also outlined with ink. This therefore outlines the area of interest in the white matter for morphometric analysis. Neurones within the white matter were counted in parallel columns beginning two counting box widths away from the inked cortical boundary (to ensure exclusion of lamina VI cells), moving systematically to the periventricular surgical resection margin (Figure 1a). 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 six pilot cases and controls (Figure 2) all positively labelled cells were counted in the white matter and categorised according to approximate nuclear size of greater or less than 10 microns diameter.

Using this method it took over one week to count one case which was considerably labour intensive. Therefore a further experiment, where either six

Figure1 : Temporal lobectomy specimens in patients with epilepsy

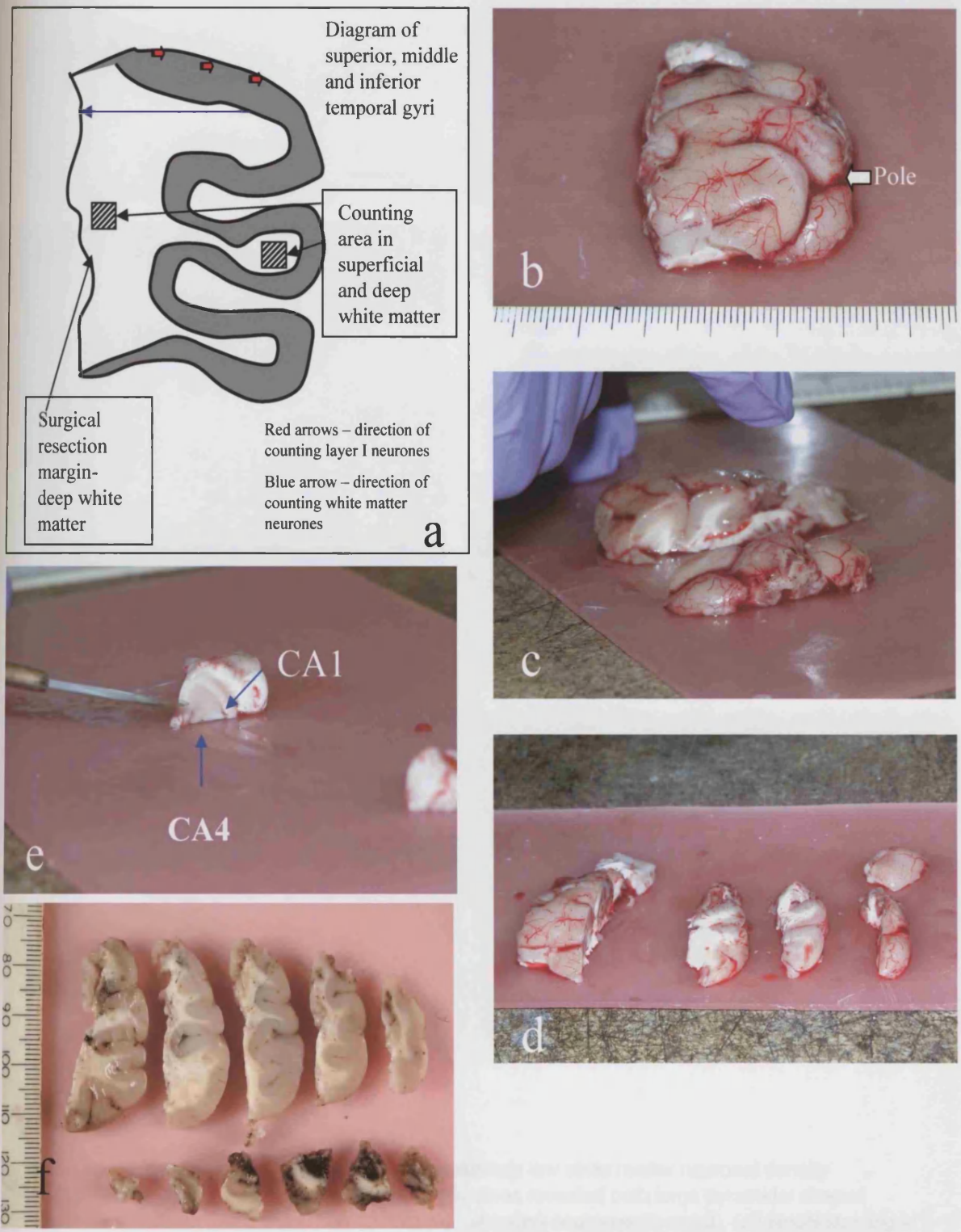
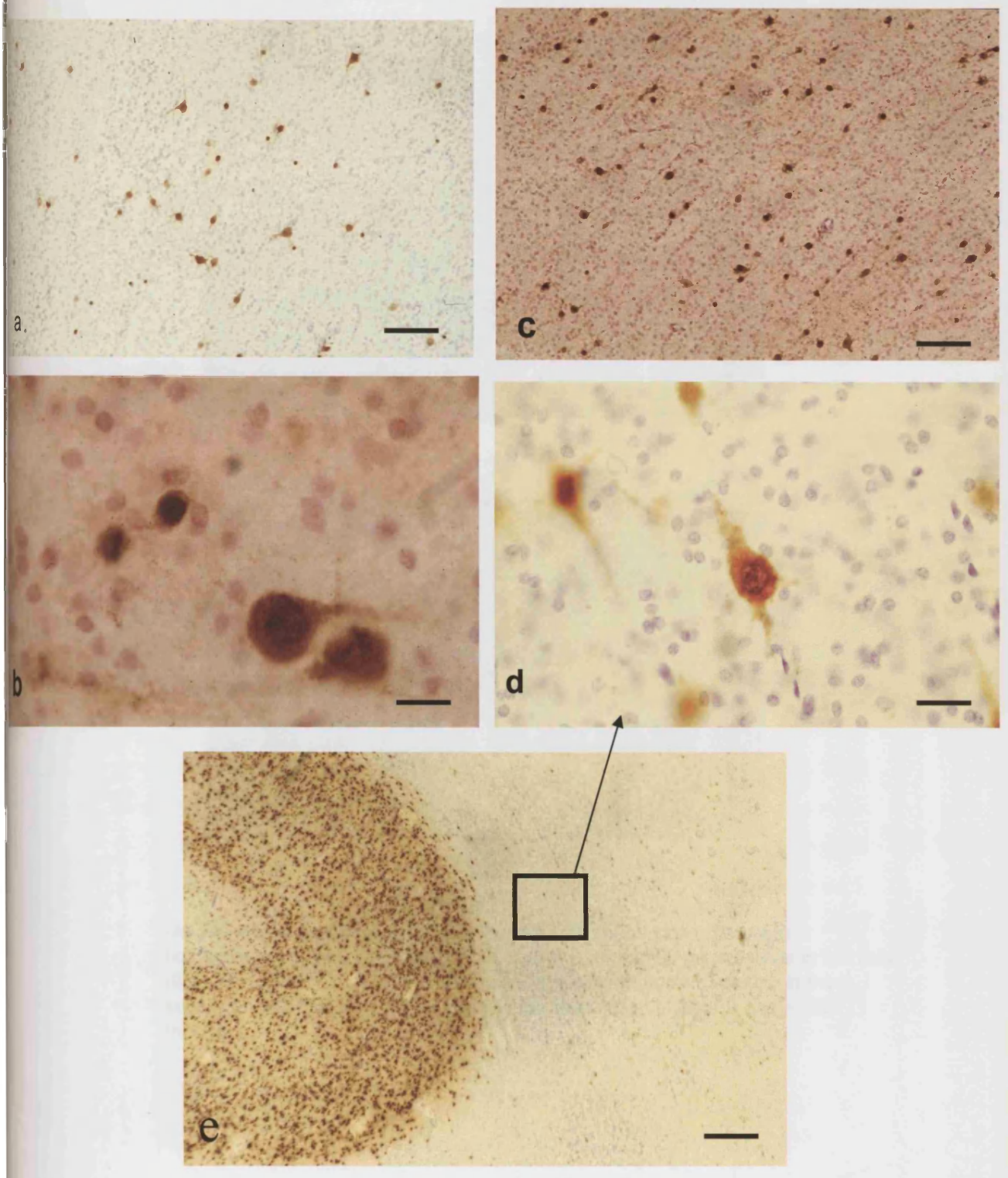
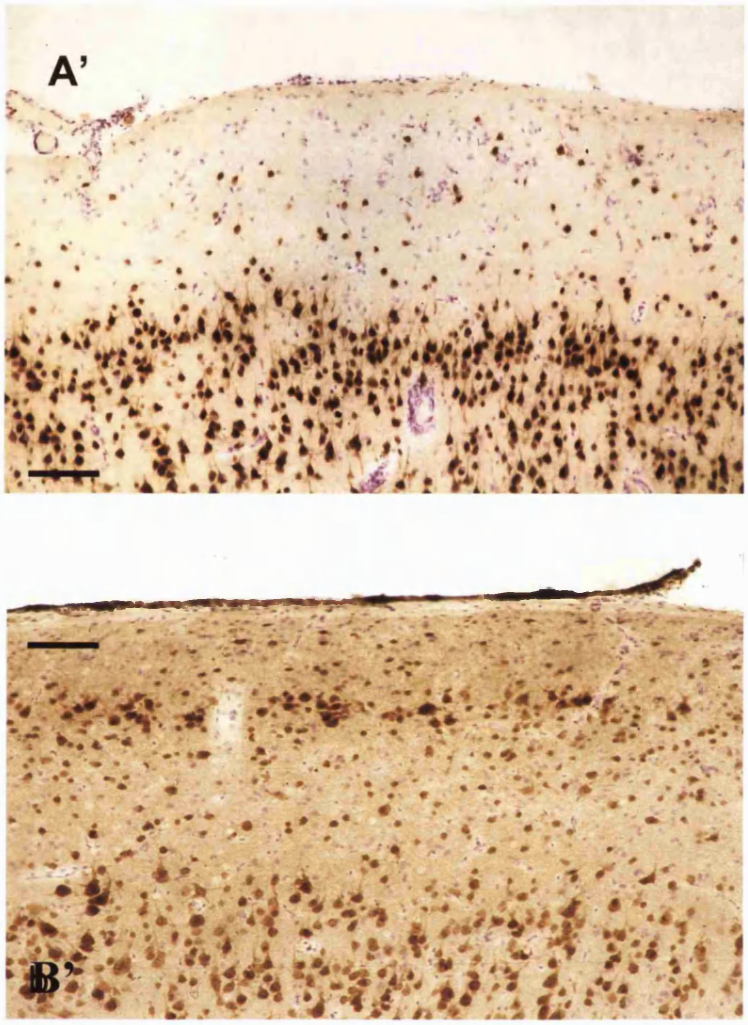


Figure 1: a) Diagram representing a coronal section of temporal lobectomy specimen depicting superior, middle and inferior temporal gyri. Arrows and boxes illustrate the sampling methods for estimating neuronal density. (b-d) Macroscopic appearances of fresh temporal lobe specimen, orientated (b) then serially sliced at approx.4-5mm intervals in coronal plane (c,d). (e) Coronal sectioning of hippocampal surgical specimen. (f) Whole temporal lobectomy and hippocampus post fixation (anterior margin to right).

Figure 2 : NeuN Immunostaining results in temporal lobe specimens



(a) Lateral temporal lobectomy specimen with relatively low white matter neuronal density (<1800/mm3) . NeuN staining of white matter neurones revealed both large pyramidal shaped neurones and small round (less than 10 microns diameter) neurones (b and d). (c) NeuN staining in white matter in a TLE case with higher neuronal density (ND=2800 /mm3) following 3D cell quantitation. (e) White matter as viewed at low power with clear demarcation of white matter from the cortex as viewed on NeuN staining. (Bar in (a and c) is 130 microns, (b) 18 microns, (d) 30 microns and (e) 380 microns approximately).



(A') NeuN positive neurones in layer I and II in temporal lobe adjacent to hippocampal sclerosis. The majority of cells in layer I are small neurones in this field. (B') The superficial cortex in another case showing neuronal clustering in the superficial part of layer II and cell loss in the deeper part of layer II. Bar = 120 microns

or four columns of white matter were counted at random intervals across each slide (regardless of the size of section), in both cases and controls, was carried out and then repeated after an interval of several weeks. The columns were placed randomly but at equal distances across the white matter (uniform random). The justification for this sampling strategy of uniform random columns as opposed to uniform random counting frames is as follows : Uniform random counting frames (e.g., counting 1 frame in every ten after starting at a random point) would still require the counting frame to be moved systematically across the whole area of white matter. It is the constraint of moving the small counting frame systematically and manually across the large area of white matter that is the most time-consuming aspect of this procedure rather than the number of cell counts to be made, the white matter being relatively paucicellular. For example, in comparison to the hippocampus which may have only 10-20 counting boxes per subfield in total (Van Paesschen and Revesz, 1997) in the temporal lobe white matter there are over 7000 potential counting boxes. Counting in random columns therefore reduces the total number of counting boxes the microscope moves through. Using 3-D counting methods in columns has been a sampling scheme previously applied to examination of the cerebral cortex (Everall et al., 1994) and, due to the radial arrangement, albeit less distinct, of neurones in the white matter I considered this method would be an appropriate and more practical sampling scheme for the white matter. This pilot study carried out on six cases showed that counting six columns of white matter gave comparable ND estimates to counting all white matter neurones with good repeatability on recounting (Table 4). This limited white matter analysis allowed one case to be examined in approximately ten hours.

Table 4. Pilot study for 3-d analysis of white matter neurones

Pilot study of sampling strategies and repeatability of measurements for white matter neuronal densities (ND) on six lobectomy specimens. Mean ND calculated by counting six or four random (cortical to periventricular) white matter columns (see Figure 1) were compared to ND from analysis of counting entire white matter. Stronger limits of agreement (mean difference in ND ± 2sd) were achieved when counting six columns. Repeat ND measurements counting 6 columns were then carried out ; RC (repeatability coefficient) = 2sd of mean differences in ND between first and second measurement. (Bland and Altman, 1986).

Area of white matter sampled (N=6 cases)	All white matter	Six columns per section	Four Columns per section
Mean Boxes counted per case	7020	1400	950
Mean ND /mm^3	2084	2170	1890
Mean Difference (SD)	-	-86 (-215)	194 (360)
Limits of Agreement	-	-516 to 344	-526 to 914
Repeatability(RC) % RC of mean ND	-	436 20%	720 34%

CASE	ND /mm3 4 columns	ND / mm3 3 columns	Differ-ence	ND/mm3 2 columns	Differ-ence	ND/mm3 1 column	Differ-ence
1	32,338	34,285	-1947	35,869	-3531	31,156	1182
2	46,868	47,711	-843	47,774	-906	46,441	427
3	37,937	33,942	3995	35,309	2628	31,556	6381
4	28,728	30,272	1544	32,105	-3377	31,297	-2569
5	47,768	44,600	3168	56,346	-8578	46,428	1340
6	46,506	46,560	-45	47,000	-494	46,400	106
Mean RC (2.sd) %RC of mean ND	40,024	39,561	980 4656 11%	42,400	-2376 7564 18%	38,879	-1144 5944 14%

Pilot data for Cortical Neuronal Densities in six cases counting 4 to 1 columns (see section 5.5.1.6) with repeatability coefficients and expressed as a % of mean ND in all cases

5.5.1.2 White matter astrocytic densities

In GFAP/cresyl violet sections in epilepsy cases, parallel ND (neurones identified as cells with vesicular nuclei, prominent nucleoli and Nissl positive cytoplasm, irrespective of their size) and GFAP positive astrocytic densities / , mm^3 (AD) were calculated in 18 cases using the same counting methodology as above. For this purpose an astrocyte was defined as a cell with a nucleus and GFAP positive cytoplasm with delicate cytoplasmic processes. The nuclear membranes in both neurones and glial cells were considered as the cell borders for counting purposes.

5.5.1.3 White matter neuronal densities in different anatomical compartments of temporal lobe

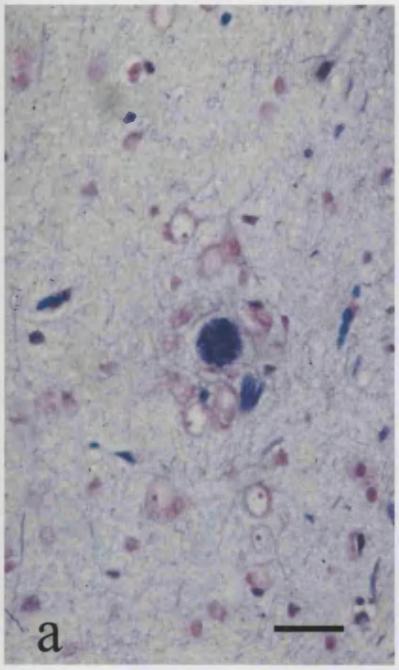
In thirteen cases, further measurements were carried out to evaluate any differences in ND between superficial and deep white matter. A NeuN immunostained section from the caudal level of the temporal lobe resection (CTL), which included part of the claustrum, was selected from each case. This level was chosen as it more often had the greatest area of white matter, including periventricular white matter and provided an anatomically comparable region between cases. Both large and small neurones were counted within an area of 10 by 10 counting boxes, in the white matter core of the middle temporal gyrus and in a similar area of deeper white matter nearer the surgical margin (Figure 1a), and the ND compared.
White matter neuronal densities were also compared between the anterior temporal lobe (pole) and the posterior temporal lobe (at the level of the claustrum).

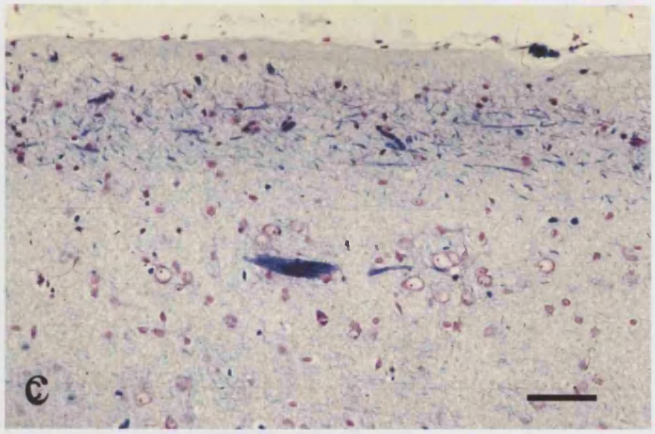
5.5.1.4 Semi-quantitative analysis of other microdysgenetic features including cortical neuronal clusters and layer I calbindin positive cells.

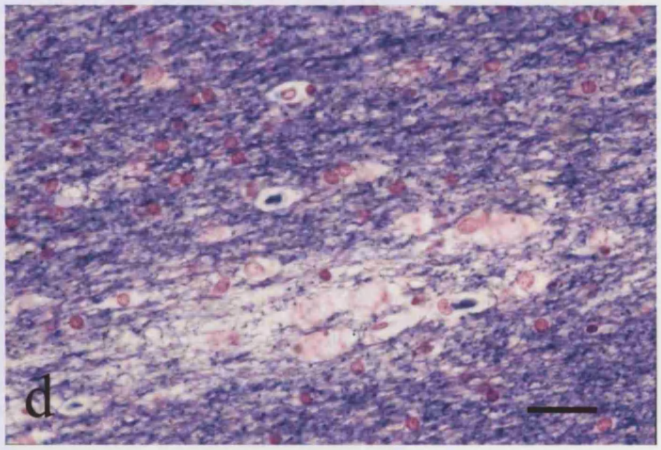
The number of neuronal clusters in the superficial cortex was also estimated on NeuN sections from caudal temporal lobe (CTL). Neuronal clusters, defined as three or more overlapping adjacent neurones without intervening neuropil, were counted in 20 consecutive fields along cortical layer II of the middle temporal gyrus using the eyepiece grid and x40 objective (total area = 5 x

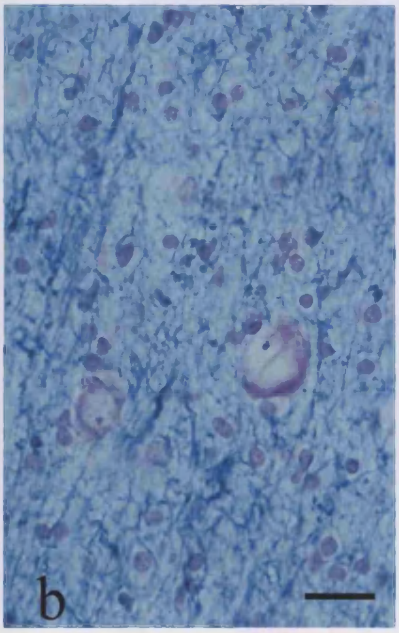
10^{-2} mm^2), beginning in the sulcal depth and moving systematically around the gyrus, in a coronal plane. The presence of abnormal tangential myelinated fibres in the superficial cortex (Figure 3c), as described in Section 6.3., was also assessed on all the LFB stained sections of each case. The number of calbindin immunoreactive cells in cortical layer I were counted along the middle temporal gyrus at the CTL level from each case. Using a 2-D cell counting method immunopositive cells were counted with a x10 objective in 20 consecutive fields (total area 10mm^2) beginning in the sulcal depth and moving the eyepiece grid systematically around in the coronal plane (Figure 1a).

Figure 3: Pathological features of MD (Microdysgenesis).

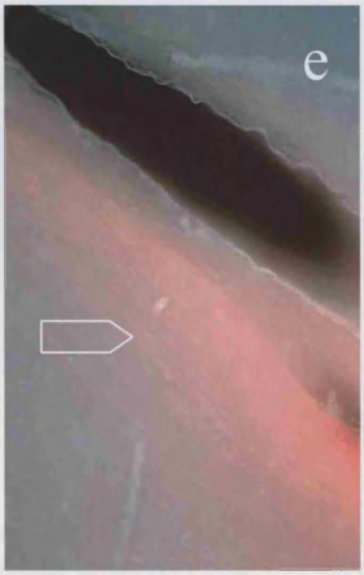








Bars : a-50 microns, b 25 microns, c 125 microns, d 40 microns.



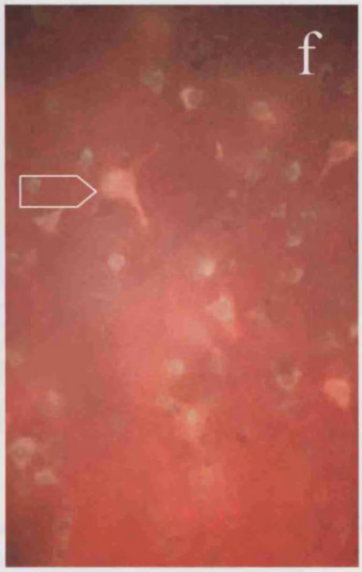


Figure 3 : Features of microdysgenesis including scattered single neurones in the white matter (b). Normal anatomical structures, as part of the claustrum in the white matter are distinguished from white matter neuronal heterotopia (d). Abnormal bundles of horizontally orientated myelinated fibres in the superficial cortex with surrounding clusters of neurones (a and c). All stained with LFB/Nissl. (e and f) show results of DiI crystal placement in superficial temporal lobe. Some diffusion of the dye along fibres in layer I is seen (arrow in e) and occasional positive pyramidal cells away from the site of crystal placement (arrow in f) where as remaining pyramidal cells are stained only with bizbenzimide.

Figure 3 continued : Features of Microdysgenesis

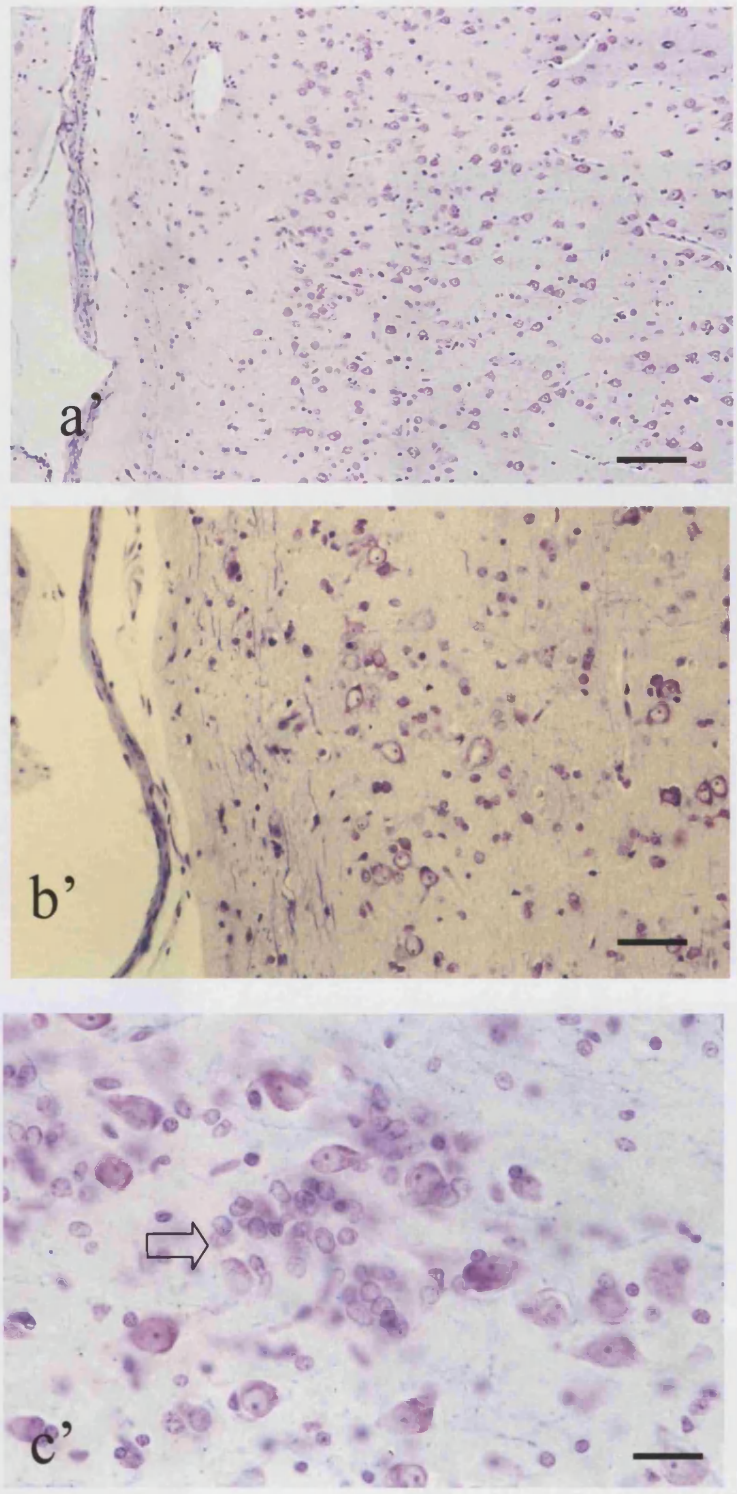


Figure 3 continued. Features of microdysgenesis: (a') Normal cortical layer I, II and III. (b') Disturbance in the normal cortical laminar architecture is seen in the superficial layers with neurones appearing larger and abnormally orientated and distributed in microdysgenesis ; distinction between layers is less apparent. (c') A glioneuronal hamartia ; microscopic aggregate of immature neurones (arrow). (All stained with LFB/CV ; Bars : a= 130, b=50, c=30 microns.

Figure 4: Neuropathological features of FCD (Focal Cortical Dysplasia)

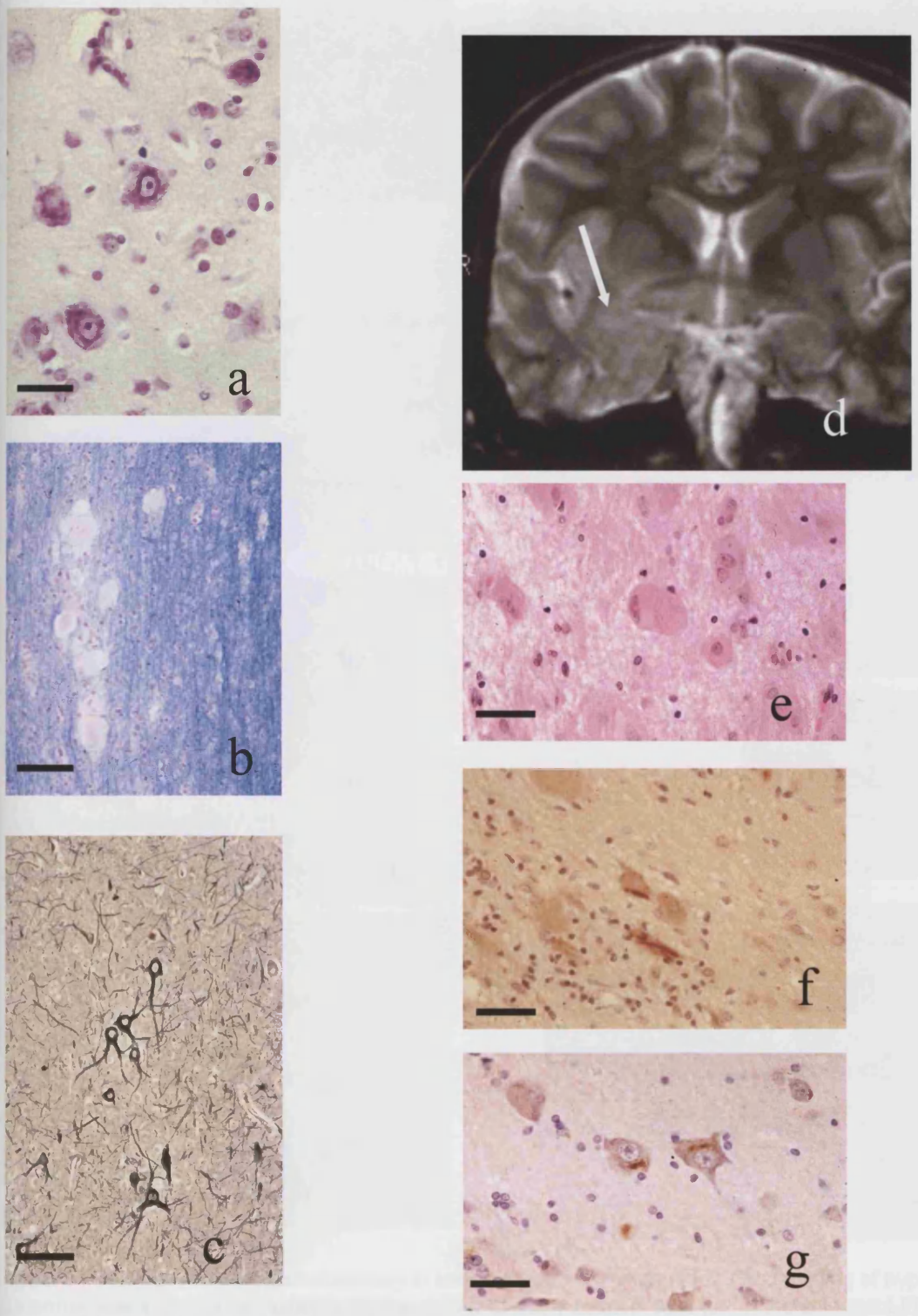


Figure 4 : FCD: The typical pathological features, including dysplastic neurones with thickened nuclear membranes in the cortex (a) and aggregates of balloon cells in the white matter (b) (both LFB/N stains). c) Bielchowsky silver impregnation of dysplastic neurones showing enhanced staining and abnormal morphology of dendrites. d) the typical radiological features of FCD on T2 MRI (see section 3.4.3.1.2.). e) Balloon cells with eosinophilic rod like inclusions and cdk5 immunopositive aggregates in balloon cells (f) and dysplastic neurones (g). Bars:a 25 , b.c 80 , e,f,g, 30 microns

Figure 5 : Calbindin Immunohistochemistry

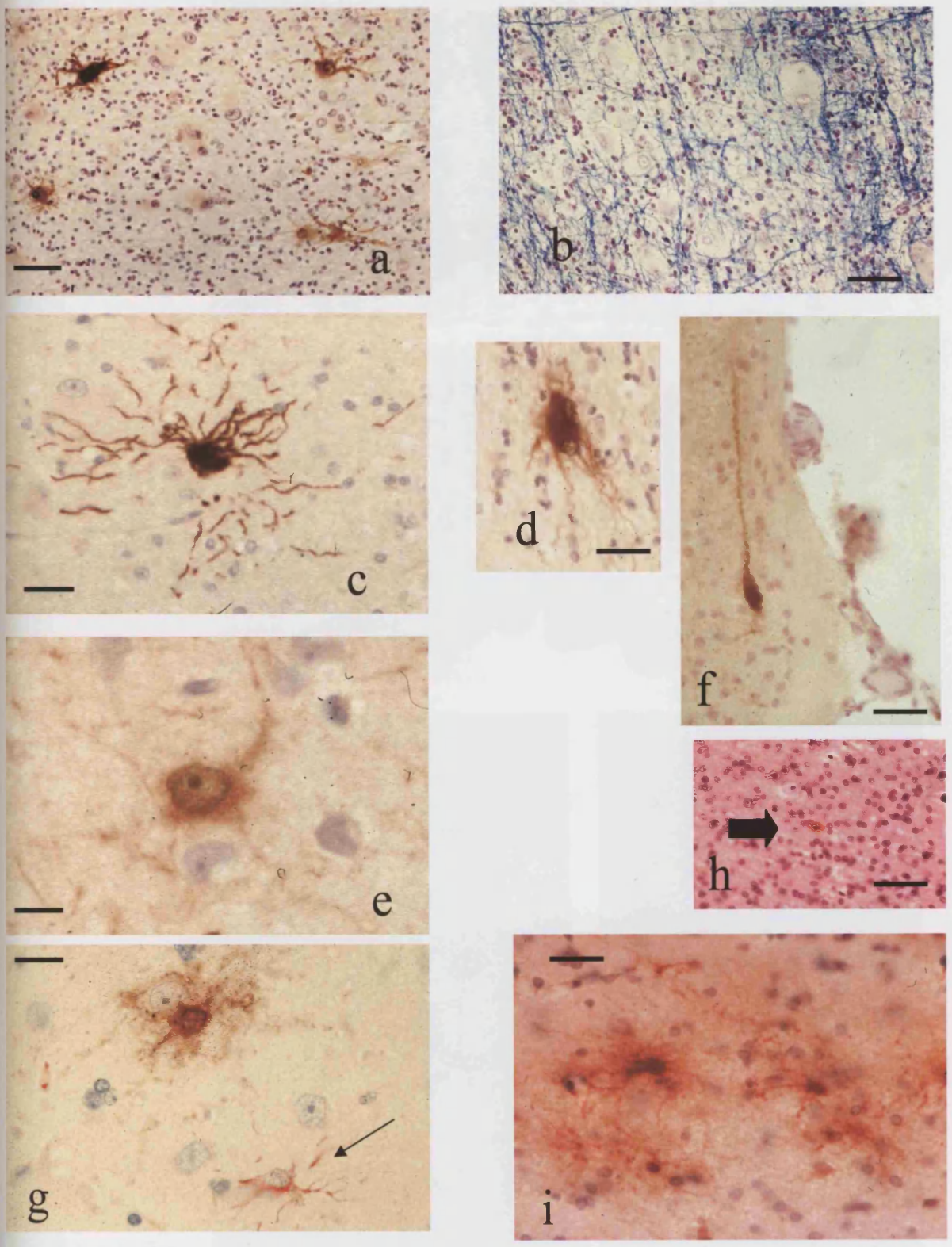


Figure 5 : Calbindin immunohistochemistry in temporal lobe specimens. In FCD, labelling of hypertrophic abnormal cells in the white matter (a) in the vicinity of many balloon cells (b) in the adjacent LFB/N stained section. (c& d) Abnormal multipolar and multinucleate calbindin positive cells in FCD and (e) weak staining of a pyramidal cell with calbindin in FCD. (f) Calbindin positive cell in layer I with the morphology of a Cajal-Retzius cell. In microdysgenesis (g) Double immunolabelling with GFAP and calbindin of astrocytes (red) and multipolar neurogliaform cell (brown) in microdysgenesis confirming the neuronal nature of the latter and (i) clusters of calbindin positive neurogliaform cells in MD. (h) A proportion of small neurones in the white matter are also calbindin positive (arrow). Bars – a,b,h – 50, c,d,f,g,I,-30, e- 18 and h -80 microns.

Figure 5 continued : Calbindin Immunohistochemistry

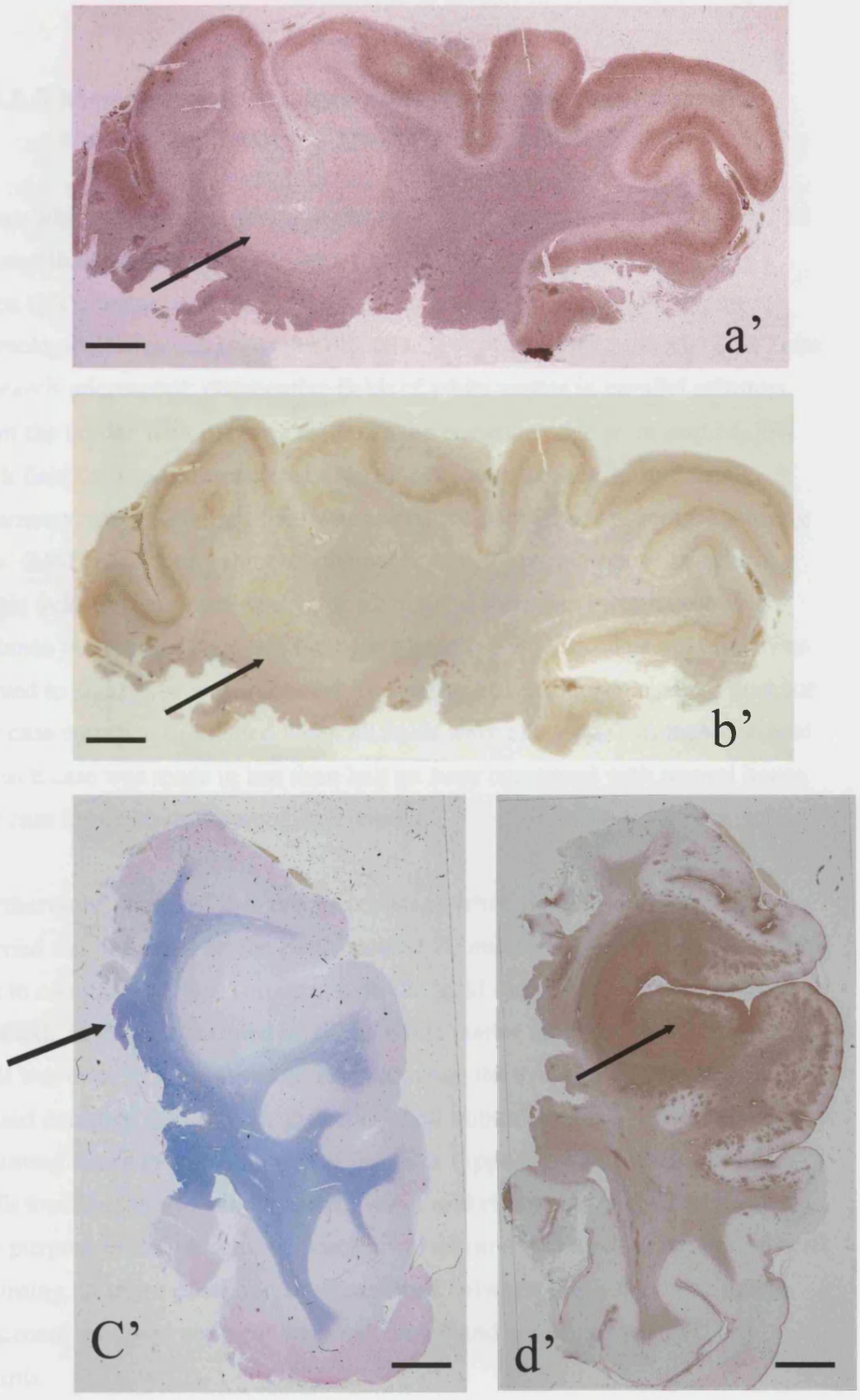


Figure 5 continued : FCD showing an area of thickened cortex with blurring of the grey-white matter boundaries on LFB/N (c') This region is extensively gliotic on GFAP stained section (d') and in calbindin stained section (a') the region of dysplasia (arrowed) corresponds to an area of dramatic reduction in labelling compared to the adjacent cortex. This is also apparent with parvalbumin immunohistochemistry (b'). Bar in each .8 cm approx.

5.5.1.5 Measurement of white matter interneuronal distance using image analysis system and 2-D cell counts

Mean white matter interneuronal distance was calculated on NeuN stained 20 micron thick sections of posterior temporal lobe (CTL) in each case using a Leica Q500 image analysis system (Leica, Cambridge , UK) and Q Prodit Stereological Software. Using an objective lens of magnification x10 on a Zeiss Research microscope, consecutive fields of white matter in parallel columns from the border with the grey matter to the resection edge were studied. In each field the mean distance between all neurones (both large and small interneurones) within that field was calculated using the 'Minimum spanning tree' (MST) tool. Using this morphometric tool, all immunopositive cells in a single field were marked on a video screen and the mean interneuronal distance between all cells was then calculated for this field. The next field was moved to and the process repeated. The average overall interneuronal distance per case was then calculated when all fields were analysed. This measurement in each case was made in less than half an hour compared with several hours per case for three-dimensional cell counts.

Furthermore, standard 2-D profile counts of white matter neurones were carried out in 8 cases on the NeuN stained 20 micron sections. This was carried out to compare 2-D cell counting with the 'gold standard' 3-D stereological method. Cells were counted in all the white matter in all sections in each case. This was done at a magnification of x20 using the eye-piece graticule as a biased counting grid of area ($0.25mm^2$). All immunopositive cells within the counting frame or touching two of the sides (upper and left) were counted. Cells touching the 'forbidden sides' (lower and right) were not included. For the purpose of cell margin, the nuclear membrane was used, as for the 3-D cell counting. In terms of labour, each case took between 6-8 hours to complete. Neuronal numbers per mm^2 were calculated and compared with 3D cell counts.

5.5.1.6 Estimates of cortical neuronal densities

On the caudal temporal lobe section from 27 cases, estimates of cortical neuronal densities were carried out using the three-dimensional cell counting method. Estimates of NeuN positive neuronal densities were carried out on the respective immunostained sections. The 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. This specified anatomical site was counted in each case, as cortical cyto-architecture is known to vary within the temporal lobe (Brodmann). Positive cells were counted in vertical columns from the cortical-white matter boundary to the pia (including layer I) at a magnification of x100 (lens aperture 1.4), using a counting box of 80 x 80 microns area and a depth of 10 microns which was moved systematically through the cortex. Identical counting rules as for the white matter were used. In the cortex up to 6 cells per counting box were present. Four randomly placed columns per section were counted following a pilot study (see Table 4 page 116), to include gyral crest, sulcus and two mid-gyral points within the cortex. The mean ND for this region of cortex was estimated.

5.5.1.7 Layer I neuronal densities (NeuN positive neurones)

Layer I (molecular layer) neuronal densities (ND) were also separately estimated in 31 cases on 20 micron thick NeuN stained sections of CTL in cases and controls (Figure 2a,b, page 114). The three dimensional cell counting method was used and identical counting rules applied. 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 (Figure 1a). All immunoreactive cells were counted as before and categorised into small and large neurones (greater or less than 10 microns diameter) and the ND for this region of the temporal lobe then calculated.

5.5.2 Study III : quantitative methods in cases with abnormal cortical myelination

Estimations of NeuN and calbindin positive cortical neuronal densities were carried out on the respective immunostained sections using the 3-D cell counting technique and an optical disector. Positive cells were counted in

vertical columns from the cortical-white matter boundary to the pia at a magnification of x100 (lens aperture 1.4), using a counting box of 80 x 80 microns area and a depth of 10 microns which was moved systematically through the cortex. Four columns per section were counted as before, to include gyral crest, sulcus and two mid -gyral points within the abnormal cortex. Identical quantitation was carried out on control cases.

In four temporal lobectomy specimens DiI tracing studies were carried out. DiI is a carbocyanine dye, a fluorescent lipophilic substance, which will label neurones and their processes in a retrograde and anterograde fashion and can be used on fixed tissues. The staining diffuses at a rate of 400 microns / day and hence long incubation periods are recommended (Godement et al., 1987). Tissue from fresh temporal lobe was used in two cases and formalin fixed tissue (fixed for 5 days) from a further two cases. In all cases a coronal section of temporal lobe including the superior, middle and inferior temporal lobe gyrus and underlying white matter was used. DiI crystals were placed at intervals on the cortical surface from two cases (one fixed tissue, one fresh) and DiO crystals in the subcortical white matter. In the other two cases Di I and O crystals were placed in the opposite anatomical compartments. The crystals were placed with a microelectrode needle using a dissecting microscope. The tissues were incubated in paraformaldehyde at 37^{0}C or 6 months minimum. The tissue was embedded in 5% agar and sections cut at 65-100 microns on a vibratome (courtesy of Dr A. Hannan, Oxford University). The sections were counterstained with bismenzamide to reveal chromatin in cell nuclei. The sections were coverslipped and viewed with a fluorescence microscope (Leica) (Figure 3e,f, page 119).

5.5.3 Study IV: quantitative methods in analysis of cytoarchitectural changes in hippocampal sclerosis

5.5.3.1 Semi-quantitative grading of hippocampal sclerosis

The severity of neuronal loss in hippocampal CA1, CA2 and hilar subfields was assessed semi-quantitatively and graded using a modification of previously proposed systems for HS (Davies et al., 1996, Watson et al., 1996). Grade 1 – Mild neuronal loss and gliosis in the hilum (end folium sclerosis); Grade 2 -

Mild, visually perceivable pyramidal cell loss in CA1 (which correlates with 30% cell loss (Levesque et al., 1991); Grade 3 – severe (greater than 90%) neuronal loss in CA1 and less severe cell loss in the hilus; Grade 4 - severe neuronal loss in both CA1 and hilus; Grade 5 – Severe neuronal loss in all subfields, including GC. Pyramidal cells of CA2 in cases of Grade 3 and 4 HS appeared relatively preserved, but analysis of this subfield was not included in this grading scheme as in many specimens surgical artefacts were present in this region making assessment difficult. For the purpose of this study all neurones within the blades of the dentate gyrus were regarded as hilar neurones with no distinction made between CA4 & 3 pyramidal, interneurones and cells of the polymorphic layer. Any marked variation in the grade of HS (regarded as a difference of 2 or more grades e.g., Grade 2 to 4) between sections from a single case along the anterior-posterior hippocampal axis was also noted.

5.5.3.2 Semi-quantitative grading of granule cell dispersion and mossy fibre sprouting

The architecture of the dentate granule cell (GC) layer in all the sections was examined for the presence of GC dispersion or disorganisation (GCD) into the molecular layer. This was assessed on the straight sections of the dentate gyrus blades at magnification x 40 in a Leica DM RB microscope using a graticule to estimate the thickness of the cell layer in three places and the mean value calculated. In six control postmortem cases the mean width of the GC layer was 75 μm (SD=25 μm, range 50-125 μm). In hippocampal resections the GC was categorized as normal *(no significant dispersion)*, mild *dispersion:* a few single neurones in the molecular layer, separated from the main distinct GC layer, overall mean width 150-250 μm (measured in regions of maximal dispersion), severe *dispersion* – striking cell dispersion with a GC layer width greater than 250 μm and loss of definition from the molecular layer. In addition, a bilaminar GC layer, if present either extensively or focally with discrete clusters of GC in the molecular layer, was recorded, as was the presence of nests of cells with the morphology of GC in ectopic locations within the hilus or CA3. Evidence of depletion of the GC layer was noted as was any variation in the appearance of the GC layer between the sections within a specimen (in the anterior-posterior axis). The width of Timms staining in the molecular layer of the dentate gyrus was also estimated using a x40 objective with an eyepiece

graticule. The width was measured in three areas where the broadest staining of mossy fibre sprouting was identified and the mean value recorded.

5.5.3.3 Stereological estimations of granule cell number

Twenty-two HS cases were selected which showed variation in the severity of GCD within different sections of the specimen but without perceivable GC loss. Further 25 μm sections were cut and stained with Luxol fast blue/ cresyl violet for GC quantitation. The number of GC was estimated using the stereological three-dimensional cell counting technique as described above. All GC were counted in 100 μm wide columns perpendicular to the GC layer using a counting box (100 μm^2, 10 μm deep) counting GC in consecutive, non-overlapping boxes through the GC and molecular layer. Columns were counted in three different regions showing maximal GC dispersion and in three regions with the least or absent dispersion. The mean number of GC per column in regions of maximal versus minimal GCD was calculated and compared. Results were also compared to identical analysis carried out on post mortem hippocampal tissue from the six age-matched neurologically normal patients.

5.5.4 Study V : Quantitatlve anaiysis of reelin-positive Cajal-Retzius cells

In all FCD and MD cases only reelin-positive subpial cells in layer I with the bipolar morphology of Cajal-Retzius cells were counted at a magnification of x20 using 2-D profile counting technique, along a pial length of 20mm, commencing at the base of a sulcus and counting consecutive fields using an eyepiece graticule. This counting method was used in preference to a 3-D counting method due to the infrequency of these cells; similar counting protocols have been used in other studies of Cajal-Retzius cells (see Garbelli et al., 2001, Impagnatiello et al., 1998). Small reelin-positive cells, probably interneurones in layer I were not quantified. In FCD cases counts were carried out both within the region of dysplasia and in the adjacent normal cortex for comparison. The number of reelin-positive cells/mm (the linear density) in each case was calculated.

Within the group with HS, twenty-six cases were selected which showed mild GCD (n=16), severe GCD or a bilaminar GC layer (n=10) (see Section 5.5.3.2). Sections were cut at 10 microns, immunostained with calretinin and reelin antibodies. Calretinin antibody (Swant, Switzerland) was applied at a dilution of 1: 400 and incubated overnight after microwave pretreatment at room temperature. The reelin antibody (Clone 142) is a well-characterized antibody and generous gift from Prof. Goffinet, (Neurobiology Unit, University of Namur Medical School, Belgium). The numbers of immunopositive cells in both calretinin and reelin sections in the molecular layer of the dentate gyrus were counted using an eyepiece graticule as a grid and a x20 objective lens. The cells were counted using a 2-D counting technique and the grid was moved systematically, from field to field, along the length of the dentate gyrus (both inferior and superior blades). All cells with bipolar neuronal Cajal-Retzius cell morphology were counted in 10 consecutive and non-overlapping fields and the mean cell number / mm^2 calculated (area density). Identical analysis was carried out on the post mortem hippocampi from four age-matched neurologically normal patients as controls and in 9 of the surgically removed hippocampi from patients with TLE and extrahippocampal mass lesions.

5.5.5 Study VI : Inhibitory interneurones in cortical dysplasia and microdysgensis

5.5.5.1 Qualitative analysis

In FCD and MD cases the distribution and morphology of the immunostained cells and fibre plexus with calbindin, calretinin, parvalbumin and NPY were assessed qualitatively using a Leica DMRB microscope. The patterns of distribution of interneuronal cells and integration in the cortical cytoarchitecture were compared with adjacent normal cortex, wherever available, and with the control cases.

5.5.5.2 Quantitative analysis of NPY fibre plexus

Additional quantitative analysis was carried out on NPY stained sections. In all sections of cortex stained with NPY, scattered single immunopositive cells and a prominent NPY fibre plexus were seen with varying density of fibres. The ratio of the length of the NPY-fibre plexus between cortical layers II and V was

estimated using an image analysis system (Leica QM500, Q-prodit stereology software). Using a x 25 objective lens a random linear grid was superimposed on a representative field of cortical layer II in each case and the number of intersections of NPY- positive fibres with this grid counted. This measurement was repeated in the underlying cortical layer V and the ratio of intersections calculated. According to Buffon's Principle [1] (see section 3.7.7) the number of intersections is mathematically related to the overall length of the structure, in this case, NPY fibre length. By calculating the ratio of intersections between cortical layers II & V any apparent differences in NPY fibre density due to variation in section thickness and intensity of immunostaining between cases could be avoided and the ratio would give a relative difference in the length of fibres between the superficial and deep cortex.

5.5.5.3 Quantitation of calbindin multipolar cells in microdysgenesis and correlation with other microdysgenetic features

The number of calbindin positive multipolar cells (small interneurones, also known as 'neuro-gliaform cells') was also estimated in temporal lobectomy cases from study II. Calbindin D-28-K (Sigma, 1 : 200) immunohistochemistry was carried out on 20 micron thick sections of posterior temporal lobe at the level of the claustrum. All calbindin positive cells with multipolar morphology in all cortical laminae of the middle, superior and inferior temporal gyri were counted and scored as : 1 –4 cells (+), 5-10 cells (++), 11-15 cells (+++), 15 or more cells (++++). The results were correlated with other microdysgenetic features as measured in study II (Section 5.5.1).

5.6 Clinical correlations and statistical methods

In study II the microdysgenetic features quantified were correlated with the clinical outcome. 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 Pearsons correlation.

In 15 patients from study II the white matter neuronal densities were compared to results from pre-operative ^{11}C-Flumazenil PET imaging. This ligand binds to $GABA_A$ receptors and, as these receptors are expressed by most neurones, this high affinity molecule is a useful neuronal marker. The results were correlated using Spearman's correlation for non parametric data.

In study III the cases with abnormal cortical myelin patterns were correlated with clinical data of epilepsy type, imaging and EEG data and with post operative follow up. Statistical analysis of mean NeuN and Calbindin cortical neuronal densities between the groups was carried out using the independent t test.

In study IV details of seizure history including age of first seizure, seizure duration (taken as the time interval between onset of habitual seizures and surgery), and any initial precipitating event for the seizures (classified as prolonged or complicated febrile seizure, meningitis, encephalitis, or trauma) were retrieved in 72 patients. Statistical analysis for comparison of clinical and pathological data was carried out using paired t test, independent t test and Pearson's correlation using SPSS software for Windows, version 9.
In studies V and VI the quantitative data (of inhibitory interneurones and Cajal-Retzius cell numbers) was correlated with other pathological and clinical criteria between groups.

6 Results

6.1 Study I : Review of all epilepsy surgical lobectomies 1993-2000

6.1.1 Range of pathologies identified

The neuropathological diagnosis in all epilepsy surgical cases carried out between 1993-2000 is shown in table 5. There were 413 cases reviewed in total. The commonest diagnosis was hippocampal sclerosis seen in 251 cases. The commonest tumour identified was a DNT, seen in 59 cases with smaller numbers of pilocytic astrocytomas, gangliogliomas and hamartomatous lesions including astrocytic hamartomas and meningioangiomatosis. (Tumours, as diffuse astrocytomas, WHO grade II or more, presenting with a short history of epilepsy were not included in this analysis). Old infarcts including ulegyric lesions were seen in 7 cases and old contusions or cortical scars in 7 cases. Gliosis alone, including subpial Chaslin's gliosis was seen in 9 cases. Taylor type focal cortical dysplasia characterised by giant neurones, dysplastic neurones and balloon cells were seen in 8 cases.

In approximately 20% of cases microdysgenetic-like cortical abnormalities were recorded in the surgical reports in association with hippocampal sclerosis, for example 'an excess of neurones in the white matter' or an 'excess of neurones in the subpial region consistent with microdysgenesis'. These were not recorded as a specific category in the results as the purpose of this project is to establish the diagnostic criteria for this abnormality. Chronic encephalitis consistent with the diagnosis of Rasmussen's encephalitis was observed in 8 cases. In 23 cases a dual potentially epileptogenic pathology was recorded and in three cases a triple pathology : for example in one case Rasmussen's encephalitis together with a cavernoma and hippocampal sclerosis was recorded. The results are also shown in a box blot in figure 6.

Table 5. Pathological diagnosis in surgical series for treatment of focal epilepsies 1993-2000

Diagnosis	Number of cases
DNT	59
Astrocytomas (WHO grade I including pilocytic)	13
Gangliogliomas	5
Meningioangiomatosis	2
Hamartomas	3
Vascular malformations	28
MCD (not otherwise specified)	8
FCD	8
Old infarcts including ulegyia)	7
Contusions / scars	7
Gliosis	9
Normal	3
Hippocampal sclerosis	251
Rasmussen's chronic encephalitis	8
Cystic lesions	2
Total cases	413

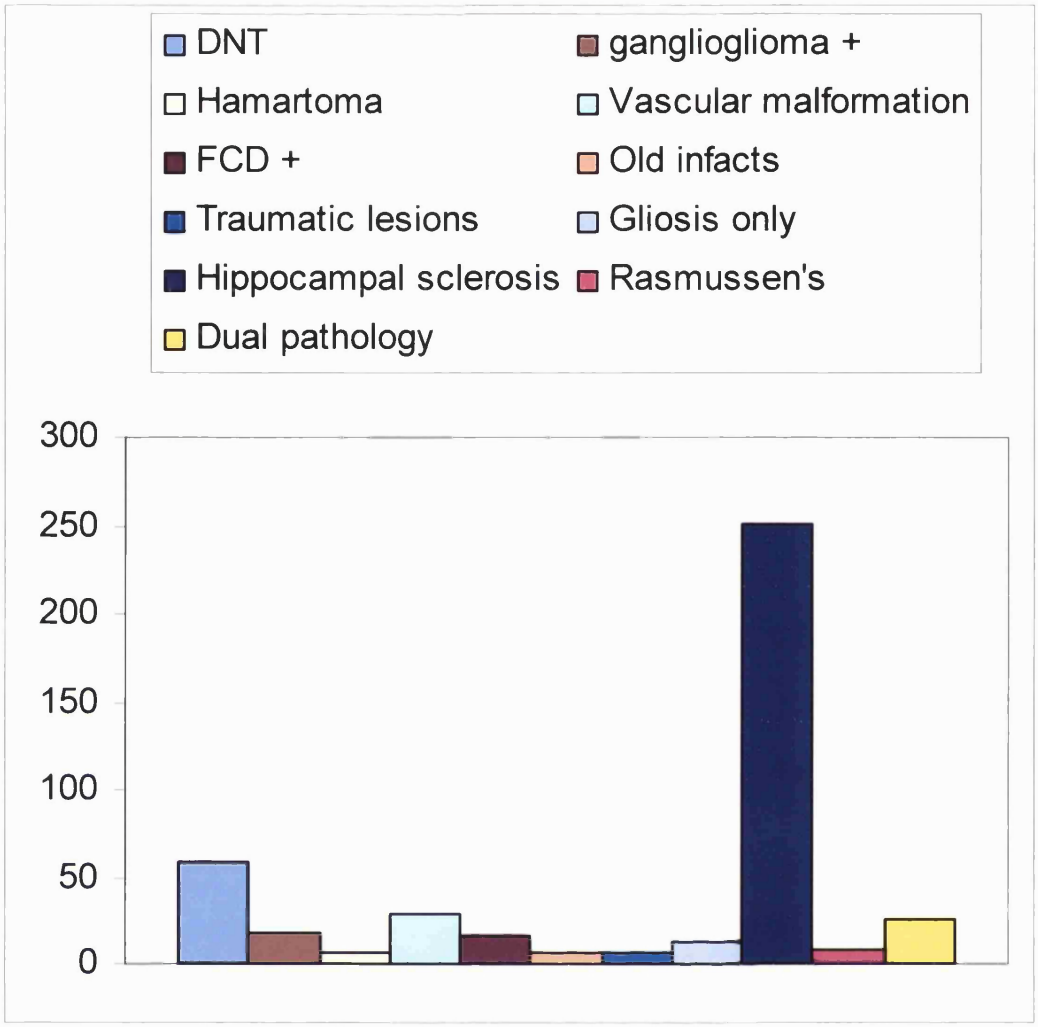


Figure 6. The number of cases in each main diagnostic category for epilepsy surgical resections carried out between 1993-2000 at NHNN. The commonest pathology is hippocampal sclerosis with dysembryoplastic neuroepithelial, tumours (DNT), vascular and cortical malformations forming a smaller proportion of cases. 'Ganglioglioma +' ; gangliogliomas and other lower grade tumours as pilocytic astrocytoma associated with a history of intractable partial epilepsy; 'hamartomas' include 2 cases of meningioangiomatosis ; 'FCD+' includes cases of focal cortical dysplasia-Taylor type and other focal malformations of cortical development excluding microdysgenesis; 'gliosis only' cases show no significant pathology.

6.1.2 Comparison of present series with other large epilepsy pathoiogy series

In the table below the results of this epilepsy surgical series are compared with two previously large published studies (see section 3.2). In all series hippocampal sclerosis is the commonest lesion identified.

NEURO-PATHOLOGICAL LESION	UNIVERISTY OF BONN Based on 541 temporal lobe resections. (Blumcke et al., 1999)	INSTITUTE OF PSYCHIATRY, LONDON Based on 234 temporal lobe resections. (Bruton, 1988)	INSTITUTE OF NEUROLOGY, LONDON Based on 413 resections (1993-2000)
Hippocampal sclerosis	37 %	45 %	61%
Uncertain/no pathology	12 %	28 %	0.8%
Tumours	25 %	11 %	19.1%
Cortical malformations excluding microdysgenesis	13 %	5 %	4.6%
Vascular malformations	5.%	1 %	6.8%
Gliosis	4 %	-	2.2%
Others (including Rasmussen's encephalitis, old scars, dual pathologies etc.)	4 %	10 %	5.8%

6.1.3 Pathologicai diagnosis and surgicai outcome

The post-operative outcomes for all patients undergoing epilepsy surgery from 1994 to 1999 at the National Hospital were compared to all patients with a diagnosis of hippocampal sclerosis confirmed on pathology for the same period. This is presented in table 6. In 75% of all epilepsy surgery patients there was a class I surgical outcome based on the Engel scale (see appendix 1). In patients with hippocampal sclerosis there was a 78% Class I outcome.

Table 6 : Surgical outcomes following epilepsy surgery. Minimum follow-up two years.

Post operative seizure outcome Engel Class	All focal epilepsy surgery cases from 1994-1999 (n=150)	All hippocampal sclerosis cases from 1994-1999 (n=109)
I	112 (75%)	86 (78%)
II	23 (15%)	13 (12%)
II	9 (6%)	6 (6%)
IV	6 (4%)	4 (4%)
Total	150	109

6.1.4 Comparison of percentage of dual pathology cases and clinical outcome with TLE series from Bonn

In addition further detailed comparisons of the diagnosis in patients undergoing temporal lobectomy were compared to the large surgical series from Bonn particularly with regard to clinical outcome and the precise incidence of dual pathology (for reference see Blumcke et al., 2002). Similar incidence of dual pathology and similar outcome data was observed.

	Bonn n = 572/725§	London n = 384
AHS (%)	357 (62.4%)	251 (65.4%)
Focal lesions (%)	141/143§ (24.6 %)	95 (24.7%)
Dual pathology (%)	38 (6.6 %)	26 (6.8%)
No pathology (%)	36/10§ (6.3 %)	12 (3.1%)
AHS only		
Age	34.0 +/- 8.6 years	31.9¤ +/- 9.1 years
Age at onset	11.3 +/- 6.8 years	9.6¤ +/- 8.1 years
Duration	22.8 +/- 9.1 years	23.3¤ +/- 10.8 years
Outcome	77.5% / 12.5% / 10%$	78%/12%/10%#

Age = age of patients at surgery. Age at onset = age at onset of spontaneous seizure activity. Duration = Duration of seizure disorder until surgical treatment.

§ 153 additional patients were collected in the Bonn series with seizure onset originating only in the lateral temporal lobe. In these samples, the surgical specimens did not contain the hippocampus.

¤Clinical data for patients with AHS operated during the period 1996-1998

$ Post surgical outcome have been recorded in a series of 40 patients for a period of more than 12 month. The numbers (in %) refer to Engel class I (seizure free) / class II (one or two seizures a year) / class III-IV (seizure reduction of 75% - 50%), respectively.

Post surgical outcome in 104 AHS cases operated between 1994-1999 with minimal follow period of 2 years.

6.2 *Study II : Results from white matter neurones in temporal lobectomies*

6.2.1 Comparison of white matter neuronal density with NeuN and Nissl staining in epilepsy cases

Mean temporal lobe white matter ND in Nissl stained sections from 18 cases was 1010 / mm^3 with a wide variation between epilepsy cases (range 440 to 1751 / mm^3) (Table 7). NeuN immunoreactive cells showed both nuclear and cytoplasmic positivity, many being smaller cells with little cytoplasm and nuclear diameter of less than 10 microns (Figure 2b, page 113) ; these were not distinguishable from glial cells on the Nissl preparation. ND measurements with NeuN from 31 cases were significantly greater than with Nissl in all epilepsy cases (mean 2164/mm^3, range 1212 to 3448 /mm^3) ($P<0.001$) although there was correlation between both measurements in all cases ($P<0.01$) (see figure 7 and 8). The proportion of small (<10mm) to total NeuN positive white matter neurones varied between 35% to 68% (mean 47%) in epilepsy cases (figure 9). For values of individual cases see Appendix 2.

6.2.2 Comparison of white matter neuronal densities between cases and controls

In control cases the mean white matter ND on NeuN sections was 1660 / mm^3 (range 620 to 2990 mm^3) which was significantly lower than in epilepsy cases ($P<0.05$) although there was overlap between the ND in both groups (table 7). In control cases NeuN also highlighted both large pyramidal and smaller (<10 mm) 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 ND 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 PM intervals and fixation times this was not significant ($P =0.16$ and 0.09). In controls and epilepsy cases there was no correlation with white matter ND and age of patient.

Table 7. Table of results from study II

Results of quantitative analysis of temporal lobectomy specimens. ND = neuronal density with Nissl staining and NeuN marker, AD = astrocytic density. Mean values (and standard deviation) given. *Indicates significant differences between means in 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.

Groups	White matter ND (NeuN) / mm^3	White matter ND (Nissl)/ mm^3	White matter ND (small <10mm, NeuN) /mm^3	White matter AD (GFAP) /mm^3	Cortical ND (NeuN) /mm^3	Cortical layer I ND (NeuN) /mm^3	Cortical layer I Calbind in ND / $10mm^2$	Cortical layer II neuronal clusters / $0.05mm^2$
All cases (n=31)	2164 $ (604)	1010 (343)	1040 (380)	8940 (3193)	41,596 (7214)	10,436 (2952)	13.8 (5.3)	24
Seizure free outcome (n=17)	2359*$ (697)	1165 (386)	1170* (433)	9670 (3315)	43,395 (7177)	11,247* (3165)	15.8* (5.5)	30* (21)
Not seizure free (n=9)	1941* (368)	850 (271)	839* (197)	8319 (3677)	39,742 (8675)	8626* (1851)	10.5* (4.2)	16* (6)
Controls (n=15)	1660$ (772)		941 (550)			9556 (3819)		

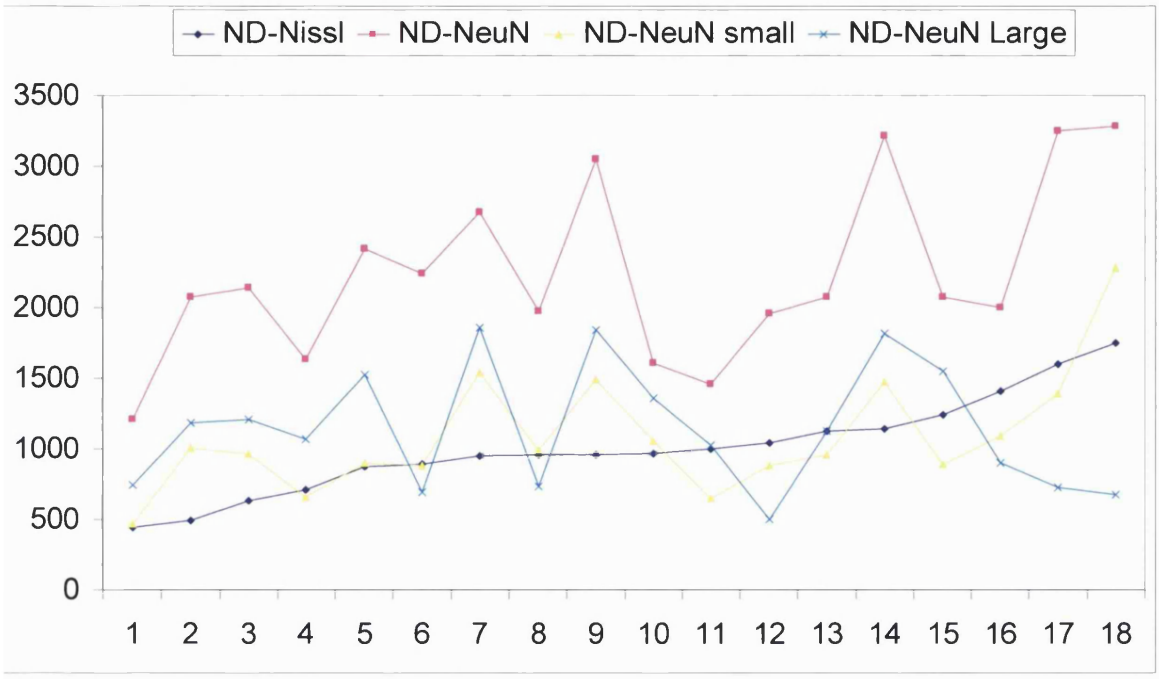


Figure 7 : Values of white matter neuronal densities (ND) in cases studied with Nissl, NeuN immunostaining with separate values shown for neuronal densities of small (<10 micron diameter) neurones and larger (>10 micron diameter) NeuN positive neurones. NeuN method gives higher values of white matter ND whereas value for Nissl ND is similar in range to large NeuN ND values ; the smaller neurones are difficult to identify as distinct from glial cells in Nissl preparations.

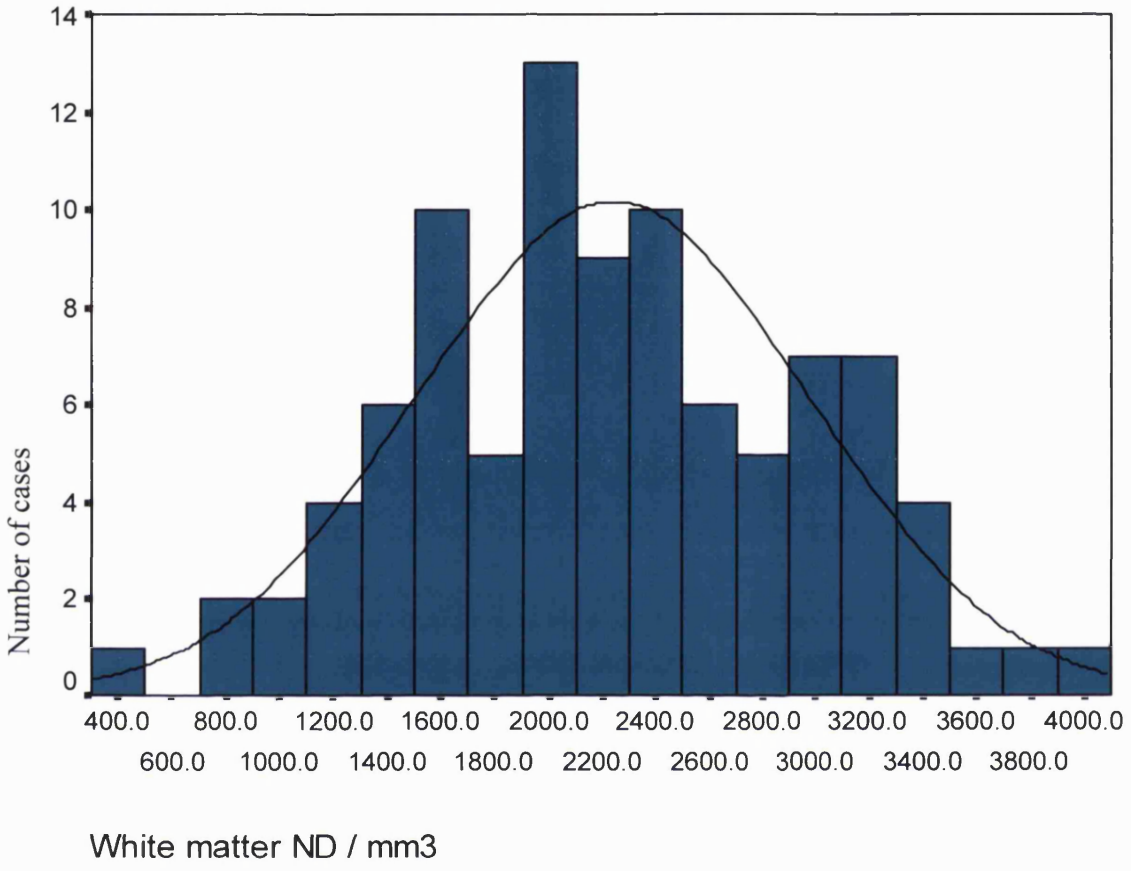


Figure 7 b : Histogram of distribution of results of stereological quantitation of white matter neurones in TLE in over 81 cases in NeuN immunostained sections from the original 31 temporal lobes and 50 further lobes analysed. The mean ND is 2238 / mm3 and the SD 735. The distribution of ND appears normal and no bimodal distribution revealed.

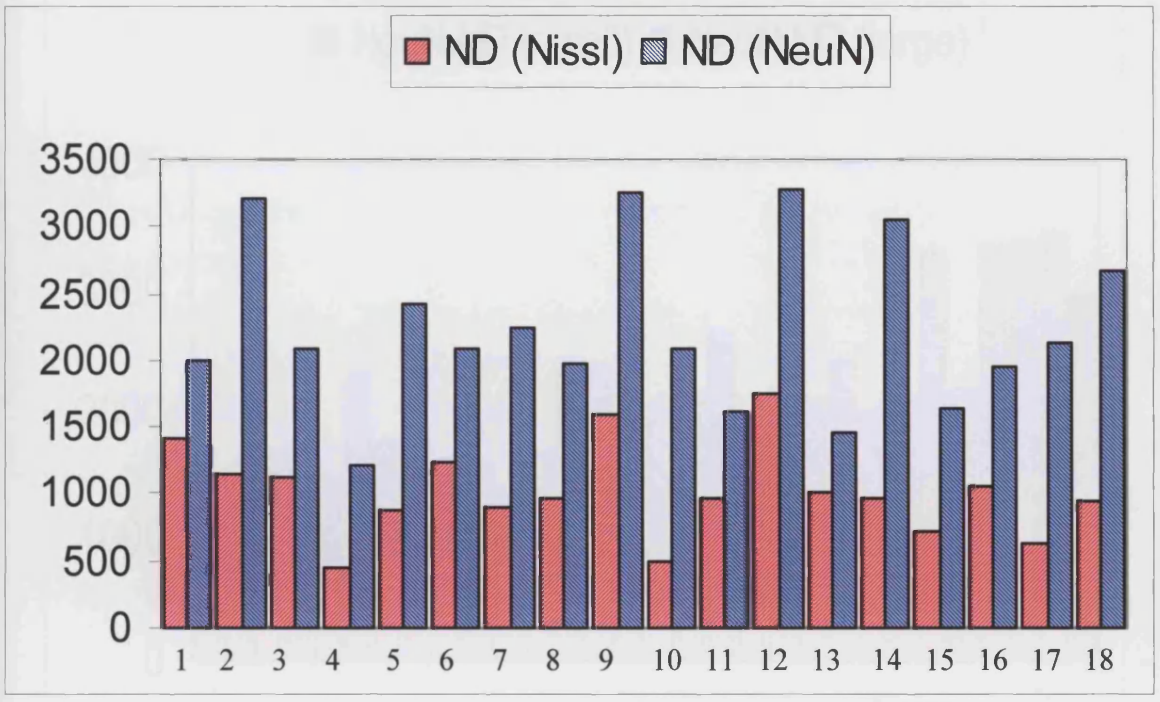


Figure 8 :White matter neuronal densities as measured in 18 cases using Nissl and NeuN immunohistochemistry. There was a correlation between the densities measured using these different methods but higher neuronal densities were always obtained in each case with the NeuN.

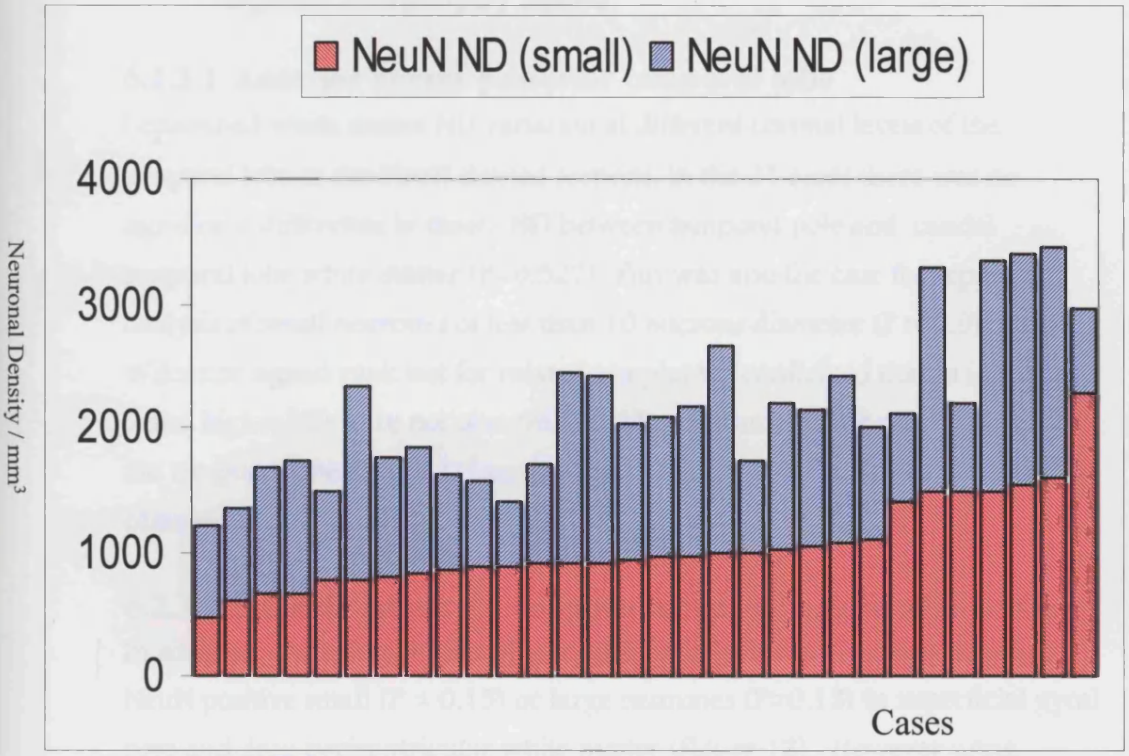


Figure 9 : The proportion of white matter neuronal densities made up of small and large neurones (> and < than 10 microns diameter) in each case. In nine cases over 50% of the neurones in the white matter were small neurones

6.2.3 White matter neuronal densities in different anatomical regions in epilepsy cases

6.2.3.1 Anterior versus posterior temporal lobe

I 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 less than 10 microns diameter (P = 0.9). Using Wilcoxon signed rank test for related samples we confirmed that in individual cases, higher ND were not observed in either the anterior or posterior part of the temporal lobe (P=0.72) (see figures 10 and 11 and for values in individual cases see appendix 3).

6.2.3.2 Superficial versus deep white matter neuronal densities

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 (Figure 12) . However using Wilcoxon signed rank test, in a significant number of cases (ten of thirteen) higher densities of small neurones were present in the deep compared to gyral white matter (P<0.05) and the percentage of small neurones in the superficial white matter was significantly lower compared to the deep on paired t tests (p=0.019)(Figure 13) whereas large neurones were more evenly dispersed (P=0.16). (For values in individual cases see appendix 4).There were no differences in mean white matter ND between right versus left temporal lobectomies (P=0.8).

6.2.4 White matter gliosis in relation to cortical and white matter neuronal densities

In all epilepsy cases a variable degrees of astrocytic gliosis of the white matter was evident on GFAP immunostaining (Figure 14). However there was no correlation between the degree of gliosis as measured by the density of GFAP positive astrocytes (AD) in the white matter and the ND in either Nissl (P=0.2) or NeuN stained sections (P=0.38) (Table 7). 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 cortical gliosis (see Figure 2b, page 114).

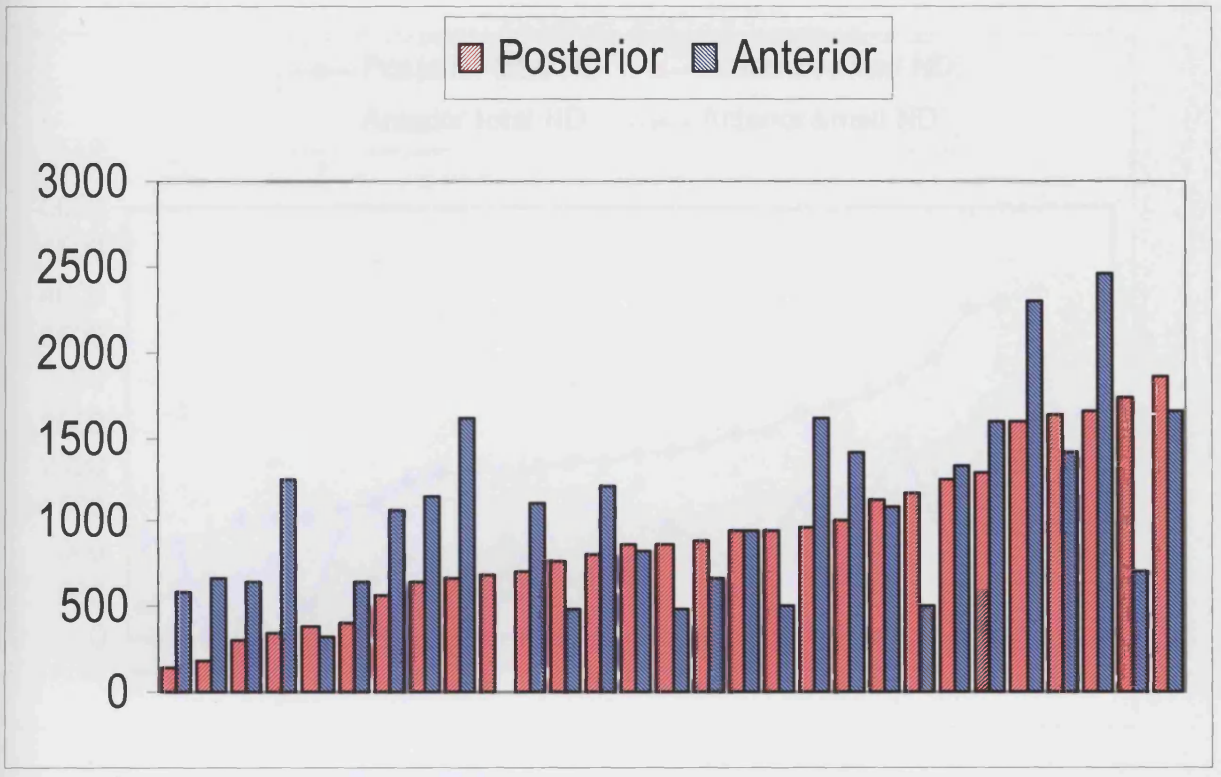


Figure 10 : The densities of small white matter neurones (<10 microns) in the anterior and posterior temporal lobe white matter. There was no significant difference in the density of small neurones between cases in these different anatomical regions.

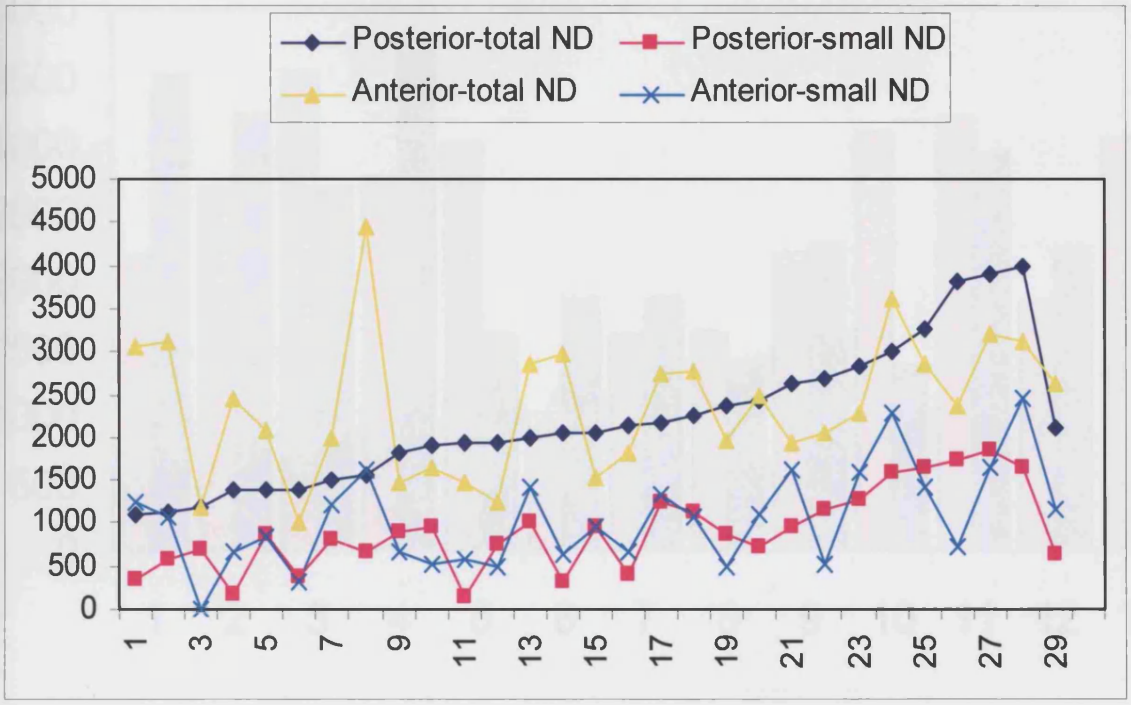


Figure 11. Neuronal densities (per mm^3) in the posterior and anterior temporal lobe white matter for all NeuN positive neurones and small (less than 10 micron diameter) positive neurones. There was no significant difference in values between regions.

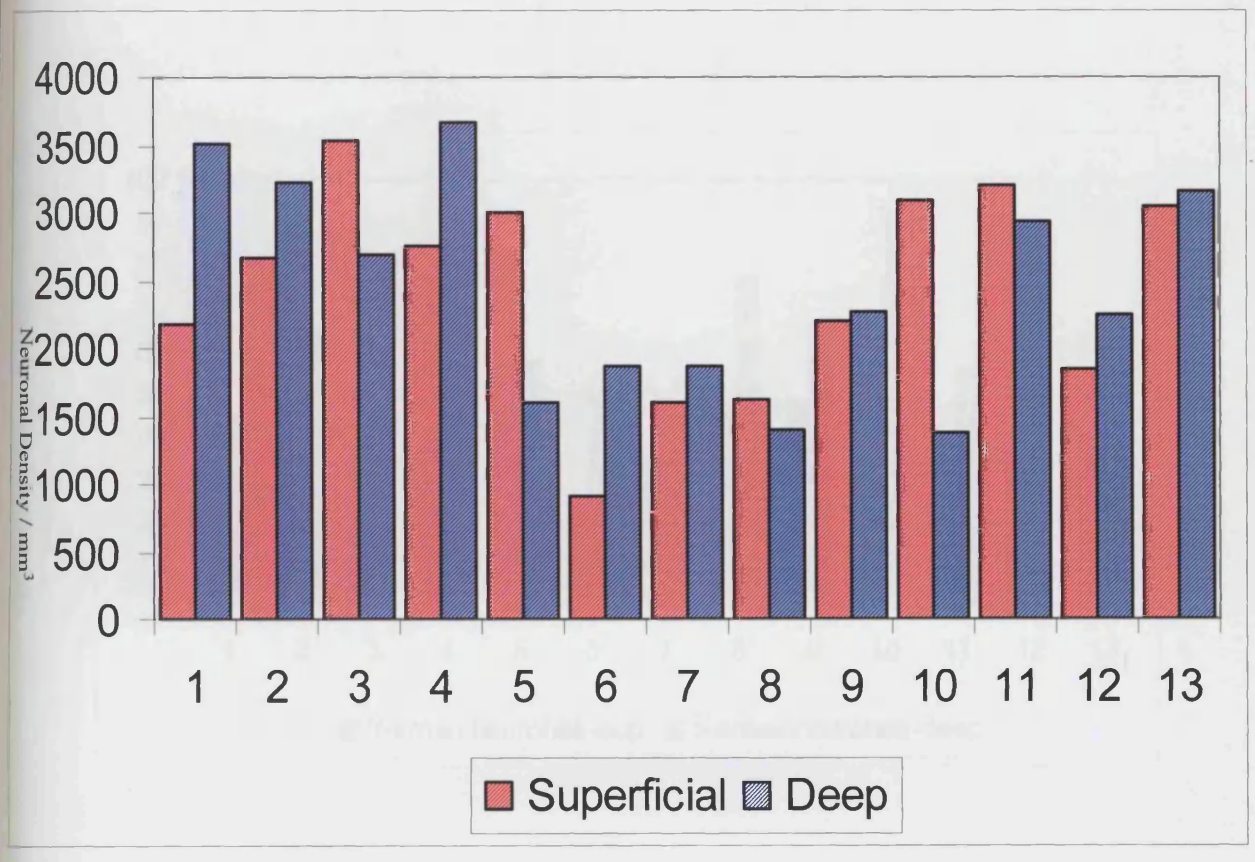


Figure 12 : NeuN white matter neuronal densities in the deep white matter and superficial white matter in 13 cases. There was no significant difference in the mean densities between the superficial and deep white matter in these cases.

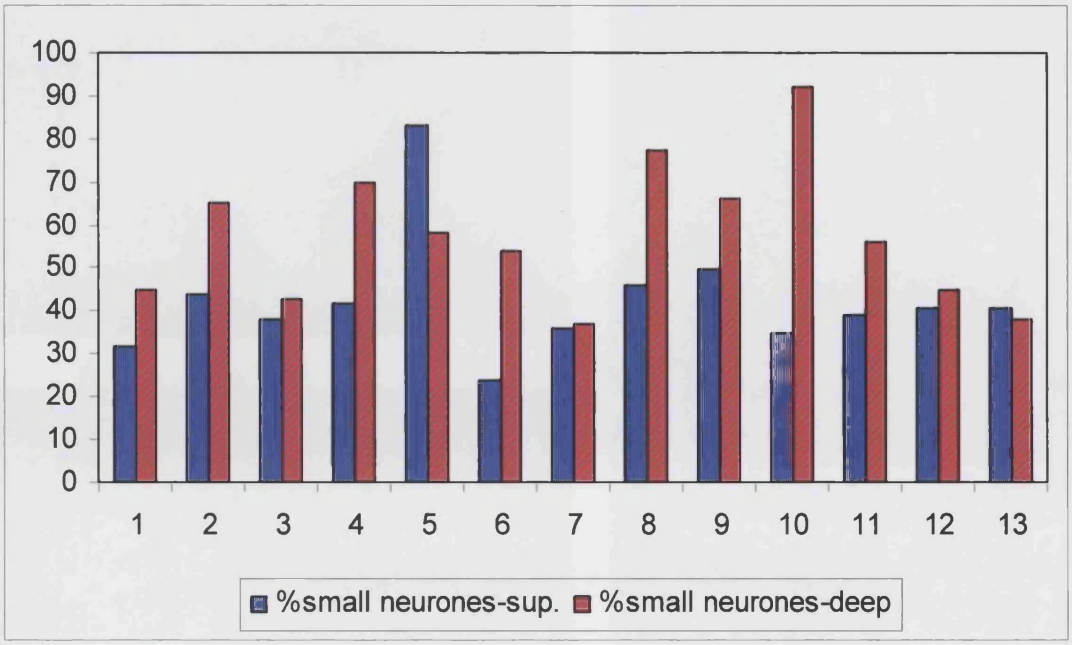


Figure 13 : Percentage of small neurones (of the total neurones) in different anatomical areas of temporal lobe, the superficial white matter (sup.) compared to the deep white matter in 13 TLE cases. In a significant number of cases the percentage of small neurones was higher in the deep white matter.

Figure 14 : Patterns of GFAP immunohistochemistry in temporal lobe epilepsy

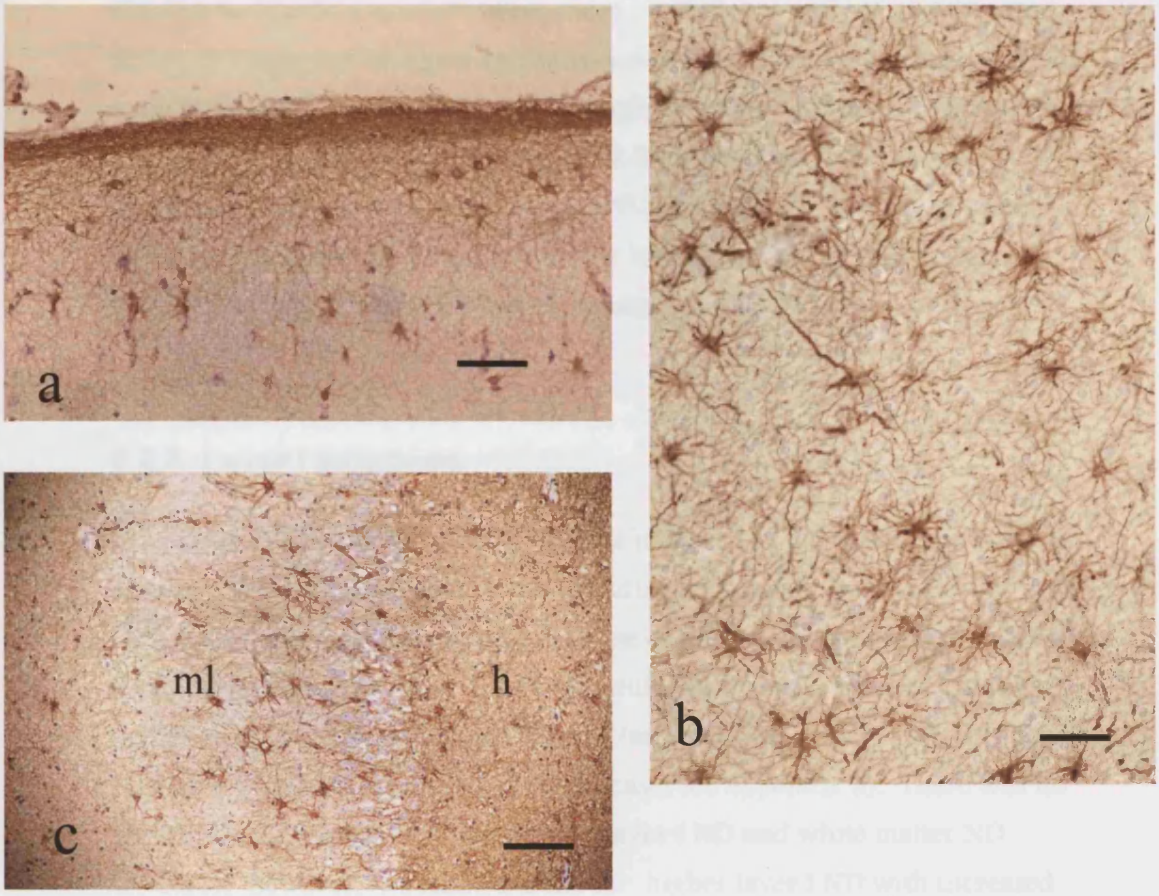


Figure 14 : Chaslin's type subpial gliosis was seen in the majority of temporal lobe epilepsy specimens (a). Gliosis of the white matter is also seen to a variable degree as illustrated in this figure with prominent numbers of reactive astrocytes (b). Gliosis is typically seen in the hippocampus (c) , in this field involving the hilus and molecular layer marked h and ml. Bar in a and c 130 and in b 50 microns

This was reflected in the wide range of middle temporal gyrus cortical ND (28,716 to 53,750 / mm^3) between cases, with a mean ND of 41,596/mm^3 (Table 7).There was no significant correlation between middle temporal gyrus cortical ND and white matter ND, although I observed a trend for higher white matter ND with higher cortical ND (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). (See appendix 8 for values in single cases).

6.2.5 Layer I neurones

In NeuN stained sections labelling of cells in layer I of the cortex was noted in all cases (Figure 2a& b ; page 114) 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/mm^3 (range 4,117 to 16,400/mm^3) (see table 7 and for values in individual cases see appendix 5). There was no significant correlation between cortical layer I ND and white matter ND (P=0.11); however, we noted a trend for higher layer I ND with increased density 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 / mm^3 (range 2500 to 15,128 mm^3) which, although lower, was not significantly different from epilepsy cases (P=0.4) (Table 7).

6.2.6 Layer I Calbindin positive neurones

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 (Figure 5f, page122). They were seen in epilepsy cases but also in controls. There was an impression of increased numbers of these cells in the sulci , a phenomenon also observed in the developing brain (Meyer and Goffinet,1998), but no clusters of these neurones were noted. Quantitation showed variations in their number between cases (table 7, and for values in individual cases see Appendix 6) 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 (Figure 3c, page 119) 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 ($P<0.001$).

6.2.7 Other microdysgenetic features including neuronal clustering

With regard to other cortical microdysgenetic features, in six cases abnormal cortical myelinated fibres were present (fig 3a, page 199) with prominent neuronal clustering (Fig 3c, page 119). In the remaining 25 cases more variable degrees of abnormality of cortical myelination and neuronal clustering were present ; semi-quantitative analysis of these features however showed no correlation with layer I ($p=0.4$) or white matter ND ($p= 0.9$).

6.2.8 Correlation of white matter neuronal density measurements using 3-d cell counting, 2-d cell counting and interneuronal distance

The results of the rapid method of assessing neuronal numbers in the white matter, using an image analysis system and measuring interneuronal distance showed a strong inverse correlation between white matter interneuronal distance, as measured on posterior lobe NeuN stained sections and ND on the same sections obtained with three dimensional cell counting ($p=0.007$). For values in individual cases see appendix 7 and Figure 15.

Furthermore there was correlation between ND obtained using three dimensional and standard two dimensional cell counting techniques ($p=0.048$) on NeuN stained sections of posterior temporal lobe.

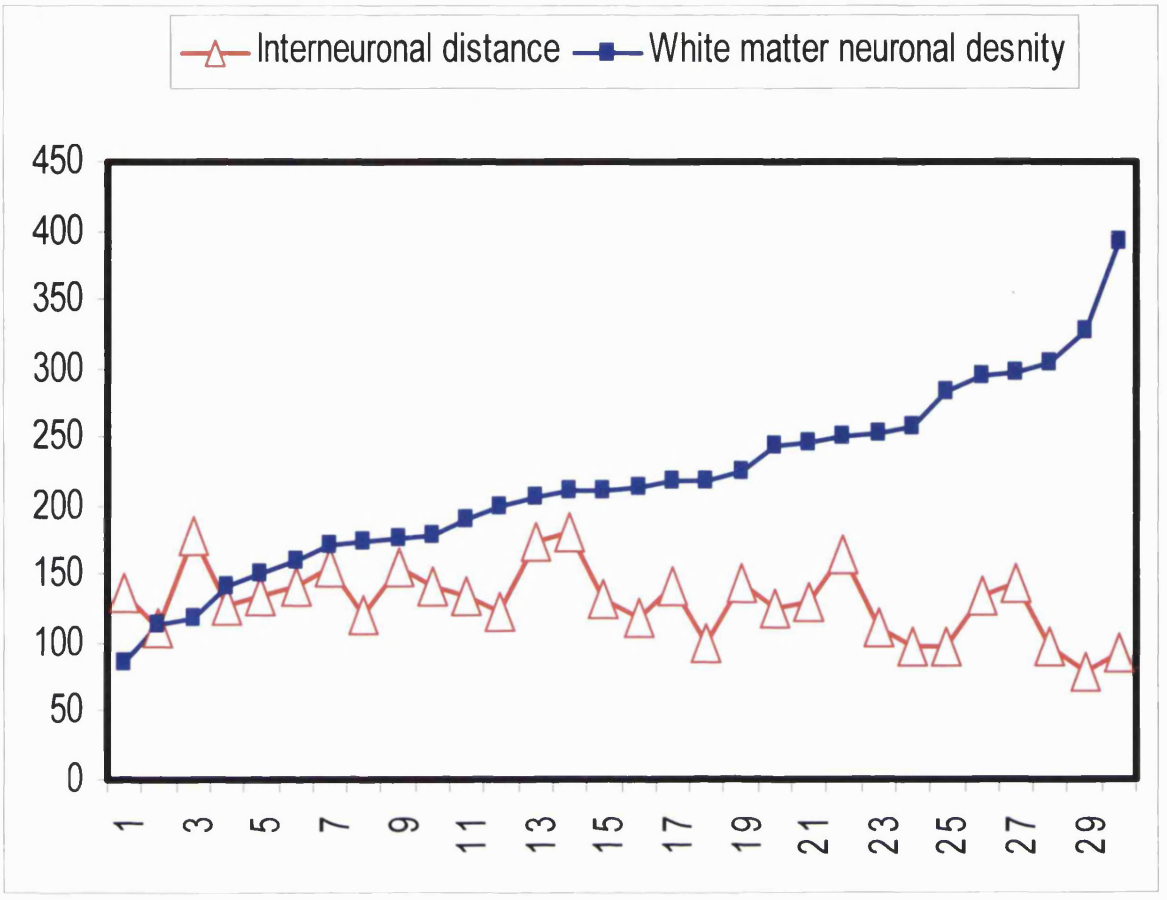


Figure 15 : White matter interneuronal distance (microns) plotted against the white matter neuronal density / 10^{-1} mm^3. An inverse correlation was shown.

6.2.9 Correlation between microdysgenetic features and ciinical 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 neuronal densities were significantly different between seizure free group and controls (P<0.05) but not between non-seizure free group and controls (P=0.4) (table 7). Furthermore, there was a significant difference in the presence of other microdysgenetic features in the seizure free compared to 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 7, Figure 16).

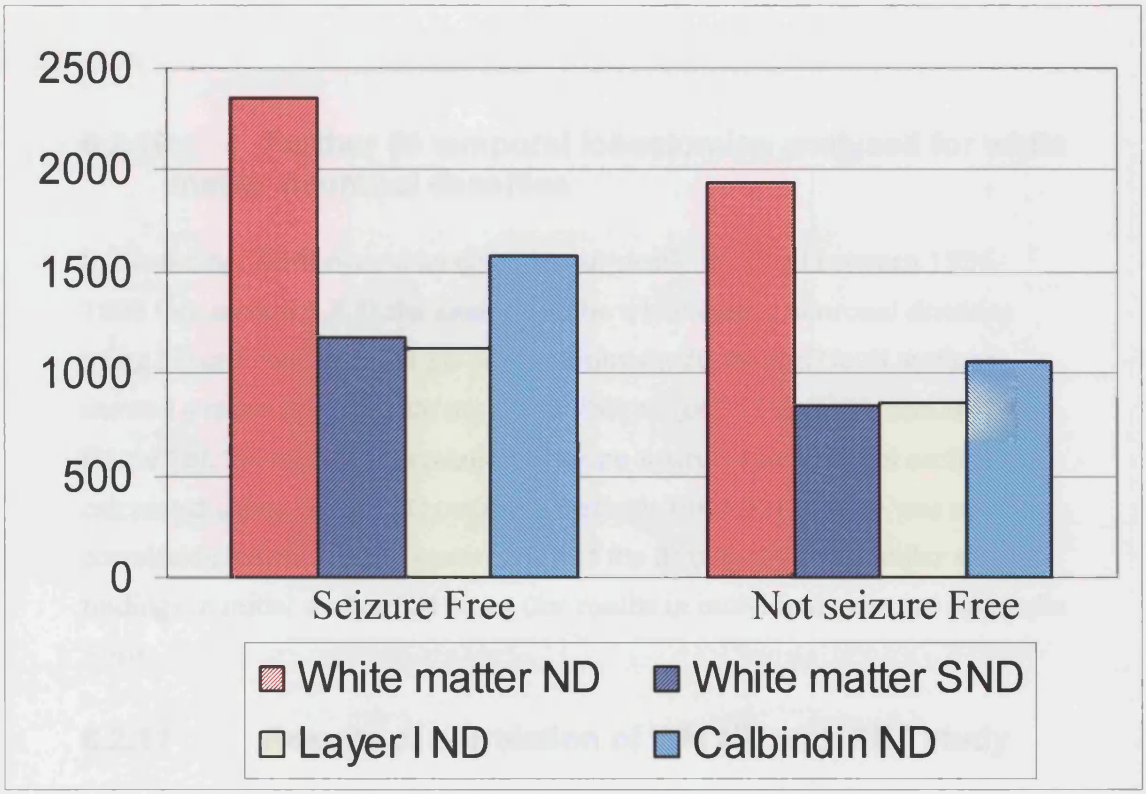


Figure 16 : Box plot illustrating the quantitative analysis according to different clinical outcomes : seizure free or not seizure free. In the seizure free group significantly higher white matter NeuN neuronal densities (ND/mm^3) of both all and small neurones (SND), higher layer I neuronal densities(ND/ $10^{-1}mm^3$) and calbindin cell numbers in layer I /10^3 mm^2

6.2.10 Further 50 temporal lobectomies analysed for white matter neuronal densities

In the further 50 temporal lobectomies surgically removed between 1996-1998 (see section 5.3.2) the analysis of the white matter neuronal densities using 3D cell counting and 2D cell counting on 20 micron NeuN sections showed a mean ND of 2238/mm3 (SD 735; range 777 to 3953/mm3) (see Figure 7b). There was a correlation between neuronal numbers in each case calculated using 3D and 2D counting methods ($p<0.001$). There was no correlation between white matter ND and the degree of gliosis similar to findings in initial study of 31 cases (for results in individual cases see Appendix 2(ii)).

6.2.11 Results of correlation of WM ND with PET study

In 15 patients with HS who underwent pre-operative 11C-Flumazenil PET studies the results were correlated with white matter neuronal densities as assessed by cell counting. For this purpose the white matter was categorised as having few, moderate or many neurones based on 2-D cell counts and 3-D cell counts with ND scores of >1800/mm3 (few), 1800-2900/mm3 (moderate), >2900/mm3 (many). There was a positive correlation with 11C-Flumazenil PET binding and white matter neuronal densities ($p<0.001$) (Spearman's correlation) (Hammers et al., 2001).

6.3 Study III identification of abnormal patterns of cortical myelin in temporal lobectomy cases

In an initial analysis four patients with abnormal patterns of cortical myelination were identified (from review of cases1996-1998) ; these findings were published (Thom et al., 2000). Following complete review of all temporal lobectomies 1993-2000 a further 6 cases have been identified all with similar features.

6.3.1 Clinical features

The four patients first described had an age range 26 – 58 years and underwent temporal lobectomy with hippocampectomy for intractable

TABLE 8 Clinical data of patients in study III

Case	Age (at seizure onset)	Age (at surgery)	Seizure Type	FC or IPI	EEG	MRI	Post surgery follow up
1	3 years	31 years	SPS, CPS SGS, SE	Possible FC	Right sided activity	Right HCS	Seizure free at 15 months
2	3 years	29 years	SPS, CPS, SGS	FC	Bilateral ictal and interictal changes	Left HCS	Not seizure free at 8 months
3	3 years	26 years	SPS, CPS, SGS	FC	Widespread abnormalit-ies	Right HCS	Seizure free at three months
4	4 years	58 years	SE SPS, CPS	Encephalit-is age 4.	Bilateral interictal and ictal changes	Previous partial left hippocam-pectomy 1964. (HS)	Not seizure free at 9 months

SPS = simple partial seizures, CPS = complex partial seizures, SGS = secondary generalised seizures, SE = status episodes, FC = febrile convulsion, IPI = initial precipitating injury, HCS = hippocampal sclerosis.

temporal lobe seizures at the NHNN between 1996 and 1998. The clinical details including the seizure histories of the patients are presented in Table 8. In all the cases magnetic resonance imaging revealed unilateral hippocampal sclerosis with no identifiable neocortical malformation. EEG findings, however, showed evidence of bilateral and more widespread abnormalities in three of the patients.

6.3.2 Neuropathological features

In all four cases additional cyto-architectural abnormalities were present in the temporal neocortex, principally involving superficial cortical layers. Abnormal clusters of up to eight small neurones were present in layer II, observed in both the Nissl and NeuN stained sections (figure 2b, page114 and 3a, page 119). There was poor demarcation between layers I and II but other features described in microdysgenesis, including columnar alignment of nerve cells and an excess of nerve cells in the white mater, were not apparent. Abnormal thick bundles of myelinated axons were present, running parallel to the pial surface in the superficial part of layer II (Figure 3c, page 119) in close proximity to the neuronal clusters. In one case (case 1) the fibres appeared within the centre of a neuronal cluster (fig 3a, page 119). The axons within these bundles were also highlighted on silver stained and neurofilament and phosphorylated neurofilament immunostained sections.

6.3.3 Cortical laminar architecture

There was evidence in the microdysgenetic temporal lobes of widespread loss of nerve cells from layer II of the cortex, particularly well demonstrated in NeuN stained sections (fig 2b, page 114) as this is an antibody recognised to enhance visualisation of cortical cytoarchitecture (Wolf et al., 1996). This finding was supported by quantitative analysis which showed a reduction in mean cortical neuronal density on the NeuN stained sections in microdysgenesis cases compared to control temporal lobes ($p=0.03$) (Table 9). GFAP immunostaining also demonstrated a moderate to severe cortical gliosis in addition to Chaslin's subpial and white matter gliosis (Figure 14a), although the degree of gliosis in these cases was not formerly quantified. Hippocampal sclerosis was confirmed in all four microdysgenesis cases as well as in six of the eight control surgical temporal lobes; mild to moderate cortical gliosis was also present in all controls but without evidence of laminar nerve cell loss.

Group	Microdysgenesis n=4	Control TLE without microdysgenesis n=8	Significance
Mean cortical Calbindin neuronal densities / mm3 (SD)	2250 (461)	2528 (703)	p=0.49
Mean cortical NeuN neuronal densities / mm3 (SD)	32,799 (2,995)	38,613 (4,628)	p=0.033

Table 9. Results of Calbindin and NeuN cortical neuronal densities in microdysgenesis cases and controls (Study II)

6.3.4 Distribution of calbindin positive neurones in these cases

Calbindin positive neurones appeared preserved in the microdysgenesis cases ; they were of normal morphology, predominantly located in cortical layers II and III and were not forming neuronal clusters. Quantification studies showed no significant difference between mean calbindin cortical neuronal densities in microdysgenesis and control cases (p = 0.49) (Table 9) although slightly higher neuronal densities were observed in [the temporal lobe epilepsy] controls. A further finding was the presence of frequent '*arachniform*' or '*neurogliaform*' calbindin positive cells in the microdysgenetic cortex, which had extensive ramifying dendritic processes (fig 5i, page 122). These cells were present mainly in the deeper cortical layers, were not observed in the white matter and were occasionally present in cortical layer I. Double labelling with calbindin and GFAP confirmed that these cells were GFAP negative (Figure 5g, page 122). Similar cells were occasionally observed in the surgical temporal

lobes without microdysgenesis. At post surgical follow up, only two of the four patients with microdysgenesis were seizure free (Table 8).

6.3.5 Results of DiI tracing studies of superficial fibres

DiI and DiO crystals were placed in proximity to the subpial region and in the white matter in an attempt to trace the direction and extent of these abnormal fibre tracts, where present, within the superficial cortex and if there was any connection with white matter neurones (see section 5.5.2). The reasoning behind this was that during corticogenesis the subplate cells and layer I cells are in close proximity and the subplate cells may be the source of any abnormal layer I fibres or they may represent abnormal thalamo-cortical projections as has been seen in animal models.

In the four cases where crystals were placed, no abnormal myelinated fibres in layer I were identified on the adjacent LFB/N stained sections. Unfortunately, limited penetration of fibres was observed with DiI/O crystals following six months incubation which is likely to relate to the maturity of the myelin in adult tissue. Diffusion of DiI along thin axons in layer I was seen for variable distances adjacent to crystal placement (Figure 3e&f, page 119). No diffusion of dye-tracers placed in the white matter was observed to extend to fibres in layer I. In cases with abnormal cortical horizontal myelinated fibres within polymicrogyria immunohistochemical positivity with parvalbumin has however been noted (personal observation) ; this may support an extra-cortical origin for these fibres.

6.4 Study IV: Results of Cytoarchitectural changes in hippocampal sclerosis

6.4.1 Patterns of hippocampal sclerosis

The *classical pattern* of HS (grade 3 and 4) was present in over 90% of cases (Table 10) with neuronal loss predominantly in CA1 and hilar regions and with sparing of CA2 subfield and GC (Fig.17a-d). In 2.8% of the cases severe HS (grade V) involved significant cell loss in all subfields and in 1.6 % an *'end folium'* pattern of cell loss was observed (grade 1) with cell loss in the hilum but imperceptible cell loss in CA1. In patients with classical HS, a variation in the grade of cell loss in the anterior-posterior axis was marked in 4% of cases.

Table I0 Results of analysis of cases of hippocampal sclerosis cases : patterns of neuronal loss and granule cell dispersion

Category (Number of cases)	Percentage of HS cases in each category for the period 1993-2000				
Grade of HS (N=183)	Grade 1 **1.6%**	Grade 2 **5%**	Grade 3 **57.2%**	Grade 4 **33.4%**	Grade 5 **2.8%**
Presence of GC dispersion (N= 174)	GC depletion **4%**	Normal GC layer **8.6%**	Mild GC dispersion **47.4%**	Severe GC dispersion **40%**	Percentage of all cases with bilayer arrangement **10.3%** or clusters of GC in ML **34.3%**

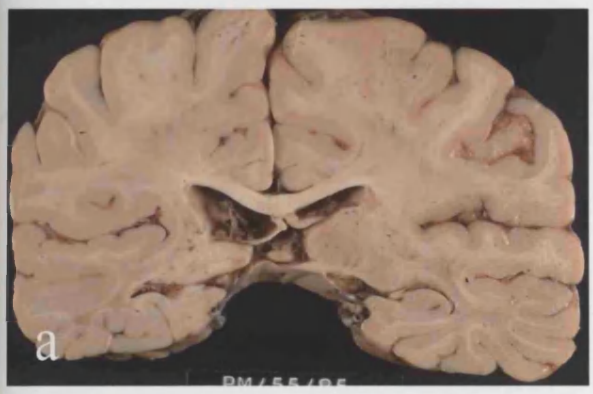
6.4.2 Results of Timms staining and patterns of granule cell dispersion

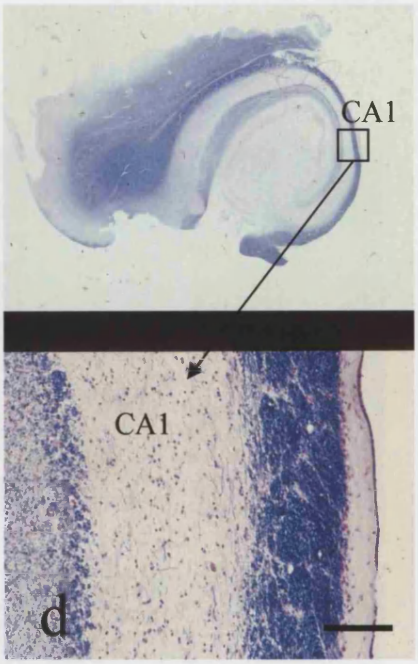
In 73 cases of HS where Timms staining was carried out mossy fibre sprouting in the supragranular layer (fig 17e&f, page162) was demonstrated in all cases with classical HS but only in one of two cases with end-folium pattern of HS. Infragranular Timms staining was more often, but not exclusively, seen in cases with severe GCD. Severe dispersion of GC into the molecular layer (GCD) was seen in 40% of the specimens. A bilayer pattern of the GC layer was seen in 10% with discrete clusters or groups of GC in the molecular layer in a further 34% (Table 10, Figure 17 c page 162 &163). Marked variation in the severity of GCD within a specimen in the anterior-posterior axis was noted in 23% of cases. Heterotopic clusters of cells with morphology consistent with GC were seen in either the hilus or adjacent to CA3 sector in 18% of cases; this was noted both in cases with mild or severe GCD (fig 17c, page 164).

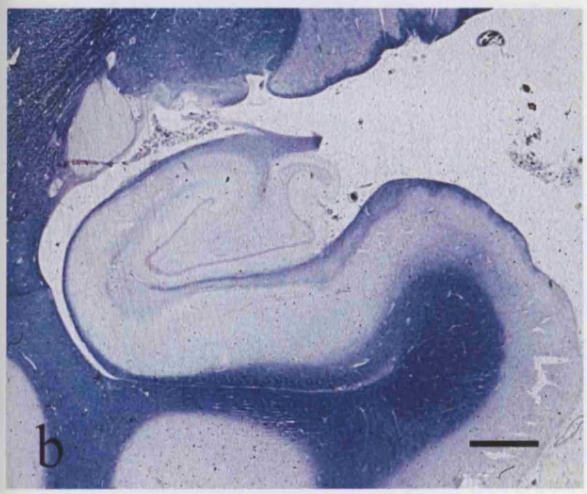
6.4.3 Cytoskeletal abnormalities in hilar neurones

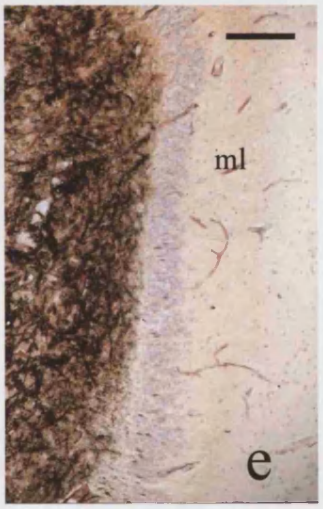
Cytoskeletal abnormalities in residual hilar neurones were noted in 55% of cases. These included enlargement or ballooning of the cell body and processes, with intense Nissl and silver staining and cytoplasmic accumulation of neurofilaments, both phosphorylated and non-phosphorylated as revealed using immunohistochemistry. In some cases only occasional hilar cells demonstrated these features whereas in others, larger numbers of positively labelled neurones and cellular processes were seen (figure 17b, page 164). There was a correlation between the severity of GCD and: severity of hippocampal sclerosis (grades 1-4, $p<0.001$) (figure 18), the presence of cytoskeletal abnormalities in hilar neurones ($p<0.001$) and the width of Timms staining in the molecular layer ($p<0.001$). In 31% of cases an abnormal myeloarchitecture within the hippocampus was also noted, including horizontal fibres in the dentate GC and molecular layers and a condensation of fibres in the end folium. In two cases thick bundles of myelinated fibres were observed traversing the GC layer (figure 17a, page 164).

Figure 17 : Cytoarchitectural patterns in hippocampal sclerosis









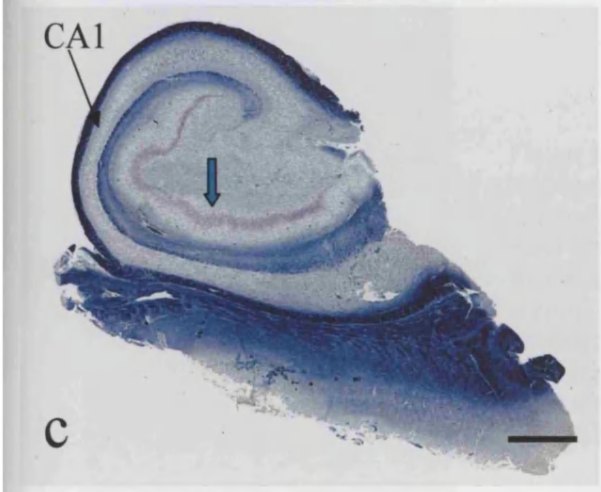


Figure 17 : macroscopic appearances of hippocampal sclerosis at post mortem with atrophy of the left mesial temporal structures (a). LFB/N stained section of normal hippocampal formation (b) and (c) from a patient with hippocampal sclerosis showing narrowing of CA1 (arrow) and dispersion of the granule cell layer (green arrow). Severe cell loss is seen in CA1 field (d). All LFB/N stained. (e) and (f) show Timms staining of a normal and HS specimen respectively, with sprouting of the mossy fibres into the molecular layer (ml) in HS (arrows). Bars : b=0.3cm, c=0.2 cm, d=110 microns, e and f = 130 microns.

Figure 17 continued : Cytoarchitectural abnormalities in hipppocampal sclerosis.

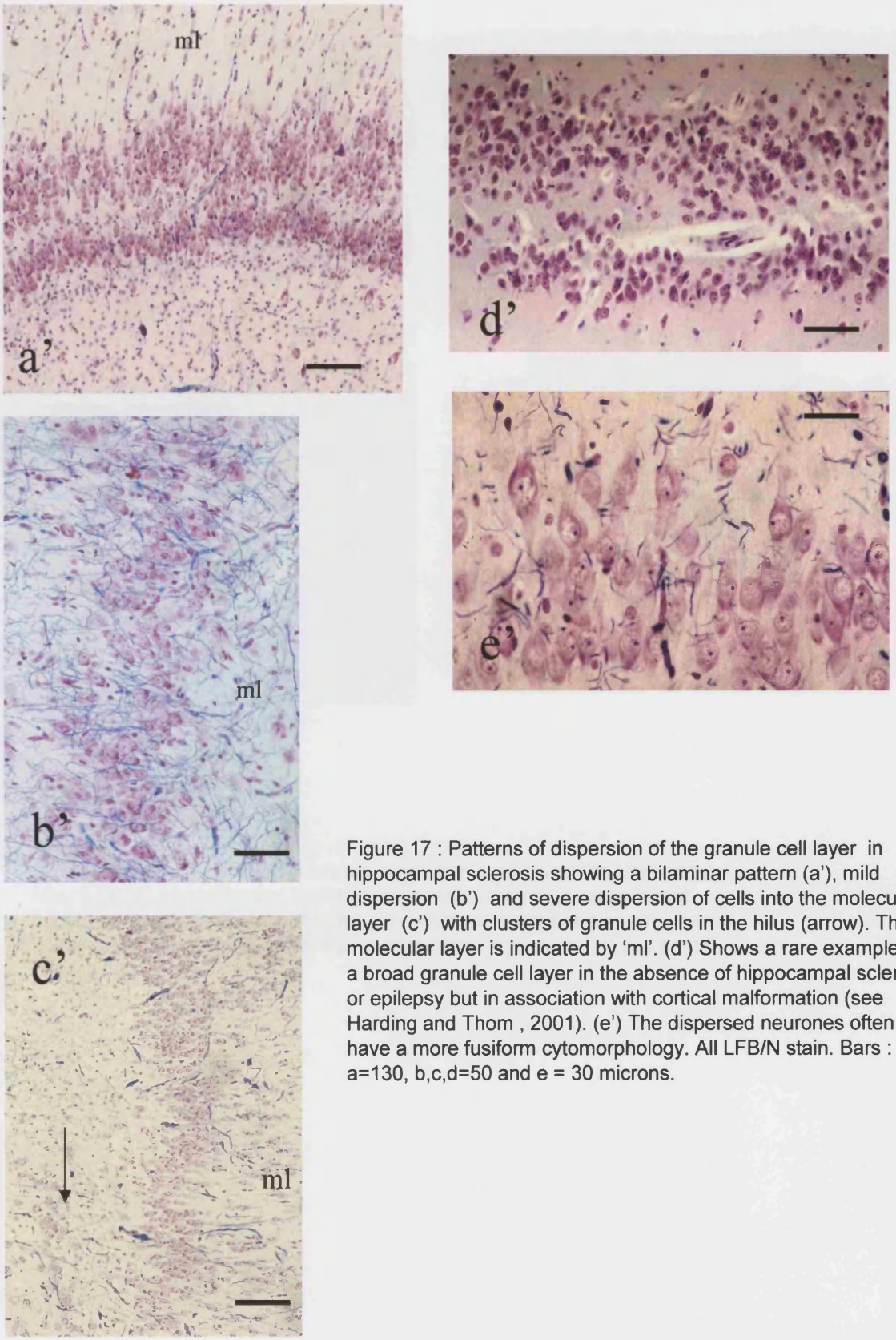
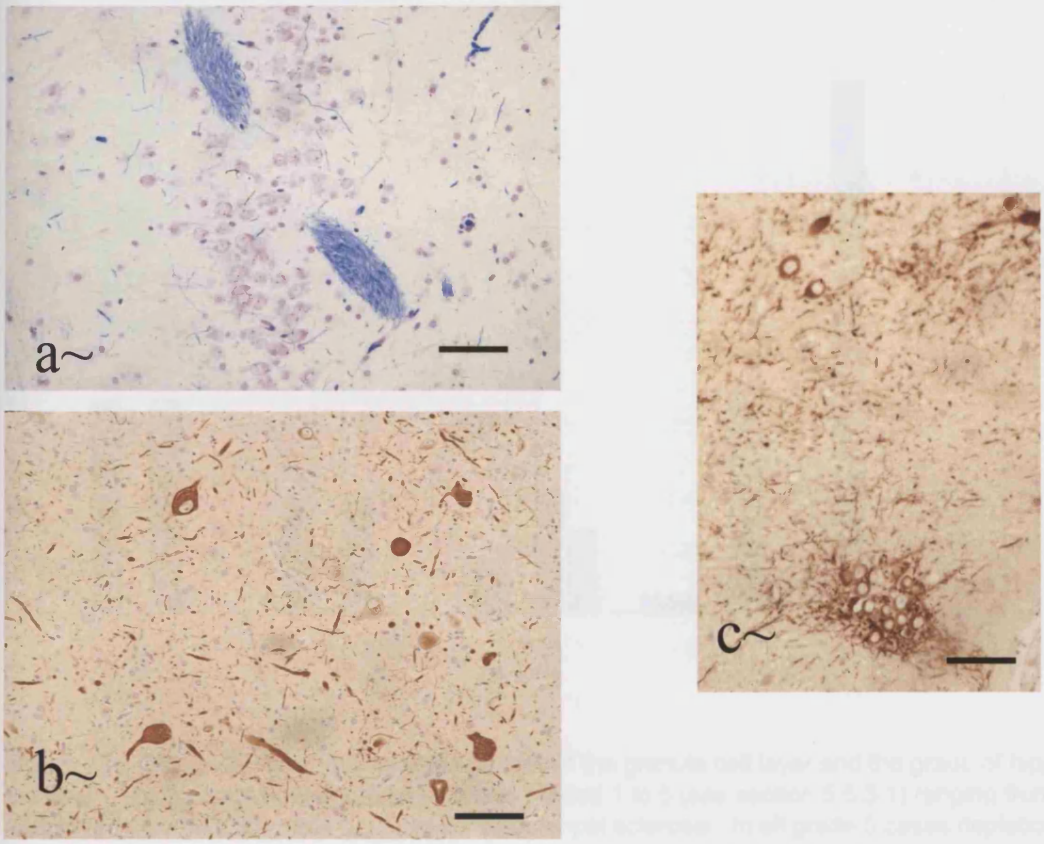


Figure 17 : Patterns of dispersion of the granule cell layer in hippocampal sclerosis showing a bilaminar pattern (a'), mild dispersion (b') and severe dispersion of cells into the molecular layer (c') with clusters of granule cells in the hilus (arrow). The molecular layer is indicated by 'ml'. (d') Shows a rare example of a broad granule cell layer in the absence of hippocampal sclerosis or epilepsy but in association with cortical malformation (see Harding and Thom , 2001). (e') The dispersed neurones often have a more fusiform cytomorphology. All LFB/N stain. Bars : a=130, b,c,d=50 and e = 30 microns.

Figure 17 continued : Hippocampal cytoarchitectural abnormalities



In (a~) abnormal myelinated fibres are seen coursing through the hippocampal hilus (LFB/N). Neurofilament staining shows residual strongly positive and hypertrophic cells in the hilus (b~) and (c~) clusters of granule cells are seen in ectopic locations in CA3 with MAP2 staining. Bars ; a and c = 110 and b = 50 microns.

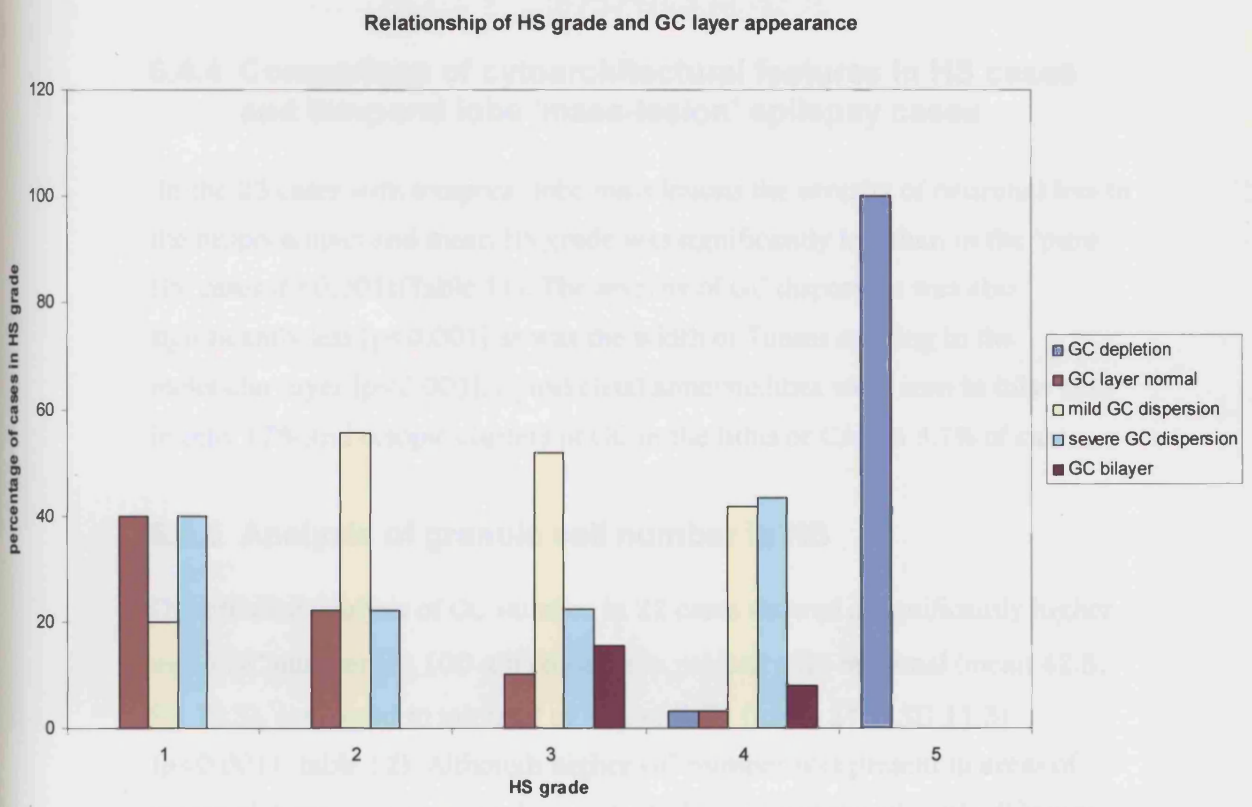


Figure 18 : The relationship between the pattern of the granule cell layer and the grade of hippocampal sclerosis. The hippocampal neuronal loss was graded 1 to 5 (see section 5.5.3.1) ranging from grade 1- end folium sclerosis, to grade 5 – severe hippocampal sclerosis. In all grade 5 cases depletion of granule cells was observed. In lesser HS grades the severity of granule cell dispersion correlated with the degree of hippocampal sclerosis and GC bilayer pattern were only seen in grade 3 and 4 of hippocampal sclerosis.

6.4.4 Comparison of cytoarchitectural features in HS cases and temporal lobe 'mass-iesion' epilepsy cases

In the 23 cases with temporal lobe mass lesions the severity of neuronal loss in the hippocampus and mean HS grade was significantly less than in the 'pure HS' cases [P<0.001](Table 11). The severity of GC dispersion was also significantly less [p<0.001] as was the width of Timms staining in the molecular layer [p<0.001]. Cytoskeletal abnormalities were seen in hilar cells in only 17% and ectopic clusters of GC in the hilus or CA3 in 8.7% of cases.

6.4.5 Analysis of granule cell number in HS

Quantitative analysis of GC number in 22 cases showed a significantly higher mean GC numbers in 100 μm columns in regions with maximal (mean 42.8, SD 15.3), compared to minimal or absent GCD (mean 24.1, SD 11.3) (p<0.001) (table 12). Although higher GC number was present in areas of maximal dispersion compared to controls this was not significantly different (Table 12) and in only 8/22 cases did granule cell numbers exceed the maximum number seen in controls. The GC number in areas of minimal dispersion was however significantly lower than controls.

6.4.6 Correlation of pathological features in HS with clinicai features

The mean age of first seizure was 4.8 years (range 0-32 years) and the mean duration of seizures until surgery 24.8 years (range 0-54 years). Initial precipitating factors included a prolonged or complex febrile seizure (16.2%), head injury (8.8%), and meningitis or encephalitis (8.8%). There was no correlation with age of first recorded seizure or duration of seizures and either the grade of HS or the severity of GCD. There was no significant difference in the mean grade of HS, or presence of GCD for cases with any type of initial precipitating event compared to HS without a precipitating event.

Table 11 : Comparison hippocampal sclerosis cases with temporal lobe mass lesion cases

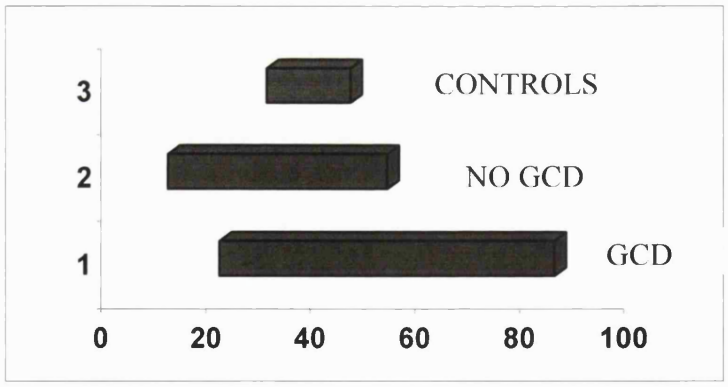
	Temporal lobectomies and hippocampectomies with extrahippocampal 'mass lesions' N= 23	Hippocampal sclerosis cases N= 183	Significance
Hippocampal sclerosis grade (Mean, range)	1.2, 1-3	3.3, 1-5	P<0.001
Normal granule cell layer	56%	8.6%	P<0.001
Severe dispersion or disorganisation of GC layer	8.6%	40%	P<0.001
Percentage of cases with cytosketal abnormalities in hilar cells	17%	55%	
Percentage with Timms positive sprouting into the ML	57%	98%	
Mean width of Timms positive sprouting in ML(microns)	43	214	P<0.001
Clusters of GC in the hilus or CA3	8.7%	18%	

GC = granule cells , ML = molecular layer

Table 12 : Results of granule cell quantitation in hippocampal sclerosis cases

Group	Mean GC number in 100 micron radial column	Range	Standard deviation	Significance Within epilepsy group* With controls ♠
Epilepsy : Region of GC dispersion (n=22)	42.8	21-85	15.3	P<0.001*
Epilepsy : Region with no GC dispersion (n=22)	24.1	11-53	11.3	P<0.001♠
Controls (n=6)	37.15	30-46	5.4	

Estimation of granule cell numbers in 100 micron radial columns in epilepsy cases and controls (GC = granule cells)



Range of values for granule cell number in hippocampal specimens

6.4.7 Correlation of HS grade and granule cell dispersion and microdysgenetic features in the temporal lobe

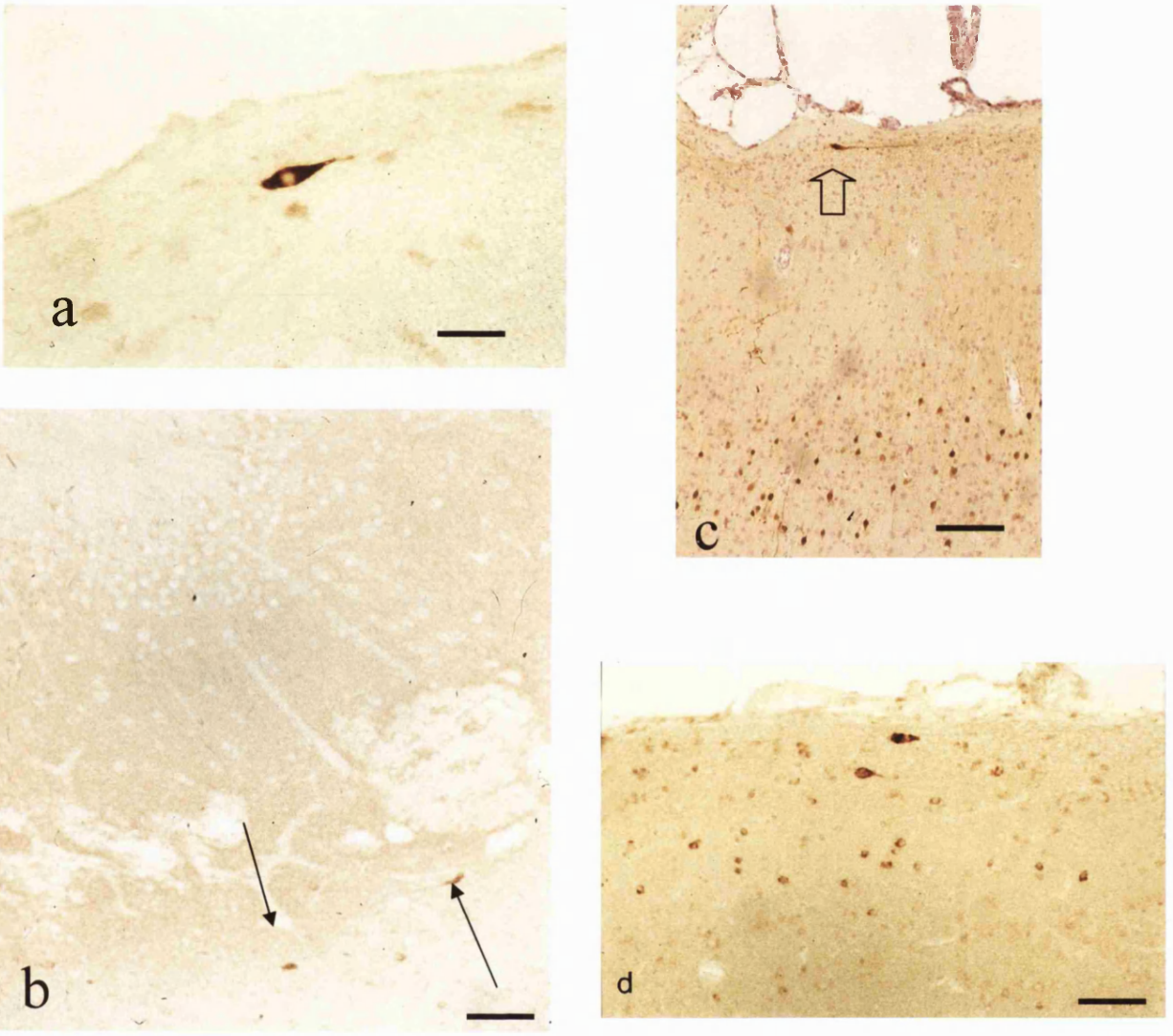
There was no correlation between the degree of dentate granule cell layer dispersion in the hippocampus and temporal lobe layer I or white matter ND ($p=0.3$ and 0.1 respectively).

6.5 Study V – Cajal-Retzius cells

6.5.1 Cajal-Retzius cells in focal cortical dysplasia and microdysgenesis

Reelin-positive cells with the typical morphology of Cajal-Retzius cells were identified in layer I in all FCD, MD and control sections (Figure 19a). In addition, smaller reelin-positive cells were observed in the molecular layer in all cases and occasionally in the deeper cortex and white matter although these were not quantified. Quantification of reelin-positive Cajal-Retzius cells in MD showed mean values of 0.47/mm (SD 0.3), significantly greater ($p<0.05$) than in adult controls (mean 0.26/mm , SD 0.13) although overlap in the range of values between the two groups was noted (Figure 20). In FCD cases a marked variation in the number of these cells in the region of dysplasia was noted with an apparent absence of reelin-positive cells in one case (mean 0.62/mm, range 0-2/mm, SD 0.66). Similar variations were noted in the adjacent normal cortex (mean 0.65/mm, range 0.23-1.52/mm, SD 0.44) (Fig 20). The mean reelin-positive cell number in the FCD cases was higher, but not significantly different, to controls. There was no correlation in MD cases between layer I calbindin and reelin-immunopositive cell numbers (see figure 21 and for values in individual cases see appendix 9).

Figure 19 : Reelin Immunohistochemistry



Reelin immunohistochemitrsy highlights bipolar cells with the morphology of Cajal-Retzius cells in cortical layer I of the temporal lobe (a). Similar cells are seen in the molecular layer of the dentate gyrus (b) and on staining with Calbindin in the neocortex (arrow) (c). Reelin staining in the neocortex aslo highlights populations of smaller cells in layer I which are probably interneurones in addition to larger neurones (d). Bars : b and c =130, d = 50 and a = 30 microns.

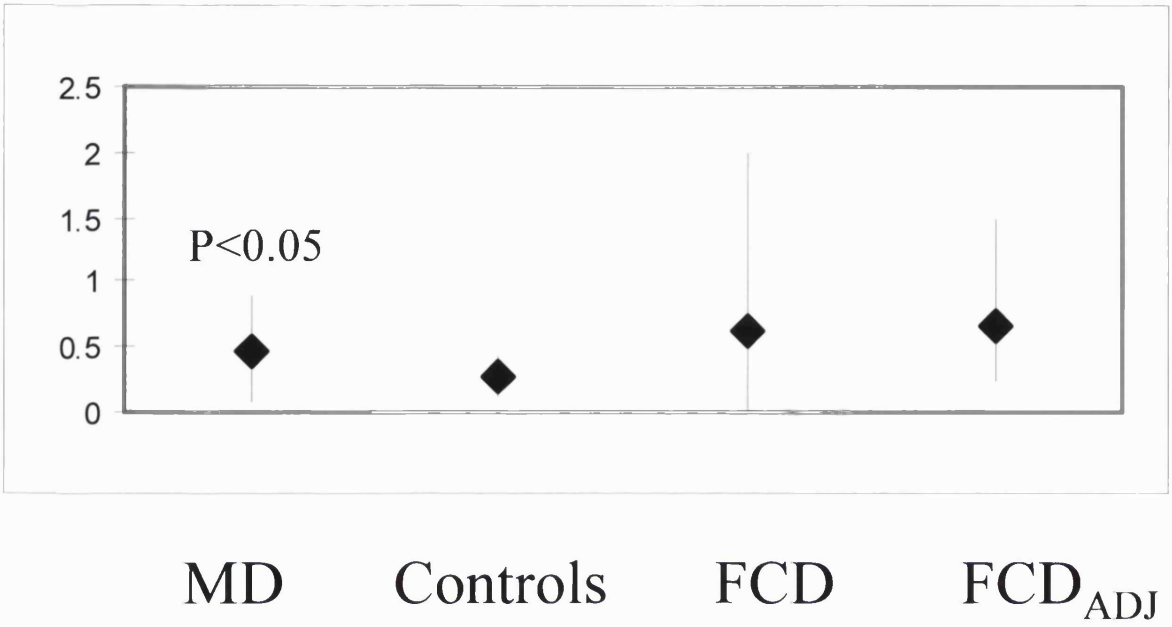


Figure 20 : Line graph of the range and mean values (diamond) of the number of reelin positive cells in microdysgenesis cases (MD), controls, in focal cortical dysplasia (FCD) and normal cortex adjacent to FCD (FCD_{ADJ}). There were significantly more cells in microdysgenesis cases than in the controls ($p<0.05$).

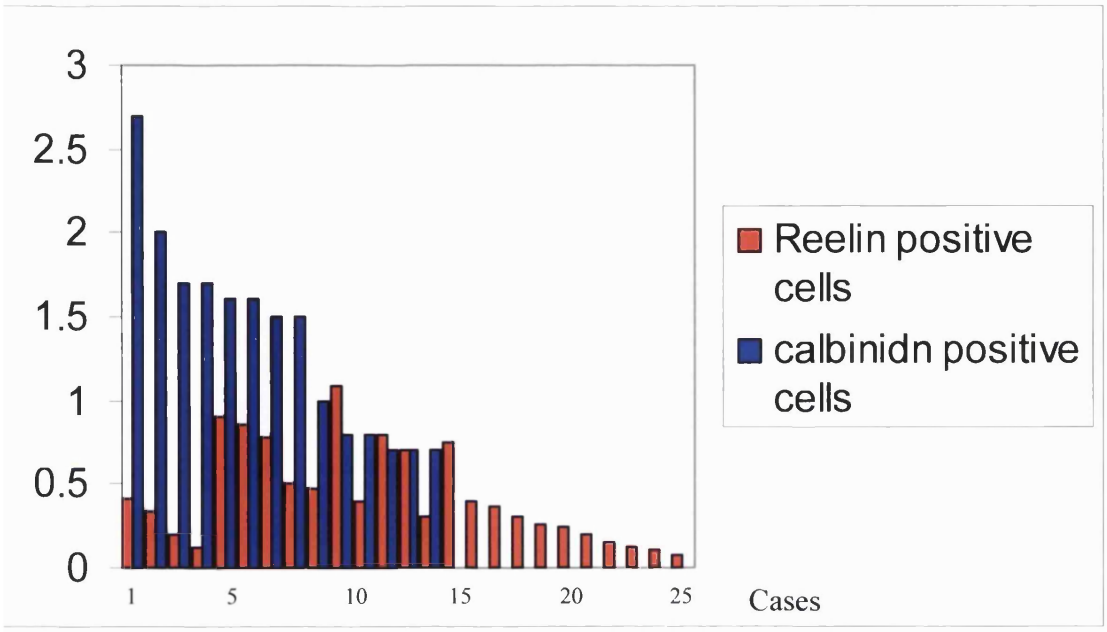


Figure 21. Box plot of the numbers of reelin and calbindin immunopositive cells in layer I in microdysgenesis cases.

6.5.2 Cajal-Retzius cells in hippocampal sclerosis

Quantitation of Cajal-Retzius-like cells in the dentate gyrus molecular layer with calretinin immunohistochemistry showed fewer positive cells in cases with severe GCD or a bilaminar GC layer than in cases with mild GCD but this was not significantly different (Table 13). There was also no significant difference in the number of reelin-positive Cajal-Retzius-like cells in these two groups (figure 19b).

Similarly reelin-positive cells were seen in control post mortem hippocampi although in significantly lower numbers ($p<0.05$) and reelin positive cells were also noted in the CA1 subfield and subiculum in the majority of cases and controls. Furthermore there were significantly fewer reelin positive cells in the hippocampi from patients with extrahippocampal lesions compared to HS ($p<0.001$). There was a correlation in the HS cases between the number of Cajal-Retzius-like cells in the molecular layer in each case with reelin and calretinin immunohistochemistry ($p<0.05$). There was no correlation between the number of reelin-positive cells and the grade of hippocampal sclerosis, duration of seizures or the width of Timms staining in the molecular layer.

Table 13 : Results of Cajal-Retzius cell quantitation in hippocampal sclerosis cases

	Controls (n=4)	HS cases (n=26)	TLE Extrahippocampal lesions (n=9)	HS cases with severe granule cell dispersion or bilaminar pattern (n=8)	HS cases with mild granule cell dispersion (n=14)
Mean reelin positive CRC /mm2 (range, SD)	1.4* (0.4-3.6, 1.5)	4.4*# (0.57-10, 2.5)	1.31# (0.4-3.2, 0.99)	4.7	4.5
Mean calretinin positive cells / mm2 (range, SD)		3.3# (0.4-9.2, 2.7)	1.62# (0.66-2.13, 0.54)	2.2	3.4
Mean duration of seizures / years (range)	NA	23 (16-54)		20	25
Mean HS grade (range)	NA	3.2 (3-4)	1.6 (0-4)	3.25 (3-4)	3.1 (3-4)
Mean width of Timms staining / microns (range)	NA	280 (80-600)		330 (100-600)	150 (80-200)

Results of counts of reelin and calretinin positive Cajal-Retzius-like cells (CRC) in the dentate gyrus molecular layer in controls, hippocampal sclerosis cases (HS) with and without severe granule cell dispersion and TLE mass lesion cases . Significant difference between hippocampal sclerosis and controls* ($p<0.05$) and between hippocampal sclerosis and TLE-extrahippocampal lesions# at $p<0.001$. NA - not applicable

6.6 *Study of inhibitory interneurones in focal cortical malformations*

6.6.1 Calbindin immunohistochemistry

6.6.1.1 In Focal Cortical Dysplasia

In control sections and adjacent normal cortex from the test cases, small calbindin (CB)-positive bitufted neurons were located predominantly in superficial cortical layers II and III, with a similar distribution seen in all age groups and in temporal and frontal cortical regions, as previously described (Ferrer et al., 1992, Conde et al., 1994). In seven of the FCD cases intensely labelled, hypertrophic and multipolar CB-positive cells were seen, with cell soma up to 40 microns in diameter (Fig 5a-d, page 122, Table 14). These cells were present in all cortical layers and were often more numerous in the white matter in proximity to balloon cells (Fig 5a&b). They were observed mainly within the region of FCD but, in one case (case 3 table 14), they predominated at the margins of the dysplasia. They were noted in both frontal and temporal lobe specimens and in all age groups. Some CB-positive cells resembled balloon cells and occasional multi-nucleated cells were observed (Fig 5d). The abnormal processes of these multipolar cells were observed on occasion in close proximity to dysplastic neurones. In ten of the FCD cases an obvious reduction in the normal small CB-positive cell population in the superficial cortex in the region of dysplasia was noted (Table 14, Fig 5a. page 123), although these interneuronal populations were preserved in the marginal normal cortex. In one case (Case 4, Table 14),

Table 14 : Results of inhibitory interneurone populations in focal cortical dysplasia

9 Case	Age(years) /site	Calbindin	Parvalbumin	Calretinin
1	32/Frontal	↓ HMC in cortex and white matter	↓	↑ in white matter Abnormal multipolar cells
2	36/Temporal	Probable ↓ No HMC	↓	→
3	27/Temporal	↓ HMC at margins of dysplasia, predominantly in layers II-1V	↓	→
4	9/Frontal	↓ (patchy) Many HMC in all cortical layers	↓ PV puncta around dysplastic neurones	↓
5	6/Frontal	Probable ↓ Many HMC	→	↓ Hyperplastic cells at margins of FCD
6	1/hemipsherectomy	→ No HMC	→	↓
7	10/Frontal	↓ Many HMC	→ Puncta around dysplastic neurones	→ Processes around dysplastic cells
8	15/Frontal	→ HMC present	→ Processes around dysplastic cells	↑ in white matter near balloon cells
9	1/Frontal	↓ No HMC	↓	↑
10	9/Frontal	↓ HMC in white matter, deep cortex and layer I.	↓ Puncta around dysplastic cells at margins of FCD	↓ Occasional balloon cells positive
11	1/Frontal	↓ No HMC	↓	→ Occasional dysplastic cell weakly positive
12	8/Frontal	Probable ↓ No HMC	↓	→

Results of distribution and morphology of calcium binding protein labelled interneurones in the region of focal cortical dysplasia (FCD). ↓=reduction in normal interneuronal populations compared to controls, → = cell number and distribution appeas normal, ↑= increase in cell number. HMC= abnormal hypertrophic / multipolar calbindin positive cells.

within an area of dysplasia, regions of CB-positive cell preservation alternated with areas of depletion.

6.6.1.2 Calbindin immunohistochemistry in Microdysgenesis

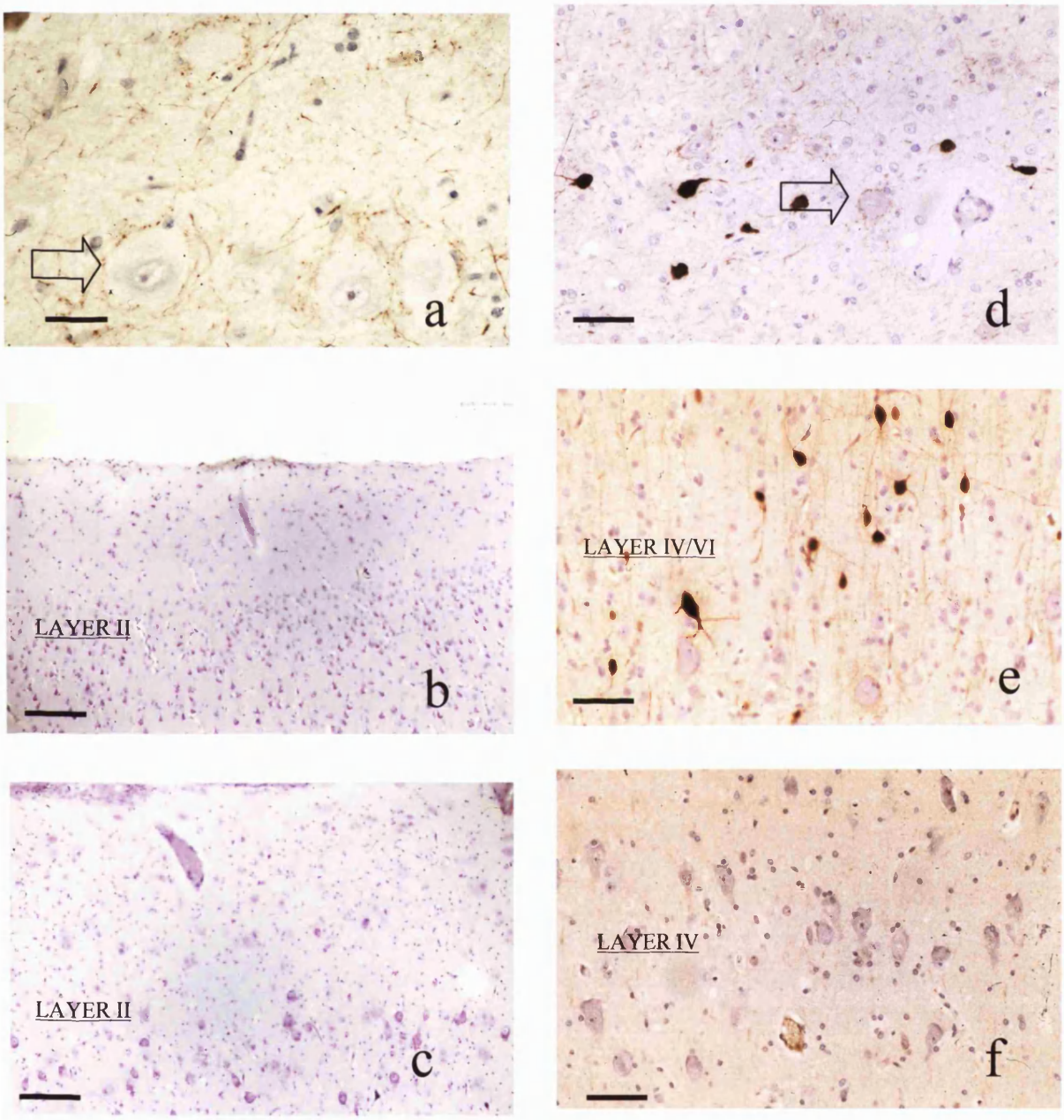
In MD cases normal CB-positive interneurones were preserved but in all cases additional small multipolar CB positive cells were seen in all cortical layers, sometimes forming small clusters (Fig 51, page 122). Abnormal multipolar, hypertrophic CB-positive cells were not seen in any of the control cases.

Variable numbers of these small multipolar 'neurogliaform cells' were seen in the temporal cortex in epilepsy patients ; they were primarily located in cortical layers III and IV, although present in some cases throughout the cortex including layer I and the white matter. In one case, groups of these cells were present forming a layer in cortical layer IV. The numbers of neurogliaform calbindin positive cells in the posterior temporal lobe were semiquantified and scored + to ++++ but there was no correlation between this score and white matter ND ($p=0.89$), layer I ND ($p=0.52$) or cortical ND ($p=0.68$). There was no correlation the number of layer I calbindin positive cells (see section 6.2.6) and the numbers of neurogliaform calbindin positive cells ($p=0.418$). (For results in individual cases seen Appendix 6)

6.6.2 Parvalbumin and calretinin immunohistochemistry

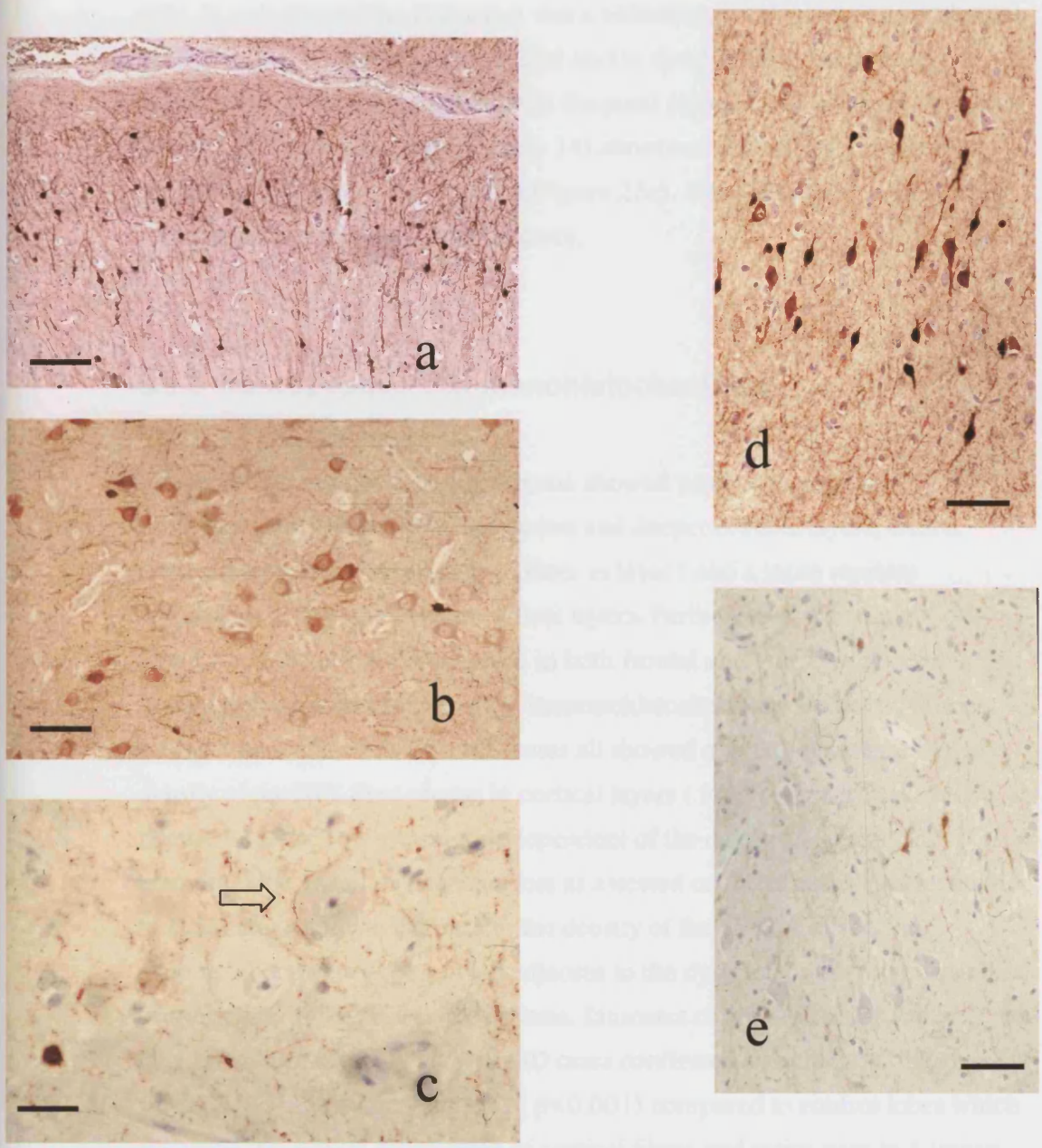
In control cases and normal cortex, parvalbumin (PV)-positive cells and fibres predominated in the mid cortical regions (layers II to IV) including small bitufted and larger multipolar cells (Fig 22e) as previously described in frontal and temporal cortex (see section 3.3.4.2). Calretinin (CR)-positive cells and fibres were present in all layers reaching greatest density in the superficial cortex (layers I and II) (Fig 23a). In eight FCD cases, including all adult FCD cases, an obvious reduction in PV-positive interneuronal number was seen in the region of dysplasia compared to adjacent normal cortex (Fig 22f, table 14). In four cases prominent PV positive puncta were present around dysplastic neurons (Figure 22d), particularly at the margins of the dysplastic region and reminiscent of peri-somal basket cell terminals as reported in normal temporal lobe (Ferrer et al., 1991).

Figure 22 : Parvalbumin immunohistochemistry



Parvalbumin immunohistochemistry demonstrates positive puncta around dysplastic neurones in focal cortical dysplasia (a and d). In areas of architecturally normal appearing cortex adjacent to dysplasia (b) normal parvalbumin cell populations are seen (e). A common observation in the region of dysplasia (c) was a reduction in the number of parvalbumin positive cells (f). Bars : a=30, b and c = 130 and d,e,f,= 50 microns.

Figure 23 : Calretinin Immunohistochemistry



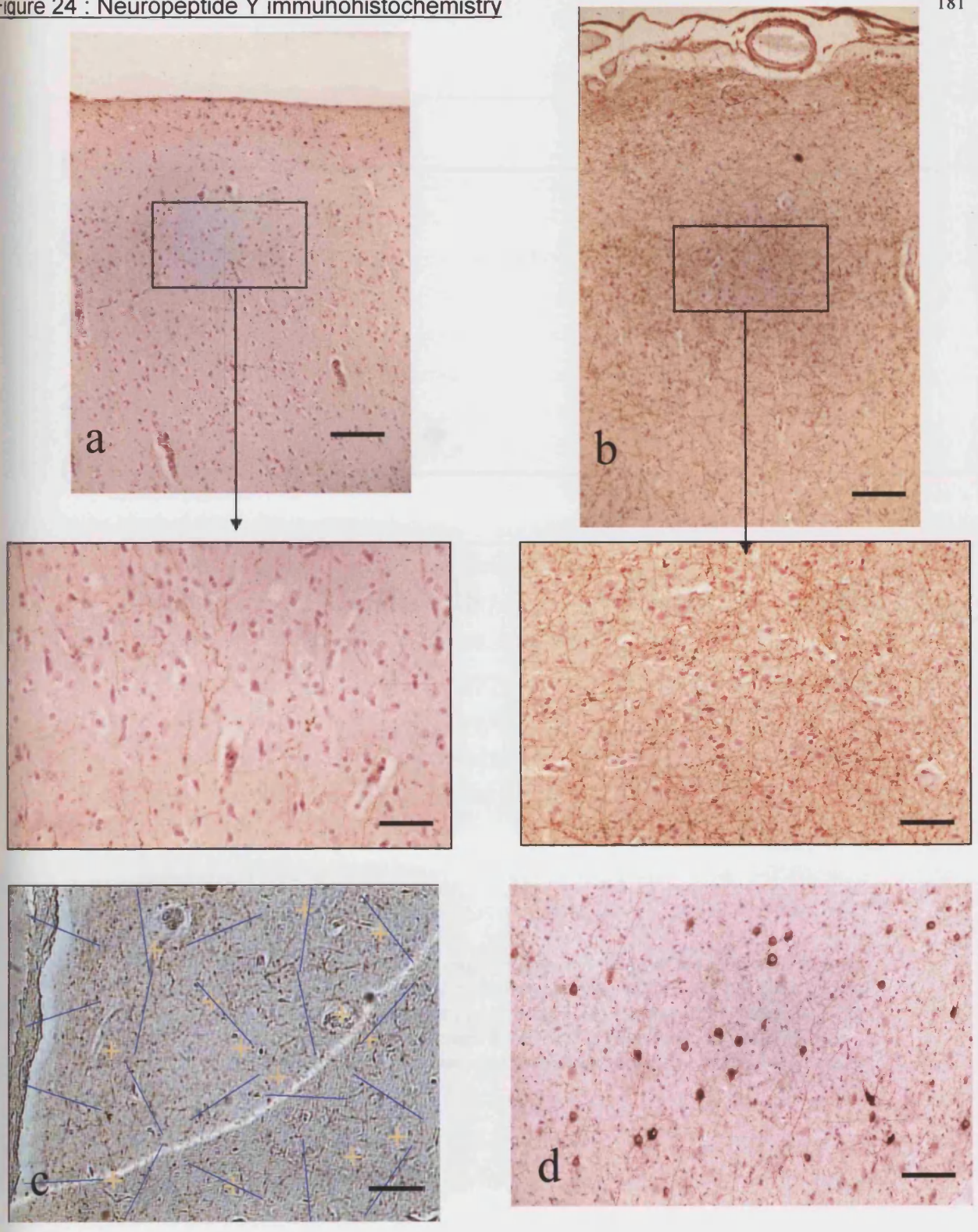
Calretinin immunohistochemistry in microdysgenesis (a) shows a pattern in this cases similar to that in normal temporal neocortex with small bitufted neurones and processes predominantly located in the superficial cortical layers. In some cases enhanced labelling of pyramidal cells was seen (b). In FCD calretinin positive puncta were occasionally observed around dysplastic neurones (arrow) (c). In other cases of FCD both increased (d) and apparent reduction (e) in normal calretinin cell populations were seen. Bars a= 130, b,d,e = 50 and c = 30 microns

Five MD cases also showed a focal reduction in the numbers of PV-positive cells. In only four of the FCD cases was a reduction in CR-positive cells obvious in the region of dysplasia (Figure 23e) and in three cases the cell density appeared to be focally increased with frequent cells present in the white matter (Table 14). In one case (case 8, table 14) abnormal CR positive puncta were present around dysplastic neurons (Figure 23c). Normal CR-immunostaining patterns were observed in all MD cases.

6.6.3 Neuropeptide Y immunohistochemistry

NPY staining patterns in controls tissue showed positive neurones predominantly within the white matter and deeper cortical layers, with a horizontal plexus of NPY positive fibres in layer I and a more random orientation of fibres in deeper cortical layers. Furthermore, a similar distribution of neurones was noted in both frontal and temporal neocortex and in all age groups, in keeping with immunohistochemistry studies by other groups (see section 3.3.4.2). MD cases all showed a striking increase in the density of the NPY fibre plexus in cortical layers I to III compared to controls (Figure 24a&b). This appeared independent of the degree of superficial temporal lobe gliosis or neuronal loss as assessed on GFAP and NeuN sections. In FCD cases a similar increase in the density of the NPY plexus in the superficial cortex was also noted adjacent to the dysplasia, with more variable findings within the region of dysplasia. Estimates of the ratio of NPY fibre length between layer II and V in MD cases confirmed significantly higher ratios (mean 3, SD 1.3, range 1.8 to 6.1 ; $p<0.001$) compared to control lobes which showed a more even distribution of cortical fibres and ratios near to 1 (mean 0.98, SD 0.32, range 0.6 to 1.5) (Figure 25). NPY fibre length ratios were also significantly higher in the cortex adjacent to FCD (mean 2.06, SD 0.7, range 1.14 -3.6, $p<0.001$) than in controls. In four FCD cases an obvious increase in NPY-positive cells within the region of cortical dysplasia was noted and in one case (Figure 24d) intense labelling of many dysplastic neurones was observed (For values of NPY in individual cases see appendix 10).

Figure 24 : Neuropeptide Y immunohistochemistry



Neuropeptide Y staining in normal temporal neocortex shows occasional positive processes as shown in high magnification insert of cortical layer II (a). In microdysgenesis an increased density of the NPY fibre plexus network is seen in the superficial cortex as shown on high magnification insert (b). Using Buffon' needle principle-see section 3.7- (c) the length of the fibre plexus in NPY sections was estimated and confirmed to be higher in microdysgenesis. In some cases of focal cortical dysplasia dramatic labelling of dysplastic neurones was noted (d). Bars : a,b,c,d = 130 microns, inserts of a and b =50 microns.

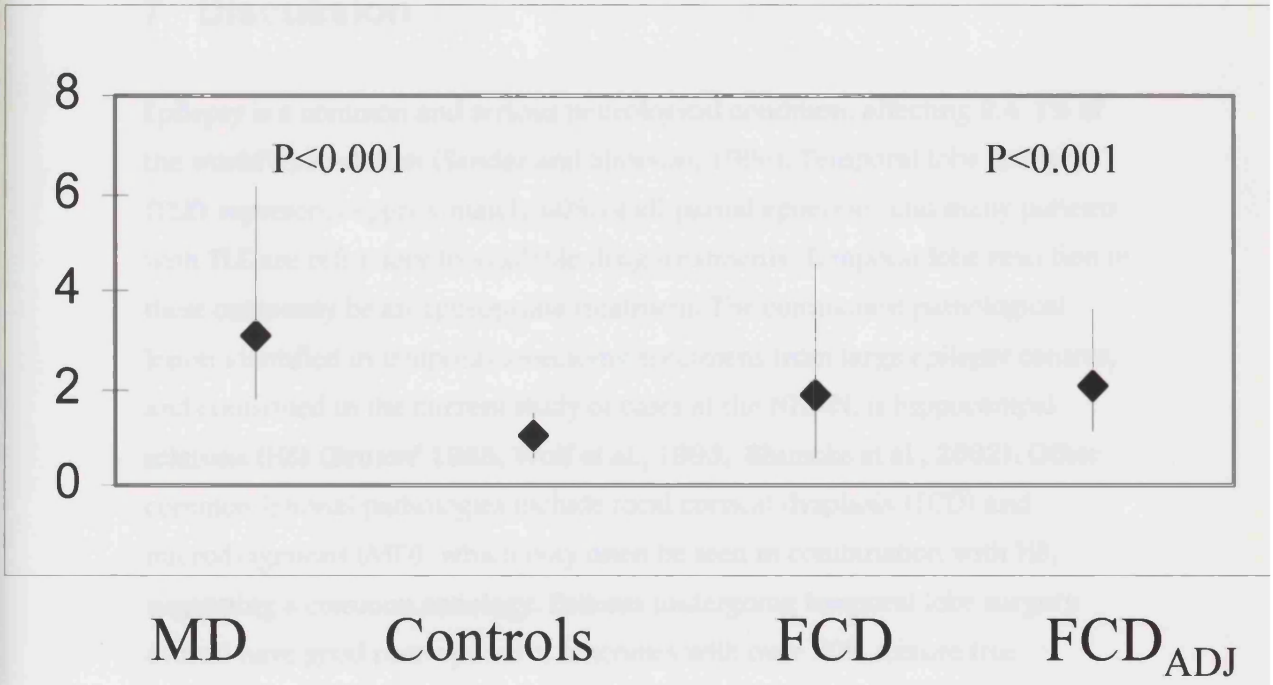


Figure 25. Line graph illustrating the mean values and range of the ratio of NPY fibre length between cortical layer II and V in microdysgenesis (MD), controls, focal cortical dysplasia cases (FCD) and in normal cortex adjacent to focal cortical dysplasia (FCD $_{ADJ}$). Significantly higher ratios were seen in MD and in cortex adjacent to FCD than controls where the mean ratio was near one (see Appendix 10 for values in individual cases).

7 Discussion

Epilepsy is a common and serious neurological condition, affecting 0.4-1% of the world's population (Sander and Shorvon, 1996). Temporal lobe epilepsy (TLE) represents approximately 60% of all partial epilepsies and many patients with TLE are refractory to available drug treatments. Temporal lobe resection in these cases may be an appropriate treatment. The commonest pathological lesion identified in temporal lobectomy specimens from large epilepsy centres, and confirmed in the current study of cases at the NHNN, is hippocampal sclerosis (HS) (Bruton' 1988, Wolf et al., 1993, Blumcke et al., 2002). Other common lesional pathologies include focal cortical dysplasia (FCD) and microdysgenesis (MD), which may often be seen in combination with HS, suggesting a common aetiology. Patients undergoing temporal lobe surgery overall have good post-operative outcomes with over 70% seizure free following surgery (Engel et al., 2003 , Shaefi and Harkness, 2003) suggesting that the resected lesion identified was initiating or sustaining the seizures. In the current series of cases from the NHNN we observed a 75% Class I outcome in all epilepsy surgical cases (of which over 60% were HS) and a 78% class I outcome where the diagnosis was HS.

Although the response to surgery is favourable, particularly for HS, the aetiology of HS, MD and FCD, as well as the mechanisms causing the seizures, are still largely unknown. Clinical and experimental evidence so far points to a mal-developmental origin for these lesions. Temporal lobectomies are being increasingly carried out for the treatment of patients with medically refractory seizures and this material has provided a unique resource for the further study of these lesions. The presence of microscopic malformations and microdysgenesis, as an additional finding to HS, are still regarded with scepticism by many and in one recent textbook of surgical pathology it is stated : "How often (microdysgenetic) changes are encountered in the lateral temporal lobe depends on the experience and persistence of the observer and his or her threshold for accepting minor variations in cytoarchitectonics as bona-fide abnormalities". My aim was to further the understanding of the developmental neuropathological aspects of these lesions and, with the application of stereological and immunohistochemical techniques, to clarify the diagnostic criteria for microdysgenesis.

7.1 Current concepts and confusion regarding microdysgenesis

The term "microdysgenesis" (syn.: architectural dysplasia, mild cortical dysplasia) (Honavar and Meldrum, 2002 ;Wolf et al., 1993; Garbelli et al., 2001) seems a very suitable term to describe the MRI occult, cortical cytoarchitectural dysgenetic abnormalities, observed in a proportion of patients with epilepsy, which lack the dysplastic neuronal and glial elements that characterise FCD. In the initial descriptions of microdysgenesis in patients with idiopathic generalised epilepsy (Meencke and Janz, 1984), the cytoarchitectural disturbances observed predominated in the cortical layers that are the earliest and last to be formed. Increased numbers of neurones were noted in layer I (which develops from the marginal zone) and the subcortical white matter (which develops from the subplate) and ill-defined boundaries between layers I and II were noted, the final layer formed in normal corticogenesis. Some of the abnormalities described are not specific to epilepsy (Akbarian et al., 1993; Kaufmann and Galaburda, 1989) and some features described as microdysgenetic, for example, 'a persistent columnar organisation of cortical neurones' (Armstrong ,1993), may reflect normal anatomical cytoarchitectonic variation within the temporal lobe. Microdysgenetic-like malformations however, have been shown in animal models of temporal lobe epilepsy (Tsuuji et al., 2001). The prevalence of microdysgenesis in human TLE surgical series remains largely unknown, mainly due to a lack of standardised diagnostic neuropathological criteria used between epilepsy centres, and therefore the exact influence of these abnormalities on patient's outcome remains also undetermined. The main features consistently attributed to microdysgenesis in previous published works (Meencke and Janz, 1984; Armstrong 1993; Hardiman 1988; Emery et al., 1997; Kasper et al., 1999; Nordburg et al., 1999) are as follows :

- **An excess of neurones in the white matter**
- **An excess of neurones in layer I**
- **Abnormal cortical laminar organisation including clustering of cells**

Analysis of these components in lateral lobe resections from patients with temporal lobe epilepsy and hippocampal sclerosis formed the main part of this study.

7.2 Stereological counts in this study confirm higher mean white matter neurone densities in TLE than controls

The origin of neurones in the temporal lobe white matter is debated. In normal adult human white matter the majority of neurones are pyramidal cells, considered to represent remnants of subplate neurones (Chun and Shatz 1989; Meyer et al., 1992). These neurones have important roles during development, including establishing cortical architecture, gyrification and guiding thalamic connections (Molnar et al., 1998, Kostovic and Rakic, 1990). Their persistence into adult life and functional roles in mature cortex are less well understood although widespread connections of these neurones with layer I has been described in rat cortex (Clancy and Cauller, 1999). Normal subplate neurones assume a variety of morphologies during development including pyramidal and multipolar forms (Mrzljac et al., 1988; de Azevedo 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 my 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). I was also able to demonstrate using this antibody a significant difference in white matter ND between epilepsy cases and controls, although there was some overlap between the groups as noted in previous studies (Hardiman et al., 1988; Emery et al., 1997).

In studies on surgical temporal lobe tissue ideal control tissue is normal temporal lobe tissue, which also has been surgically removed, for example, tissue removed adjacent to a tumour, with similar fixation times and processing methods. Access to such tissue is limited and the control tissue we used comprised both surgical and post mortem temporal lobectomies in which there is a variable post mortem delay and fixation times which may affect tissue volume and antigenicity. However, we did not find a significant correlation between PM interval or fixation time and ND in our cases and, in addition, ND in surgical and PM controls was not significantly different, justifying our including of PM cases as controls. Our 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 control patients was older than that of 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.

White matter neuronal densities have been the most studied aspect of microdysgenesis in AHS and the majority of quantitative studies using 2-D cell counting methods confirm an excess of white matter neurones in TLE patients compared to controls and with overlap between the two groups (Hardiman et al., 1988; Emery et al., 1997; Kasper et al., 1999). Stereological cell counting allows a more accurate assessment of temporal lobe white matter neurones. In my study, the cell density ranged from 444-1751/mm^3 in Nissl stained sections of TLE similar to the range of 440-1950 in the only other published stereological study of 8 patients (Bothwell et al., 2001) suggesting results using this method are reproducible between laboratories. The range of white matter neuronal densities in NeuN sections was from 1212 to 3448/mm^3 and in a study of 50 further temporal lobe specimens, using NeuN and identical stereological cell counting method, a similar range of neuronal densities was found with a normal distribution (see Figure 7b).

7.3 NeuN immunostaining highlighted small white matter interneurones which may also be of significance in epilepsy and overlooked in previous studies

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. 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 microns or greater and showed a range of 2 to more than 15 neurones /2 mm^2 in epilepsy patients using two dimensional cell counting (Hardiman et al., 1988). Emery quantified cells with prominent nucleoli and nuclear diameter greater than 12 microns, finding neuronal densities of 4.11 ± 1.86 per mm^2 in a similar patient group; however ,they acknowledged 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 TLE 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 (de Azevedo 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. The smaller neurones in the white matter may represent GABAergic neurones, and some may express calbindin and parvalbumin proteins (data not shown). This may indicate that these neurones originate from tangential migration. Further study to more fully characterise the nature,

origin and connectivity of white matter neuronal subtypes will be fundamental to our understanding of their possible contribution to epilepsy.

7.4 White matter neuronal densities did not vary in different anatomical regions of the temporal lobe

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 previously demonstrated 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 (Meencke, 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 to the gyral core white matter in a significant number of cases in epilepsy. These findings suggest that future quantitative analysis of white matter MD could be restricted to one coronal section of temporal lobe but should include both superficial gyral and deep periventricular white matter.

7.5 The density of white matter neurones appears to be independent of the severity of the gliosis.

It has been suggested that increased ND observed in the white matter in TLE may be merely an epiphenomenon, i.e., the 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 TLE on neuroimaging are likely to be gliosis or myelin loss (Mitchell et al., 1999). Cortical and white matter gliosis is commonly observed in surgical resections. We estimated the severity of white matter gliosis by quantifying the density of GFAP positive, reactive astrocytes (AD) 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 cortical neuronal loss, both of which may correlate with macroscopic atrophy.

7.6 The presence of microdysgenetic features is associated with a more favourable outcome with HS than HS alone

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 recognised 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 histologically identified in the temporal lobe (Hardiman et al., 1988), although in that study the presence or absence of co-existing adjacent hippocampal sclerosis is unknown. My findings may support a different pathogenetic mechanism for HS in association with MD (i.e., a developmental cause) compared to other causes of HS which may be secondary to an exogenous insult and associated with a worse prognosis due to subtle involvement of the other hippocampus.

7.7 Establishing quantitative criteria for microdysgenesis

What constitutes the pathological condition of microdysgenesis is a very pertinent question. All the microscopic features described in microdysgenesis (including single white matter neurons, cortical clustering of neurons and neurones in layer I) may be present in some normal brains and their significance in epilepsy has been much debated. There are no pathognomonic morphological criteria, as the dysplastic neurones in Taylor's Focal Cortical Dysplasia, which can easily discriminate microdysgenesis from normal and many investigators have resorted to quantitative methods of analysis. These studies to date all confirm an excess of white matter neurons in epilepsy, which 'overlap' with the normal range. Many biological measurements in disease groups overlap with control ranges , but this does not exclude a developmental pathology in the study group. For microdysgenesis in epilepsy, at what point white matter neurons are present in 'pathological numbers' is a problematic issue.

If the ongoing controversy regarding the significance of microdysgenesis in epilepsy is to be resolved, then the starting point is surely to provide reliable, reproducible potentially diagnostic quantitative data. Studies to date have employed two-dimensional cell-counting methods with their inherent biases. In the recent study by Kasper and colleagues for example (Kasper et al., 1999) neurons in the white matter were counted per 'high power field' without information of the size of the field area, how many fields were counted and how the white matter was sampled. It is unlikely that other groups could reproduce their method and therefore their value of >10 neurones per high power field in the epilepsy group is virtually meaningless. Methodical stereological approaches, in contrast, can provide more accurate and reproducible data. A stereological study of white matter neurons in temporal lobe epilepsy also published recently (Bothwell et al., 2001) showed near identical ranges of white matter neuronal densities in Nissl stained sections (mean neuronal density 1160/mm3, range 440-1950/mm3) to the present study (mean neuronal density 1010/mm3 , range 444-1751/ mm3, Thom et al ., 2001) suggesting that a stereological approach can give measurements that are reproducible between laboratories.

The incidence of microscopic malformations or microdysgenesis in TLE has been variably reported between 10-15% (Kuzniecky et al., 1999), 16.7% (Nordberg et al., 1999) or 'in the majority of surgical specimens' (Armstrong and Mizrahi, 1997) ; different pathological criteria used are likely explain these differences. A more exact definition of microdysgenesis, as derived by quantitative analysis, would give more consistent information regarding its incidence for future clinico-pathological correlations. From our preliminary data, if we consider white matter ND > 2360/mm^3, small white matter ND >1170/mm^3, layer I ND >11,250 /mm^3 and layer I calbindin ND >1.5/mm^2 as positive criteria for microdysgenesis, then in the present study group 30% of patients with none of these criteria, 53% with 1 to 2 criteria and 67% with 3 to 4 criteria were seizure free following surgery. It is likely however, that there are different 'types' of microdysgenesis involving specific cortical layers (see Section 7.9) and that all the constellation of features described in microdysgenesis are not always encountered in a single case.

In the future, automated quantitative methods, such as image analysis, could be incorporated into routine practice to carry out measurements and provide more accurate information regarding the incidence of microdysgenesis as well as potentially useful prognostic information for both patient and clinician. In this study I have shown, for example, that measurement of interneuronal distance using an image analysis system in the white matter strongly correlates with the neuronal density as measured by 'gold-standard' 3-D cell counting. This method takes a small fraction of the time taken for arduous cell counting and may prove a more practical tool in the diagnostic laboratory.

My data are based on a sample of only 31 patients and a larger clinico-pathological study which is currently in progress (see Section 8), could lead to further refinement of the diagnosis of microdysgenesis as well as clarifying its real significance. With my data a correlation between white matter neuronal densities and 11-C flumazenil binding on PET imaging was seen. This suggests that microdysgenesis may become detectable *in vivo* and future modern MRI modalities may also be able to detect these changes and ongoing quantitative pathological and imaging correlative studies will be important. In our initial

study we have shown a positive correlation between microdysgenetic features and good outcome, in contrast to other studies, for example in the study by Kasper (Kasper et al., 1999), where a negative correlation between white matter neuronal density and outcome was noted. In Kasper's study only 44% of patients had classical hippocampal sclerosis in contrast to 100% of patients in our study group. The presence or not of hippocampal sclerosis and the surgical approach is likely to have had considerable bearing on post-operative seizure outcome independently of the white matter neuronal densities and this may explain the differences in outcomes observed. It also emphasizes the importance of the presence or not of a second epileptogenic temporal lobe pathology and standardizing study groups when investigating the significance of white matter neurons if the controversy of microdysgenesis is to be resolved.

7.8 Identification of abnormal patterns of cortical myelo-architecture as a marker for microdysgenesis

The presence of abnormal myelinated nerve bundles in the superficial cortical laminae of the temporal lobe has not been previously reported in the setting of microdysgenesis. We identified such fibres in close proximity to abnormal neuronal clusters; no calbindin positive inhibitory interneurones were identified in these clusters which were composed of small neurones lacking a pyramidal morphology. One possibility is that the nerve fibres have originated from these abnormal neuronal aggregates; if they represent abnormal cortical-cortical projection fibres we can speculate that they may be of functional significance in terms of both local seizure genesis and/or propagation. Alternatively, they may represent thalamo-cortical projections. However, in experiments using dye-tracing techniques we were unable to establishthe origin or pathway of these fibres.

Similar, tangentially orientated myelinated fibres are recognised to occur in the cortex of polymicrogyria (Harding and Copp, 2002). In more severe cortical malformations extensive abnormal bundles of cortical myelinated fibres have been identified reminiscent of driftwood and termed in the original report as 'driftwood cortex' (Rebeiz et al., 1968). This may suggest an overlap between these malformations, both thought to arise from an insult occurring in the later stages of cortical development (Mischel et al., 1995). Subpial layers of myelinated axons have also been reported in focal cortical dysplasia (Janota

and Polkey, 1992) and were considered to represent inappropriately projecting fibres from the abnormal and malorientated nerve cells of the malformation. In a personal study of a case of polymicrogyria, FCD and heterotopia similar abnormal horizontal bundles of fibres were shown to be parvalbumin positive; therefore a subcortical or thalamic origin of these fibres rather than local origin is a further possibility. In the New Zealand Black mouse model, which has a microdysgenetic-like malformation with aggregates of neurones in layer I, abnormal fibres projecting into these abnormalities have been shown to originate from extracortical site using DiI tracing (Jenner et al., 2000). Furthermore, in animal models of epilepsy abnormal fibre networks and connectivity between heterotopic cells have been shown (Chevassus-au-Louis et al., 1999b).

In the present cases of microdysgenesis, the well-orientated and organised appearance of these fibres suggest that they represent part of the primary malformation rather than regenerative axons. Furthermore, EEG findings of more widespread cortical abnormalities in three patients and the poor post-operative outcome in terms of seizure control in two, may be an indication that this malformation is more extensive.

In all the cases I also observed more widespread cortical laminar nerve cell loss, predominantly from layer II, which was supported by quantitative analysis of cortical NeuN neuronal densities (see Section 6.3.3). Similar patterns of neuronal loss have been noted to occur in a proportion of patients with temporal lobe seizures and hippocampal sclerosis (Cavanagh and Meyer, 1952). It is considered that, unlike ischaemic laminar necrosis, this pattern of cell loss represents excitotoxic, seizure-mediated neuronal loss via cortical association fibres, of which layer II and III nerve cells are the main source and recipient. It is possible that the abnormal cortical myelinated fibres identified have contributed to the degree of laminar cell loss observed in these cases by such a mechanism. It is unlikely that the layer II neuronal depletion is part of the cortical malformation (akin to the 'cell free' layer in four layered polymicrogyria) in view of the presence of a reactive gliosis and the observation that this pattern of cell loss occurs in epileptic cortex without malformation. However, in a recent study of focal malformations in temporal lobe epilepsy Tassi et al. (2002) report a reduction of neurones in layer II as a typical feature of 'architectural dysplasia'.

7.9 Our findings support that "Microdysgenesis' involving the superficial cortex (layers I and II) and 'Microdysgenesis' with white matter heterotopia are distinct entities

In the recent paper by Palmini and Luders (2002) on a proposed revision for the classification of cortical malformations they suggest that mild cortical malformations (the term they use for microdysgenesis) should be divided into Type I lesions (heterotopic neurones in layer I) and Type II (where the abnormalities occur outside layer I, including heterotopic aggregates of white matter neurones). The scientific basis for this distinction is not discussed in their paper, but from the findings in our study there is evidence that this subdivision has some merit. We showed no correlation between white matter neuronal densities and abnormalities in layer I including: layer I neuronal densities, number of Cajal-Retzius cells, the presence of abnormal cortical myelinated fibres in layers I-II and neuronal clustering in layer II. However, there was a correlation between the number of Cajal-Retzius cells in layer I and the presence of neuronal clusters and the presence of abnormal myelinated fibres in the superficial cortex. This would suggest that 'Layer I microdysgenesis' is distinct from 'White matter microdysgenesis' and that these entities should be kept separate as they may have different aetiologies and functional significance in temporal lobe epilepsy.

7.10 Cytoarchitecturai evidence for a deveiopmental basis of Hippocampal sclerosis

Hippocampal sclerosis is the commonest lesion identified in temporal lobe series in epilepsy. It has been the subject of intensive scrutiny over the last decades and yet its exact aetiology remains elusive. There is epidemiological evidence that an injury occurring early in life precipitates the process of neuronal loss (Mathern et al., 1995c, Kuks et al., 1993) but the theory of underlying aberrant hippocampal development has been also speculated upon. The combination of HS with neocortical malformation is noted in some patients with epilepsy as is malformation of the hippocampus in isolation or as part of a more widespread process (Baulac et al., 1998, Raymond et al., 1994a and

1995, Ho et al., 1998, Levesque et al., 1991). In patients with hippocampal sclerosis alone, various cytoarchitectural abnormalities have been reported in neuropathological studies suggesting an underlying malformation, although their frequency, significance and temporal relationship to the process of HS are not well defined. I aimed to address this by reviewing these abnormalities in a large surgical series of hippocampal resections for epilepsy.

7.11 Greater hippocampal neurone loss is confirmed in the absence of temporal lobe mass lesion but no correlation of neuronal loss with duration of seizures is shown.

In 183 HS specimens the *classical pattern* was seen in over 90%, with neuronal loss predominant in CA1 and hilar subfields whereas *end-folium* and *severe* hippocampal sclerosis were seen in only 1.6% and 2.8% of cases respectively. In all the cases with classical hippocampal sclerosis in which Timms staining was performed, mossy fibre sprouting into the molecular layer was confirmed. Mossy fibre reorganization has been proposed as one of the major epileptogenic mechanisms in HS (Sutula et al., 1989, Babb et al., 1991). Furthermore, in keeping with previous observations (El Bahh et al., 1999), infragranular Timms staining was more prominent in cases with severe GCD.

In an earlier large study of HS, end-folium sclerosis was reported in 4% of 122 cases and classical and severe HS in 57% and 39% respectively (Bruton, 1987) (see Table 15). The smaller number with severe HS in the present study may reflect differences in pre-operative assessments and patient selection. By contrast, in the second smaller group of patients in my study with temporal lobe mass lesions, significantly less hippocampal neuronal loss was demonstrated, which has also been the finding in previous studies (Mathern et al., 1997a). I did not find any correlation between the severity of neuronal loss in the hippocampus and clinical parameters including whether there was an initial precipitating event of any sort, age of onset of first seizure and the duration of seizures prior to surgery. This contrasts with previous studies, which have suggested that greater hippocampal neuronal loss is present where there is a history of a precipitating event, particularly if it is a seizure (Mathern et al., 1995c), and with earlier age of onset of seizures (Davies et al., 1996).

Table 15: Comparison of patterns of hippocampal sclerosis in our series with other large series

Series of Hippocampal sclerosis specimens	**Institute of Psychiatry (Bruton, 1988) n=107**	**Institute of Neurology (Present series) n=183**
Classical Hippocampal sclerosis (neuronal loss predominantly in CA1 and CA4)	57%	90%
End-folium sclerosis (neuronal loss predominantly in CA4)	4%	1.6%
Severe Hippocampal sclerosis (neuronal loss in all fields)	39%	2.8%

7.12 Granule cell dispersion appears to be dependent on degree of hippocampal pyramidal cell loss

Disorganization of the GC layer in HS was first recognized by Houser (Houser 1990, Houser et al., 1992) and considered most likely to represent a neuronal migration disorder. Indeed, I have personally observed similar abnormalities bilaterally, in association with neocortical malformations and in the absence of epilepsy and HS, lending support to this theory (Harding and Thom, 2001). Similarly, in animal models of cortical malformations, abnormalities of the GC layer have also been identified. In the p35 mutant mouse, heterotopic GC are present in the molecular layer and in the hilum with mossy fibre sprouting (Wenzel et al., 2001) and in the reeler mouse dispersion of GC is also identified (Stanfield et al., 1979). In Houser's initial series of 34 cases of surgical HS, mild to marked granule cell dispersion was seen in 38% of specimens with a bilaminar pattern in 18% (Houser et al., 1992) and in a more recent study GCD was identified in 45% in a series of 20 (El Bahh et al., 1999). In the present series of 183 HS cases we identified severe GCD in 40% with a bilaminar pattern in a further 10.3%. In essence, marked organizational abnormalities of the GC layer is present in approximately one half of HS cases. In my study, the lack of correlation between the presence of GCD and either the age of onset of seizures or history of a precipitating event makes it unlikely that this laminar disorganisation is related to an insult disrupting the normally prolonged maturation of the hippocampus, which is known in humans to extend into the first few years of life.

However, I have shown a correlation between the severity of GCD and severity of neuronal loss in HS. This supports findings from previous work by Lurton and colleagues that the extent of GCD correlates with the severity of overall hippocampal neuronal loss (El Bahh et al., 1999). In addition, in Houser's original work, a correlation between GCD and neuronal loss in the hilar polymorph cell layer was noted (Houser et al., 1992). These findings would seem to confirm that the degree of GCD is related to the extent of hippocampal damage suggesting this is a secondary phenomenon occurring in the evolution of HS. Disorganisation of the GC layer was significantly less commonly observed in the mass lesion epilepsy group, which also showed less severe hippocampal neuronal loss, supporting the hypothesis that GCD is more closely

linked to the pathological process of HS rather than a manifestation of severe temporal lobe seizures.

7.13 Stereological quantitation supports enhanced GC neurogenesis in HS

A possible explanation for the common finding of GCD in HS has arisen from studies of animal models of epilepsy. Increased neuronal proliferation in the dentate subgranular zone has been shown with newly generated cells being identified in ectopic locations, such as the hilum and molecular layer of the dentate gyrus (Parent et al., 1997). Neurogenesis of GC is also known to continue into adulthood in humans (Eriksson et al., 1998, Singh Roy et al., 2000) and it is therefore possible that GCD in HS is also a result of enhanced proliferation induced by seizures. There is some evidence to support the hypothesis that proliferation of granule cells occurs in humans with epilepsy, albeit at low turnover rates (Del Bigio, 1999) and expression of immature proteins as nestin has been shown in GC in young patients with temporal lobe epilepsy supportive of ongoing cell genesis in epilepsy (Blumcke et al., 2001).

Quantification of GC number in areas of dispersion in adults is another approach to demonstrate an increased cell number. We estimated mean GC number in 100μm perpendicular columns through the GC and molecular layer using an established stereological three-dimensional cell counting method. This analysis is based on the presumption that GC migrates in a radial, rather than tangential, direction. I showed significantly more GC in areas with maximal dispersion compared to areas with no dispersion and although higher numbers of GC were seen in areas of dispersion compared to control hippocampi, this difference was not significant. Furthermore in only 8/22 cases were actual GC numbers in areas of maximal dispersion higher than the maximal number observed in controls. Comparison of GC number in areas without dispersion to control values did, however, show a significant reduction in number confirming GC loss. It is likely that areas of GCD are also vulnerable to the mechanisms of epilepsy-mediated neuronal loss. Therefore the observation of excess neuronal numbers in these regions of GCD may be of greater significance than initially apparent, and suggestive of neurogenesis masked by

superimposed neuronal loss. Further evidence in support of GC neurogenesis in HS comes from the identification of clusters of cells with apparent morphology of GC in other ectopic locations, such as the hilum or CA3, in 19% of my cases as opposed to only 8% of epilepsy cases with mass lesions. In Houser's studies, nests of hilar GC were identified in 2 of 15 cases and their presence considered to indicate a failure of normal granule GC migration during development rather than an anatomical anomaly (Houser, 1990, Houser et al., 1992). However, in light of the more recent experimental findings, it is perhaps more likely that GC in the hilar region and CA3 in HS also represent newly generated cells in response to seizures as it has been shown that new GC can migrate far from their presumed site of origin (Sherman et al., 2000). In animal models there is considerable interest regarding the potential integration of newly generated GC into the hippocampal network and their functional contribution to hippocampal reorganization and mossy fibre sprouting (Parent et al., 1997 and 1999, Sherman et al., 2000), which may also be of relevance in human hippocampal epileptogenesis.

7.14 Hippocampal neuronal hypertrophy and altered cytoskeleton is more likely to represent an adaptive than dysplastic phenomenon in HS

Cytoskeletal abnormalities have been recently recognized in hilar neurones in HS, including abnormal dendritic ramifications and accumulation of neurofilaments (Blumcke et al., 1999b, Thom et al., 1999a). Their resemblance to the dysplastic neurones of cortical dysplasia has been commented upon, raising once more the possibility that this represents a hippocampal malformation. Hypertrophy and dendritic abnormalities of calbindin positive hilar interneurones has also been shown in HS (Magloczky et al., 2000). In the present study I identified cytoskeletal abnormalities in residual hilar neurones in 55% of HS cases, but in only 17% of mass lesion temporal lobe epilepsy cases, and there was a correlation between their presence and the extent of GCD. This would suggest that hilar cytoskeletal changes more likely reflect an adaptive cellular phenomenon as a result of altered hippocampal circuitry rather than a primary abnormality. Abnormalities in the myeloarchitecture of the hippocampus were also seen in 31% of cases. Abnormal myelinated tracts

within the neocortex have been identified in association with a variety of cortical malformations including polymicrogyria, focal cortical dysplasia, microdysgenesis and the so-called 'driftwood cortex' (dystopic cortical myelinogenesis) (Rebeiz et al., 1968, Thom et al., 2000, Janota and Polkey, 1992). Abnormal bundles of fibres are also reported in experimental cortical malformations and considered to be of relevance in the propagation of paroxysmal activity (Chevassus-au-Louis et al., 1999b). The identification of abnormal myelinated fibres traversing the dentate gyrus in two cases is intriguing and may indicate an underlying malformation. In the remaining cases, I consider the abnormal myeloarchitecture more likely the result of condensation of residual fibres in a sclerotic hippocampus or sprouting of new fibres.

7.15 'Dual pathology' cases may throw light on the pathogenesis of HS

In a proportion of patients with HS, depth electrode recordings and intraoperative electrocorticography may reflect more widespread areas of epileptiform activity involving both mesial and lateral temporal lobe region. From both neuroimaging and neuropathological studies it is well established that HS can occur in combination with a second temporal lobe epileptogenic pathology such as Focal Cortical Dysplasia (FCD) or low grade glio-neuronal tumours (Bruton 1988, Levesqueet al., 1991, Wolf et al., 1993, Raymond et al., 1994, Kuzniecky et al., 1999, Mathern et al., 1997a, Li et al., 1999). There are also occasional reports of isolated hippocampal malformations occurring with HS (Baulac et al., 1998), without HS (Thom et al., 2002a) and structural hippocampal abnormalities on MRI which appear to precede HS (Grunewald et al., 2001, Fernandez et al., 1998). In the two surgical series from Bonn and London (see Section 6.3) dual pathologies were seen in 6.6% and 6.8% of cases. There is some evidence that less severe hippocampal neuronal loss occurs when a second pathology is present (Mathern et al., 1997a, Nakasato et al., 1992) and, of course, in many 'mass lesion-associated' TLE cases, no hippocampal cell loss is perceivable; these cases provide a valuable surgical comparison group for 'pure' HS in neuropathological studies.

In dual pathologies 'kindling' of the hippocampus by the adjacent temporal lobe lesion may be one pathophysiological explanation for the observed neuronal loss and there is some evidence to support that progressive hippocampal atrophy occurs with longer duration of seizures (Fuerst et al., 2001, Kalviainen et al., 1998) although this was not supported by my data. It has been shown, however, that surgical removal of both lesions results in the best post-operative seizure outcome for dual pathologies (Li et al., 1999), implying that each contributes to the genesis of seizures. The not infrequent coincidence of dual temporal lobe pathologies, however, also raises the important question of a common predisposing mal-developmental process for both lesions. Furthermore, in a larger proportion of TLE cases, more subtle microscopic malformations such as microdysgenesis may be identified, as further evidence for an underlying temporal lobe dysgenesis which renders it more vulnerable to seizures, injury and ultimately HS.

7.16 An excess of Cajal-Retzius cells is a common factor in both HS and MD

Cajal-Retzius cells play a critical role in the control of radial directed neuronal migration and hippocampal development. Inhibitory interneurones within the cortex are likely to arise from tangential migration from the ganglionic eminence. The cellular signals and mechanisms controlling tangential cell migration are less well understood than for radial migration (Marin and Rubenstein, 2001). It is known from experimental studies that reelin protein secreted by Cajal-Retzius cells plays an important role in the final stages of radial neuronal migration, including organisation of the layering of the cortical plate and the development of architectonic patterns (Lambert and Goffinet, 2001, Rice and Curren, 2001). Cajal-Retzius cells during development may express reelin (Meyer and Goffinet., 1998), CR or CB (Zecevic and Rakic, 2001), NPY (Uylings and Dellale, 1997) and be GABA-negative or -positive (Zecevic and Rakic, 2001) suggesting that there are several subpopulations of these cells, some of which persist into adulthood (Eriksson et al., 2001). I estimated the number of residual Cajal-Retzius cells in controls and dysplasia cases. Previous quantitative studies in normal cortex have shown higher numbers compared to my data (Garbelli et al., 2001, Guidotti et al., 2000, Impagnatiello et al., 1998). This may be explained by different methodologies

used; in one study Cajal-Retzius cells were identified using calretinin antibody (Garbelli et al., 2001) and in other studies all reelin-positive cells in layer I, including small interneurones in addition to Cajal-Retzius cells, were quantified (Guidotti et al., 2000, Impagnatiello et al., 1998). I demonstrated higher numbers of reelin-positive cells in layer I of MD cases compared to my control group whereas in FCD there was a wide variation between cases. It has been suggested that Cajal-Retzius cell densities may serve as a marker to distinguish cortical dysplasia type (Garbelli et al., 2001), but the wide variation in cell densities I observed within groups make the predictive value of a single measurement less certain. The finding of increased numbers of Cajal-Retzius cell in MD may, however, indicate a potential role of these cells in the development of such lesions via reelin protein, or other factors released by these cells.

I also noted significantly higher numbers of layer I Cajal-Retzius-like cells using calbindin immunohistochemistry in MD cases in the patients with a seizure-free outcome (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 number 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 are 'diluted out' with cortical expansion whilst reelin synthesis is reduced (Zecevic et al., 1999, Meyer et al., 1998, Meyer 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-Retzius cells in mature temporal lobe neocortex may also have a functional role influencing pyramidal cell excitability through molecular layer ramifications (Ferrer et al., 1992).

In hippocampal sclerosis the potential cellular signals involved in the mechanisms of dispersion of granule cells remains to be identified; neurotrophin over-expression during seizures has been suggested as one possibility (Lurton et al., 1997, 1998). Reelin and p35 are key proteins regulating normal neuronal migration and laminar organization during mammalian cortical development. An interesting study has shown an abnormal persistence of calretinin-labelled Cajal-Retzius cells in human HS (Blumcke et al., 1996a, 1999a), including an excess of cells in the dentate gyrus molecular layer. Although this observation has not been replicated in all studies (Magloczky et al., 2000) a possible functional role of Cajal-Retzius cells and reelin in the architectural abnormalities of the GC layer in HS would seem plausible. I did not find, however, any relationship between the number of reelin-immunopositive Cajal-Retzius cells in HS cases with and without severe dispersion. I also failed to demonstrate increased expression of p35 in cases with GCD (data not shown). It is considered likely that reelin also has an essential physiological role in the adult brain contributing to the formation of neuronal circuits (Rice et al., 2001). In my study there was no correlation between reelin-positive cell number and the width of Timm's mossy fibre sprouting. There were, however, significantly more reelin-positive cells in the hippocampi of epilepsy patients compared to controls and patients with extra-hippocampal lesions confirming an apparent increase in the number of Cajal-Retzius cells in HS. As increased numbers of reelin-positive cells have been identified in layer I of the cortex in cortical malformations as polymicrogyria (Eriksson et al., 2001) and microdysgenesis (Garbelli et al., 2001, Thom et al., 2003) the likely explanation is that excess Cajal-Retzius cells in association with HS are also a hallmark or harbinger of underlying hippocampal malformation. Alternatively, Cajal-Retzius cells, as a 'dynamic' cell population (Super et al., 1997), could be recruited into the area of hippocampal injury as a secondary phenomenon. However, in my cases there was no relationship between the number of these cells and the duration of epilepsy or the severity of neuronal loss to further support this hypothesis.

7.17 A reduction in the number of interneurones in region of cortical dysplasia is a common observation

Focal cortical abnormalities such as FCD and MD are commonly identified in resections from patients undergoing therapeutic surgery for intractable

seizures (see Palmini and Luders, 2001, Taylor et al., 1971, Tassi et al., 2002). These lesions are sporadic and their precise origin and the cellular mechanisms rendering them epileptogenic are largely unknown. The pathological features, particularly the disordered cortical lamination, suggest that they represent disorders of radial neuronal migration. The majority of cortical inhibitory interneurones in the mammalian cortex are considered to arise from the ganglionic eminence via tangential rather than radial migration (Anderson et al., 2001, Lavdas et al., 1999, Marin and Rubenstein, 2001, Wichterle et al., 1999). Therefore studies of the laminar distribution, integration and morphology of interneuronal populations in FCD and MD might provide insight into the evolution of these lesions during development. In addition, although studies of dysplastic neurons in FCD suggest that they are abnormally excitable (Crino et al., 2001, Kerfoot et al., 1999, Najm et al., 2000, Spreafico et al., 1998) local deficits in inhibitory neurones could also contribute to their epileptogenicity.

Interneurones in the cortex are highly diverse, their phenotype influenced by their local afferent connections in addition to developmental factors (Gonzalez-Albo et al., 2001). The calcium binding proteins PV, CB and CR identify distinct subsets of interneurones present in all regions of the mammalian cortex (Gonzalez-Albo et al., 2001, Conde et al., 1994). In the present study I investigated the distribution of these neurones in a series of FCD and MD cases. A common finding in the majority of FCD cases was their depletion in the region of dysplasia. Loss of these interneurones was less apparent in MD cases on qualitative assessment alone, but was supported by my quantitative analysis of CB-positive cells (see Section 7.18) (Thom et al, 2000). Depletion of inhibitory interneurons has been noted in FCD in previous reports (Ferrer et al., 1992, and 1994, Garbelli et al., 1999, Spreafico et al., 1998) and may partly explain the excitatory overbalance in these lesions. This local deficit of interneurones may represent a primary failure of tangential migration or of subsequent differentiation within the region of dysplasia. The identification of normal interneuronal populations in the adjacent cortex confirms, however, that normal migration into these marginal regions has occurred. An alternative explanation is that selective secondary cell loss of interneurones has occurred, supported by similar loss of inhibitory neurones observed adjacent to other epileptogenic lesions, such as tumours or HS (Ferrer et al., 1994, Marco et al.,

1996). Such an acquired process may explain the heterogeneity of interneuronal patterns I observed both within and between cases.

7.18 Abnormal morphology of calbindin interneurones is a common observation in severe focal dysplasias of Taylor type

Abnormal morphology of some inhibitory interneurones was also a striking observation. Cytomegalic, dysplastic neurons and balloon cells, pathognomonic of FCD, are considered to represent abnormally differentiated, immature cells (Crino et al., 1997). Hypertrophy of cortical and white matter neurons and glia (Bothwell et al., 2001, Kendall et al., 1999, Marin-Padilla et al., 2002, Thom et al., 1999a) in human epilepsy tissue in the absence of malformation has also been argued to reflect altered cell metabolism. Abnormal, hypertrophic CB-positive cells were seen in the majority of FCD cases in the present series, involving both the frontal and temporal lobes in all age groups, and prominent multipolar cells were seen in MD cases. In some cases, a proximity and morphological similarity of these hypertrophic CB positive cells to balloon cells in the white matter was seen as previously reported (Garbelli et al., 1999, Spreafico et al., 1998). It is known from experimental studies that induction of CB can occur in glial cells (Mattison et al., 1995). Hypertrophic CB-positive cells and interneurones have been reported in epilepsy tissue without malformation (Magloczky et al., 2000, Marin-Padilla et al., 2002), and in a rat model of microgyria an increase in the number of CB-positive occurs (Schwarz et al., 2000). These observations suggest that CB expression can be induced or increased in response to seizures and that morphological changes in interneurones may take place. Although it is possible that the bizarre and abnormally localised CB-positive in the present series represent a primary failure of normal neuronal migration and differentiation, it is more plausible that this is induced expression of CB with secondary changes in cell morphology as a consequence of the seizures.

I also studied in detail, using quantitative methods, the interneuronal populations in MD cases which showed abnormal cortical myelination in the superficial cortical layers. Calbindin D-28-K immunostaining labels a subset of cortical GABA-ergic inhibitory interneurones and may weakly stain some

pyramidal cells; over 90% of these cells are located in layers I and II of the cerebral cortex (see Section 3.3.4.2). Other potent inhibitory cortical neurones include those that label with parvalbumin, which are more uniformly distributed between cortical layers. In view of both the primary malformative and possible secondary pathologies (neuronal loss and gliosis), mainly involving the superficial cortical layers, I carried out a quantitative evaluation of calbindi- positive cells. Quantitative analysis has not been employed in previous studies of calbindin cell populations in epilepsy, which has been a criticism (Sutula et al., 1998), but no consistent patterns have emerged to implicate alterations of this neuronal subpopulation as a common cellular basis for the epilepsy. Ferrer described a focal decrease in calbindin positivity in gliotic temporal lobe adjacent to hippocampal sclerosis and abnormal nodules of calbindi- positive cells in the molecular layer in microdysgenesis-like malformations (Ferrer et al., 1994). A reduction in calbindin inhibitory interneurones was reported in focal cortical dysplasia and interpreted as a failure of commitment of neuroblasts to become interneurones (Spreafico et al., 1998) and, in another reported case, loss of inhibitory neurones in areas of necrosis adjacent to cortical dysplasia was considered to have influenced the severity of seizures (Ferrer et al., 1992).

The distribution and morphology of calbindin-positive neurones in the cases of microdysgenesis with abnormal myelin patterns appeared as described in normal temporal lobe (Ferrer et al., 1992) and preservation of calbindin positive cells was confirmed by the quantitative analysis, with comparable cortical densities present in temporal lobes from patients with TLE but without microdysgenesis. This was despite a reduction in total (NeuN positive) cortical neuronal densities in the microdysgenesis group with abnormal myelin and is analogous to observations in hippocampal sclerosis which have shown preservation of calbindin interneurones and their apparent resistance to seizure mediated cell loss (Sloviter et al., 1991).

I also observed increased numbers of multipolar '*neurogliaform*' calbindin positive cells, mainly in the deeper laminae of the microdysgenetic cortex, which were only occasionally present in the control cases. These cells are morphologically similar to small multipolar local circuit neurones identified in calbindin studies of normal temporal cortex in man (Friede, 1989) and primate cortex (Conde et al., 1994) rather than astrocytes. Although calbindin has been

reported to be expressed by astrocytes following experimental brain injury, in the present cases few multipolar neurogliaform cells were present in the molecular layer and none in the white matter where the gliosis was also severe. These cells also bear some similarity to the increased numbers of large multipolar calbindin cells reported above in focal cortical dysplasia which were also considered not to be astrocytic in nature (Spreafico et al., 1998). In the present study double labelling with calbindin and GFAP failed to demonstrate co-expression of GFAP by these cells which also supports a neuronal rather than glial lineage. The excessive numbers of multipolar neurogliaform observed may represent an intrinsic part of the microdysgenetic malformation or a secondary adaptation of local circuit neurones in response to chronic seizures.

7.19 Alteration of NPY fibre plexus in focal dysplasias, similar to that observed in HS, is likely to be an adaptive anti-convulsant mechanism

NPY is considered to be a powerful endogenous anticonvulsant (Furtinger et al., 2001, Vezzani et al., 1999a). In animal models, NPY expression is enhanced in the hippocampus (Schwarzer et al., 1995), entorhinal cortex and temporal cortex following seizures, promoted by BDNF secretion (Vezzani et al., 1999b). Study of NPY expression in human epilepsy tissue has been restricted to the hippocampus. In hippocampal sclerosis loss of hilar NPY interneurones (DeLanerolle et al., 1989, Mathern et al., 1995b, Sundstrom et al., 2001) and increased length of NPY fibres has been shown compared to autopsy controls (Furtinger et al., 2001), particularly involving the molecular layer of the dentate gyrus (De Lanerolle et al., 1989). This is considered to reflect adaptive NPY expression to counteract excess excitation from mossy fibres. In normal cortex, immunohistochemistry studies of NPY have shown a stereotypical laminar distribution of positive interneurones in all cortical regions in different age groups (Blinkenberg et al., 1990, Brene et al., 1989, Gonzalez-Albo et al., 2001, Horung et al., 1992, Terenghi et al., 1987). A predominance of cortical over white matter neurones has been shown in frontal and temporal cortical regions compared to parieto-occipital cortex (Horung et al., 1992) and quantitative biochemical studies have suggested a variation in NPY within frontal cortical regions (Brene et al., 1989, Dawbarn et al., 1984). In parallel

with the studies in human hippocampal sclerosis I have observed a significant increase in the NPY fibre plexus involving the superficial cortex in MD and cortex adjacent to FCD compared to our control group. This was observed in both frontal and temporal cortical resections and in all age groups. The origin of these cortical fibres is likely to be from local NPY expressing neurones (Horung et al., 1992) and in some cases increased numbers of NPY-positive and dysplastic cortical neurones were noted. I consider these changes to represent adaptive phenomena to counteract excitability in cortical layer I-II, which. in the present patients with refractive epilepsy, has however been of limited clinical benefit.

7.20 Significance of neuronal enlargement in epilepsy in both malformations and hippocampal sclerosis

Significant enlargement of cortical and white matter neurones has been noted in studies of TLE and HS (Bothwell et al., 2001), as well as hypertrophy of glial cells in the temporal lobe white matter, (Kendal et al., 1999, Krishnan et al., 1994) and residual neurones in the hippocampal hilum in HS (Blumcke et al., 1999, Thom et al., 1999) including specific calbindin- positive hilar interneurones (Magloczky et al., 2000). Neuronal cytomegaly and dysplasia are characteristic and pathognomonic features of FCD and are considered to represent undifferentiated or immature cell phenotypes (Crino et al., 2002, Mischel et al., 1995). The cellular enlargement observed in HS has generally been considered to represent an adaptive response to altered metabolic demands. For example, these neurones may support an expanded dendritic arborisation and somal size may also change in response to altered afferent and efferent connections in the reorganised circuitry of AHS. Although it cannot be ruled out that these hypertrophic cells represent an immature cell type (Magloczky et al 2000) regardless of the cause, their enlargement may lead to an overestimation of neuronal densities in quantitative studies of microdysgenesis (Bothwell et al., 2001). A recent study of the cellular pathology of amygdala neurones in TLE using confocal microscopy, however, demonstrated a reduction in neuronal soma size (Aliashkevich et al., 2003).

7.21 Current position : Association of temporal lobe microdysgenesis and HS

In addition to pyramidal cells, a prominent component of small (<10 micron diameter) white matter interneurones and non-pyramidal cells are also seen in TLE using NeuN or MAP2 immunostaining. Subsets of these neurones label with antibodies for NPY, calbindin and calretinin and other antibodies indicating their likely functional heterogeneity. The excessive numbers of all white matter neurones in TLE may represent enhanced survival of subplate neurones, true 'heterotopic' cortical neurones or even newly generated neurones. Any functional significance of these cells in relation to the seizures remains to be determined and they may merely be the harbinger of an overlying cortical malformation. Although more extensive clinico-pathological studies are required my studies and others suggest there is some evidence that the presence of white matter microdysgenetic features in association with HS correlates with a marginally better post-operative outcome than HS alone (Hardiman et al., 1989, Thom et al., 2001, Choi et al.,1999).

Using calbindin, calretinin and reelin immunohistochemistry this study and others have also confirmed an excess of Cajal-Retzius-like cells in temporal lobe microdysgenetic malformations (Garbelli et al., 2001) and neocortex adjacent to HS (Thom et al., 2001) compared to controls. In the context of the previously discussed observation of excess Cajal-Retzius cells in HS specimens (Blumcke et al., 1996, 1999) this may suggest a common mechanism involving the reelin pathway linking HS and temporal lobe microdysgenesis. Reelin protein is known to be present in the mature cortex after its developmental functions are fulfilled with ongoing functional roles likely to govern formation of neuronal circuits (Rice et al., 2001). The excess Cajal-Retzius cells observed in TLE cases, as well as highlighting a potential developmental abnormality, may also be be contributing to abnormal neuronal connectivity and plasticity in epilepsy.

7.22 Hypothesis proposed for the molecular pathogenesis of the cytoarchitectural lesions observed

In recent years major advances in the understanding of molecular events controlling normal cortical development have been made, largely through the

study of diffuse and severe malformations in epilepsy such as lissencephalies and grey matter heterotopia (Gleeson and Walsh, 2000). In many cortical malformations single gene mutations are responsible. Although FCD and MD are generally considered to represent disorders of neuronal migration, proliferationand differentiation, their genetic basis and timing during development are unknown. They are sporadic lesions and representative animal models are lacking. It has recently been suggested that these focal malformations result following somatic mutations in developmental genes occurring in progenitor cells during development (Crino et al., 2002). In view of the pathological features of FCD, MD and HS, likely pathways involved include those controlling cortical layering and neuronal morphology

Neurones in the outer iso-cortical layers (II-IV) are the last to migrate from the sub-ventricular proliferation zone and have different connectivities in mature cortex compared to layer V-VI neurones with an emphasis on local cortico-cortical projections (Tarabykin et al., 2001, Aboitiz et al., 2002, Rice and Curran 1999). Normal cortical lamination, involving neuronal radial migration and cell arrest, is in part controlled by reelin protein (Gleeson and Walsh 2000, Bar and Goffinet, 1999) mediated via VLDLR, apoER2, integrin and cadherin cell receptors (D'Arcangelo et al., 1999, Senzaki et al., 1999), proteins downstream in the reelin signalling pathway, including cdk5, p35 and dab1 (Chae et al., 1997, Hammond et al., 2001) and regulators of these factors e.g., Brn-1&2 (McEvilly et al., 2002). Animal models such as the reeler mouse (lacking reelin protein) and cdk5/p35 knockouts show varying degrees of abnormal and inverted cortical lamination and, of particular relevance, disturbed myelo-architecture particularly involving the superficial layers (Gleeson and Walsh, 2000, Chae et al., 1997). Abnormal expression of proteins in this pathway or other cell-molecular signals controlling neuronal motility and radial migration e.g., Emx2 (Gangemi et al., 2001), are likely candidates in the pathogenesis of microdysgenesis and FCD with resultant dyslamination and subsequent abnormal local neuronal connections.

Neuronal size and shape are dependent on the cytoskeleton and a reflection of cell connectivity as well as differentiation. Immature cytoskeletal filaments, such as nestin and MAP1, have been demonstrated in dysplastic neurones in FCD (Crino et al., 1997, Yamanouchi et al., 1996), suggesting a failure in cell maturation. Giant neurones, similar to those in FCD, also occur in tuberous

sclerosis in relation to abnormal expression of hamartin or tuberin growth-regulating proteins (Johnson et al., 1999) ; it has been suggested these proteins are also involved in the pathogenesis of FCD (Crino et al., 2002). Abnormal metabolism and phosphorylation of neurofilaments have been demonstrated with immunohistochemistry in dysplastic neurones in FCD (Taylor et al., 2001), MD (Crino et al., 2002) and HS (Thom et al., 2002b). Abnormal neuronal morphology is associated with p35/cdk5 dysfunction in experimental systems (Rashid et al., 2001) and may relate to dysregulation of neurofilament phosphorylation. Abnormal orientation and shape of neuronal somata occurs in the p35 mutant (Chae et al., 1997). Study of the expression of these candidate genes in dysmorphic neurones in FCD, MD and HS may disclose the cause of this pathological feature.

Heterotopic masses of cortical neurones in the white matter are seen in patients with DCX and Filamin 1 gene mutations in patients with epilepsy (Gleeson and Walsh, 2000, Fox et al., 1998, Ross and Walsh, 2001). As I have discussed, it is not certain whether the excess of single white matter neurones in microdysgenesis and FCD represents residual subplate neurones or arrested cortical neurones. Therefore genes regulating radial neuronal migration, and those influencing cell survival and programmed cell death (e.g., caspase-3), are likely to be important in the pathogenesis of white matter neuronal ectopia. In addition, study of the co-expression of mRNAs indicative of cortical pyramidal neuronal lineage (e.g., Emx1) versus subplate lineage (e.g., Tbr1) (Hevner et al., 2001) in isolated white matter neurones may establish the phenotype and origin of these cells.

Cajal-Retzius cells, the early pioneer cells in the marginal zone (the future cortical layer I) synthesise reelin and LIS1 protein (which interacts with microtubules influencing the dynamics of neuronal motility) (Meyer et al., 1999). They play a pivotal role in orchestrating cortical architecture and connectivity during development, thereafter their numbers declining with maturation. In the hippocampus Cajal-Retzius cells are essential for normal afferent connections with adjacent entorhinal cortex. Increased survival of Cajal-Retzius cells is observed in the hippocampus of the reeler mouse and associated with abnormal hippocampal development (Coulin et al., 2001). Hippocampal malformations are also seen in human RELN gene mutations (Hong et al., 2000). The identification of excess Cajal-Retzius cells in human

HS and microdygenesis in this study and by other groups and our observation of abnormal Cajal-Retzius cells populations in hippocampal malformations in epilepsy without HS (Thom et al., 2002a) is further evidence to support a functional disturbance of these cells in temporal lobe epilepsy.

Granule cell ectopia of the dentate gyrus are occasionally documented in the context of widespread cortical malformations in the absence of HS (Harding and Thom, 2001). Similar abnormalities in animal models of epilepsy are linked to enhanced granule cell neurogenesis (Parent et al., 1997). My quantitative analysis of granule cells and the demonstration of nestin-positive neuronal precursor cells (Blumcke et al., 2001) also support enhanced neurogenesis occurring in human HS. Abnormal expression of genes controlling granule cell migration and positioning e.g., adhesion molecules, ApoER2, VLDLR, cdk5, p35 and cell chemokines (Lu et al., 2002, Gebhardt et al., 2002), differentiation e.g, BETAD/neuroD (Liu et al., 2000), DNA replication and proliferation (Stoeber et al., 2001) are likely to be demonstrated in such ectopic granule cell populations based on this hypothesis. Newly generated granule cells are also likely to play a role in the abnormal hippocampal function in epilepsy.

An alternative, albeit less likely hypothesis, is that the cortical cyto-architectural abnormalities observed in these pathologies represent adaptive or secondary cellular changes as a result of seizures. Pathological changes reminiscent of FCD and MD, including dyslamination and neuronal hypertrophy ('architectural dysplasias'), have been demonstrated in close association with chronic encephalitis (Hart et al., 1998) and post-traumatic lesions (Marin-Padilla et al., 2002) as well as low grade tumours such as dysembryoplastic neuroepithelial tumours (DNT) (Honavar et al., 1999) in patients with epilepsy. These changes may reflect excessive metabolic demands and altered connectivity occurring as a result of seizures on these local neurones. The resolution of these issues, i.e., distinguishing primary developmental phenomena from secondary compensatory cellular mechanisms, is fundamental to our understanding of the origins of such abnormalities and their temporal relationship to seizure activity.

Using quantitative and immunohistochemical methods I have been able to demonstrate and confirm cytoarchitectural changes in focal dysplasias and hippocampal sclerosis in patients with epilepsy that are likely to have developmental origins. Such an analysis allows a more exact neuropathological definition of these lesions to be made. This in turn will allow correlation with clinical investigations, including functional imaging, to be carried out in the future which may lead to the *in-vivo* detection of these hitherto microscopic malformations. This in turn may allow a more exact information regarding the prognosis of these lesions in epilepsy to emerge which may direct future surgical strategies. Future investigations of the molecular neurobiology of isolated cell populations from these lesions may identify abnormalities of expression of genes critical to normal cortical development.

8 Conclusions

Studies of microscopic maiformations in temporal lobe epilepsy

8.1 Study I

In my review of a large series of temporal lobectomy specimens from adult patients with intractable temporal lobe epilepsy I have confirmed that the commonest lesion is hippocampal sclerosis (62% of cases) with smaller numbers of cortical malformations (4.6% of cases, excluding microdysgenesis). In 6.8% of cases a dual pathology is present. An overall seizure free outcome is achieved in 75% of patients and in 78% of patients with hippocampal sclerosis.

8.2 Study II

The application of stereological quantitative techniques using NeuN immunohistochemistry has confirmed higher mean white matter neuronal densities in temporal lobe epilepsy cases than in controls. There appears to be no significant variation in the distribution of white matter neurones in different anatomical regions of the temporal lobe. Small interneurones in the white matter make up approximately half of the total neurones and may be of functional significance in epilepsy. The white matter neuronal densities are independent of the degree of gliosis suggesting the observed increase in density is a real phenomenon and independent of any temporal lobe atrophy occurring in the context of epilepsy. There is no correlation between white matter neuronal densities and layer I microdysgenetic features including layer I neuronal densities, Cajal-Retzius cell number and neuronal clustering . This suggests that microdysgenesis with white matter heterotopia is a distinct malformation from architectural dysplasias involving layer I.

8.3 Study III

In conclusion to this study I have documented the presence of abnormal cortical myelinated fibre tracts within temporal lobe showing other features of microdysgenesis in patients with epilepsy. We postulate that this is part of the

primary malformation and may have a role in seizure propagation and in exaggerating the secondary effects of epilepsy observed in these cases including laminar nerve cell loss. The density of calbindin positive neurones, which predominate in layer II, did not appear to be affected although prominent numbers of calbindin positive multipolar *neurogliaform* cells were present in the microdysgenetic cortex.

8.4 Study IV

Neuropathological studies of a large series of resections with hippocampal sclerosis confirmed frequent cytoarchitectural abnormalities in addition to the typical patterns of neuronal loss. In surgical specimens of HS we are limited to studying an already established disease process and the confirmation of an underlying hippocampal maldevelopment becomes problematic. With our large patient group I have, however, been able to study a wide spectrum of cases, representing varying stages of severity or snapshots in time in the progression of HS. My analysis has shown that the degree of dispersion of granule cells is closely linked to the severity of neuronal loss but not to clinical parameters such as the age of onset of seizures. Furthermore, quantitation of granule cell number suggests that dispersion may a consequence of excessive neurogenesis in epilepsy. Neuronal hypertrophy observed in hippocampal sclerosis is also closely linked to the severity of neuronal loss and may be a secondary phenomenon rather than a dysplastic cell change.

8.5 Study V

Using reelin and calbindin immunohistochemistry I have demonstrated increased numbers of Cajal-Retzius cells in microdysgenesis-like malformations but not in Taylor-type cortical dysplasia. Increased Cajal-Retzius cells in hippocampal sclerosis compared to controls has also been shown which, as well as being a potential marker for these diseases, may indicate a common developmental link between microdysgenesis and hippcoampal sclerosis.

8.6 Study VI

I have identified distinctive alterations in inhibitory neuronal populations in FCD and MD which I consider more likely to be adaptive phenomena as a result of seizures rather than a primary manifestation of the malformation. Loss of inhibitory interneurones is a common observation in focal cortical dysplasia and morphologically abnormal calbindin positive cells were a typical finding.

8.7 Future direction for studies of microscopic malformations in epilepsy following on from the findings in presented studies

1. Clarification of terminology

It is widely recognised that a unifying nomenclature for microscopic malformations and lesional pathologies identified in epilepsy surgical resections is required. At a recent European workshop in Aachen (October 2002) on Focal Cortical Dysplasia, chaired by Ingmar Blumcke, it was resolved that a uniform classifcation should be adopted based on the ILAE panel (Cleveland 2000) but that further clinico-pathological clarification is required for those lesions not specifically recognised by this panel, for example excess of white matter neurones. The adoption of a universal classification would allow the development of a European epilepsy register for tissue banking and to co-ordinate pathology based research and clarify the response of these lesions to surgery.

Based on the findings in the current study I propose the following classification scheme :

- Focal cortical dysplasia
  - Type I (with giant neurones)
  - Type II (with dysplastic neurones and giant neurones)
  - Type III (with dysplastic neurones, giant neurones and balloon cells)- This is equivalent to Taylor type FCD

- Microdysgenesis
  - Type I Laminar architectural dysplasia (without giant neurones, dysplastic cells or balloon cells)
  - Type II Excess white matter neurones (> 2,300/mm3)
  - Type III Layer I abnormalities; excessive neuronal clusters/Cajal-Retzius cells/abnormal myelination

2. The application of image analysis systems in the quantitative analysis of focal and microscopic malformations and future correlations with advanced MRI methods

Stereological cell counting techniques are time consuming and tedious and not practical tools for use in a diagnostic laboratory. I have demonstrated that using an image analysis system, measurements, for example of white matter neuronal numbers, can be made in a fraction of the time used for cell counting which correlate with stereological cell counts. Modern sophisticated image analysis systems as the Histometrix system (Kinetic Imaging) also use design based stereological tools. This is the system that I plan to use in future studies to look at larger numbers of temporal lobe lesions. It will allow me to simultaneously measure several cytoarchitectural features in malformations, including neuronal size as well as number, and provide information on layer specific distribution of neurones. Furthermore this information can be reproduced in a three dimensional format to produce a 'map' of the distribution of cyto-architectural abnormalities within the temporal lobe. Such information can be correlated with sophisticated and advanced MRI techniques as diffusion weighted imaging. This may allow pre-operative identification of previously regarded 'MRI-occult' dysplasias.

3. Molecular studies in temporal lobe malformations.

Recent developments in the molecular-genetic biology of focal lesions in epilepsy are likely to further the analysis, classification , aetiology and

prognosis of these entities. It is generally agreed that it is paramount that snap-frozen tissue is banked from each epilepsy specimen as a matter for research and future diagnosis as further tests become available. Studies of the molecular pathogenesis of microscopic lesions in epilepsy will need to integrate several approaches including the development of animal models which closely resemble the human abnormality. In vitro electrophysiology should be used to study single cell recordings in these lesions. As in most of these lesions a family history is not present, positional cloning methods to identify candidate genes will not be an appropriate strategy.

Another approach would be to quantify gene expression in the cyto-architectural abnormalities which may identify those likely to be linked to the malformation. As the cellular phenotype within these lesions is typically diverse, microdissection of single and 'pure' cell populations, identified with appropriate immunohistochemical markers, and application of microarray technology is an ideal approach to study the expression of mRNA in these lesions. This may allow the identification of mRNA and in turn protein expression that is linked to the development and epileptogenesis of these lesions. This may in turn lead to specific and targeted therapeutic interventions that may treat or prevent these malformations. Likely genes to be involved in focal dysplasias include those involved in neuronal migration, cortical layering, neuronal morphology and cell survival as outlined in section 7.22. Similarly, identification and microdisseection of abnormal Cajal-Retzius populations demonstrated in focal dysplasias may identify a functional impairment of gene expression involving the reelin pathway in these cells.

Laser capture microdissection (LCM) of tissue sections has been used increasingly for the isolation of homogeneous, morphologically identified cell populations, thus overcoming the obstacle of tissue complexity. Gene expression profiles from pure cell populations can be obtained by integrating techniques of LCM and T7-based RNA amplification with cDNA microarrays (Luo et al., 1999). Quantification of relative gene expression will provide clues to the mechanisms which alter cortical cyto-architecture. Because of the diverse cell phenotypes present in cortical malformations single cell molecular biology is an attractive and appropriate strategy to study gene expression in specific neuronal populations in delineated anatomical subregions of interest. Microarray techniques allow the simultaneous assay of the expression of

multiple mRNAs in these defined cell populations to identify which genes may be responsible for the pathological features we have described.

4. Proposed studies of granule cell neurogenesis in hippocampal sclerosis.

My studies suggest an increased in granule cell number in HS cases which may result from enhance neurogenesis. The excitatory glutamatergic granule cells (GC) of the hippocampal dentate gyrus are presumed to play a central role in the genesis of spontaneous seizures in temporal lobe epilepsy through re-organisation of the mossy fibre axons of the GC. Recent studies have confirmed regeneration in normal adult human GC and neuronal progenitor cells have been isolated from the dentate gyrus. This pool of precursor cells may have important physiological roles but it is conceivable that in epilepsy, stimulated by seizures, an increased rate of GC neurogenesis occurs leading to abnormal hippocampal reorganization and cell localisation, contributing to hippocampal hyperexcitability. We also know from animal studies that there is considerable potential for adaptation and plasticity in GC. For example induction of inhibitory cellular mechanisms by increasing basal expression of GAD has been shown. In human surgical HS tissue altered neurotransmitter receptor profiles on GC have been shown, including upregulation of $GABA_A$ and NMDA 2A&B receptors. It is plausible that such plasticity of GC may be enhanced in newly generated cells. Future studies will therfore be directed to investigate if newly generated GC can be identified in surgical HS specimens, and if their neurotransmitter and receptor profiles differ markedly from pre-existing GC, contributing to hippocampal epileptogenesis. Studies investigating
abnormal expression of genes controlling granule cell migration and positioning (eg. adhesion molecules, ApoER2, VLDLR, cdk5, p35 and cell chemokines), differentiation (eg BETAD/neuroD), DNA replication and proliferation are proposed on ectopic granule cells using microarray methodology. Newly generated granule cells, if demonstrated, are also likely to play a role in the abnormal hippocampal function in epilepsy by interaction into local neuronal networks.

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1. Thom M, Sisodiya S, Harkness W, Scaravilli F.
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13 Appendices

Appendix 1 Engel system for classification of post operative outcome

Classification of postoperative outcome used in this study (Engel, 1993)

I. Class I. Free of disabling seizures
   a. Completely seizure free since surgery
   b. Non disabling simple partial seizures only since surgery
   c. Some disabling seizures after surgery, but free of disabling seizures for at least 2 years
   d. Generalised convulsion with antiepileptic drug withdrawal only

II. Class II. Rare disabling seizures (" almost seizure free")
   a. Initially free of disabling seizures but has rare seizures now
   b. Rare disabling seizures since surgery
   c. More than rare disabling seizures after surgery, but rare seizures for at least 2 years
   d. Nocturnal seizures only

III. Worthwhile improvement
   a. Worthwhile seizure reduction
   b. Prolonged seizure-free intervals amounting to greater than half the follow-up period, but not less than 2 years

IV. No worthwhile improvement
   a. Significant seizure reduction
   b. No appreciable change
   c. Seizures worse

Appendix 2 : Values for white matter neuronal densities (study II)

	White matter neuronal densities (ND) /mm3 Nissl	White matter neuronal densities (ND) / mm3 NeuN	White matter neuronal densities (cells less than 10μm diameter) NeuN (%of total)	White matter neuronal densities (cells more than 10μm diameter) NeuN
1		**2016**	**944 (47%)**	**1072**
2	**1406**	**1997**	**1092 (54%)**	**904**
3	**1144**	**3219**	**1473 (45%)**	**1817**
4	**1124**	**2078**	**955 (46%)**	**1122**
5	**444**	**1212**	**469 (39%)**	**740**
6	**873**	**2418**	**896 (37%)**	**1521**
7		**2330**	**779 (33%)**	**1550**
8		**2174**	**1488 (68%)**	**693**
9		**1340**	**604 (45%)**	**735**
10		**1487**	**761 (51%)**	**724**
11	**1239**	**2077**	**892 (43%)**	**1185**
12	**895**	**2243**	**882 (39%)**	**1360**
13	**958**	**1974**	**992 (57%)**	**679**
14		**1938**	**817 (44%)**	**1028**
15	**1604**	**3246**	**1389 (42%)**	**1845**
16	**491**	**2076**	**1010 (49%)**	**1065**
17	**966**	**1611**	**1058 (68%)**	**504**
18	**1751**	**3282**	**2284 (65%)**	**1205**
19		**3448**	**1593 (46%)**	**1855**
20		**2144**	**1050 (48%)**	**1093**
21		**2649**	**975 (37%)**	**1673**
22		**1741**	**789 (45%)**	**951**
23		**1624**	**840 (51%)**	**784**
24		-	-	-
25	**999**	**1461**	**650 (44%)**	**810**
26		**1689**	**891 (52%)**	**797**
27	**960**	**3047**	**1490 (49%)**	**1556**
28	**710**	**1630**	**659 (40%)**	**971**
29	**1044**	**1959**	**884 (46%)**	**1015**
30	**637**	**2141**	**970 (45%)**	**1170**
31	**951**	**2676**	**1538 (57%)**	**1137**
Mean	**1010**	**2164**	**1040**	**1118**
Min	**444**	**1212**	**469**	**504**
Max	**1751**	**3448**	**2284**	**1855**
Sd	**343**	**604**	**380**	**376**

In case 24 quantitation not possible due to consistent artefacts in cut sections

Appendix 2 (Part ii). Results of white matter neuronal density analysis on further 50 cases using both three dimensional (3d) and two dimensional (2d) cell counting techniques.

Case	Case Number	White matter neuronal density (3d) / mm3	White matter neuronal number (2d) / 0.5mm2
1	13-97	2328	32
2	65-97	1043	18
3	187-97	1147	22
4	201-97	2393	44
5	356-97	2187	44
6	473-97	2717	41
7	537-97	3707	61
8	544-97	777	25
9	672-97	3953	55
10	728-97	3484	35
11	917-97	1788	23
12	932-97	3628	53
13	1008-97	2352	17
14	1027-97	3119	41
15	1050-97	2626	23
16	1066-97	3084	39
17	1084-97	2204	28
18	1115-97	1969	22
19	1169-97	3086	25
20	1179-97	1923	30
21	24-98	1772	25
22	98-98	1487	30
23	150-98	1461	24
24	271-98	1666	13
25	367-98	-	-
26	348-98	1250	20
27	350-98	3225	29
28	521-98	1818	34
29	529-98	2931	19
30	571-98	2068	20
31	593-98	1981	28
32	603-98	-	-
33	626-98	1413	15
34	637-98	2545	30
35	685-98	1574	14
36	706-98	2111	34
37	714-98	-	-
38	766-98	3008	38
39	888-98	3272	48
40	924-98	1684	14
41	947-98	1588	24

42	968-98	869	15
43	619-98	3450	60
44	25-96	2405	25
45	60-96	1290	22
46	385-96	-	-
47	296-96	2121	17
48	486-96	3050	27
49	635-96	2909	40
50	834-96	3302	39
51	921-96	1686	16
52	959-96	-	-
53	1088-96	-	-
54	1087-96	2846	43
55	1110-96	1110	32
Min Max MEAN SD		777 3953 2318.2 821	13 61 30 12.3

Artefacts in the sections (-) prevented quantitative analysis.

Appendix 3 : Values for white matter neuronal densities in anterior versus posterior temporal lobe

Case	NeuN white matter neuronal densities / mm3 (Mid temporal lobe) Total ND (Small ND)	NeuN white matter neuronal densities / mm3 (Posterior temporal lobe) Total ND (Small ND)	NeuN white matter neuronal densities / mm3 (Anterior temporal lobe) Total ND (Small ND)
1	**2258 (1129)**	**2258 (1129)**	**2767 (1081)**
2	**2966 (1780)**	**2055 (944)**	**1544 (952)**
3	**3916 (1843)**	**3916 (1843)**	**3221 (1658)**
4	**1413 (724)**	**2626 (959)**	**1935 (1612)**
5	**1718 (684)**	**1178 (684)**	**1186 (0)**
6	**2426 (710)**	**2426 (710)**	**2500 (1111)**
7	**2584 (1123)**	**1379 (172)**	**2444 (666)**
8	**2943 (1733)**	**1943 (133)**	**1470 (588)**
9	**1145 (610)**	**1388 (388)**	**1025 (320)**
10	**855 (427)**	**1379 (862)**	**2083 (833)**
11	**2133 (400)**	**2133 (400)**	**1830 (653)**
12	**2180 (995)**	**2049 (310)**	**2978 (638)**
13	**1601 (922)**	**2674 (1162)**	**2051 (512)**
14	**2511 (684)**	**1129 (564)**	**3119 (1058)**
15	**2837 (1280)**	**2837 (1280)**	**2272 (1590)**
16	**2111 (637)**	**2111 (637)**	**2635 (1148)**
17	**2464 (1619)**		**1059**
18	**2537 (1253)**	**3011 (1595)**	**3614 (2289)**
19	**3270 (1635)**	**3270 (1635)**	**2857 (1407)**
20	**1741 (967)**	**1101 (338)**	**3068 (1250)**
21	**3046 (1562)**	**2380 (873)**	**1951 (487)**
22	**2060 (969)**	**1974 (764)**	**1234 (493)**
23	**2000 (1000)**	**2000 (1000)**	**2865 (1402)**
24	-	-	-
25	**1760 (920)**	**1894 (947)**	**1645 (506)**
26	**1793 (1086)**	**1511 (813)**	**2000 (1200)**
27	**2108 (544)**	**4000 (1642)**	**3114 (2459)**
28	**1719 (596)**	**1814 (889)**	**1466 (666)**
29	**1517 (669)**	**3806 (1741)**	**2380 (714)**
30	**1894 (1052)**	**1557 (655)**	**4464 (1607)**
31	**2169 (1254)**	**2169 (1254)**	**2755 (1326)**
Mean Min Max SD	**2189** **855** **3916** **654**	**2171** **1049** **4000** **834**	**2317** **1025** **4464** **819**

In case 24 quantitation not possible due to artefacts in sections.

Appendix 4 : Values for white matter neuronal densities in superficial versus deep temporal lobe

Case	Neuronal densities Superfical White Matter			Neuronal densities Deep White Matter		
	Small ND	Large ND	Total ND	Small ND	Large ND	Total ND
1	714	1468	2182	1605	1897	3502
2	1171	1486	2657	2110	1232	3222
3	1336	2158	3524	1176	1503	2679
4	1181	1574	2755	2589	1071	3660
5	2500	500	3000	939	671	1610
6	230	692	922	1007	852	1859
7	587	1004	1591	689	1172	1861
8	751	867	1618	1075	322	1397
9	1095	1095	2190	1515	757	2272
10	1100	2000	3100	1274	100	1374
11	1268	1940	3208	1666	1269	2936
12	773	1071	1843	1011	1235	2247
13	1250	1785	3035	1288	1929	3157
Mean			2432			2444
Min			922			1374
Max			3524			3660
SD			774			800

Appendix 5 : Values for layer I neurones in temporal lobectomies

Case	Layer I NeuN neuronal densities / mm3	Layer I NeuN neuronal densities / mm3 of small positive cells (less than 10 micron diameter) [% of total]	Layer I NeuN neuronal densities / mm3 of large neurones (greater than 10 micron diameter)
1	**12972**	**10810 (83%)**	**2162**
2	**4117**	**3235 (78%)**	**882**
3	**10555**	**9444 (89%)**	**1111**
4	**5675**	**5405 (95%)**	**270**
5	**6744**	**6511 (96%)**	**232**
6	**13333**	**10000 (75%)**	**3333**
7	**7894**	**5087 (64%)**	**2807**
8	**13400**	**11900 (88%)**	**1500**
9	**8000**	**6500 (81%)**	**1500**
10	**8627**	**3921 (45%)**	**4705**
11	**11000**	**9500 (86%)**	**1400**
12	**8900**	**8600 (96%)**	**300**
13	**9487**	**9230 (97%)**	**256**
14	**9200**	**7900 (85%)**	**1300**
15	**12700**	**12000 (94%)**	**700**
16	**11700**	**11300 (96%)**	**400**
17	**6700**	**5500 (82%)**	**120**
18	**11578**	**10263 (88%)**	**1315**
19	**10700**	**9700 (96%)**	**100**
20	**9400**	**9400 (100%)**	**0**
21	**10300**	**8300 (86%)**	**2000**
22	**15300**	**14500 (94%)**	**800**
23	**13157**	**11578 (88%)**	**1578**
24	**7631**	**7631 (100%)**	**0**
25	**9210**	**7368 (80%)**	**1842**
26	**10250**	**8000 (78%)**	**2250**
27	**8139**	**5116 (62%)**	**3023**
28	**11764**	**10000 (85%)**	**1764**
29	-	-	-
30	**15400**	**14900 (96%)**	**500**
31	**16444**	**12888 (78%)**	**3555**
Min Mean Max SD	**4117** **10436** **16444** **2952**		

In one case (29) quantitation not possible due to artefacts in sections

Appendix 6 : values for calbindin positive neurones in layer I and semi-quantitative scores

Case	Calbindin positive neurones in layer I per 10 mm^2	NeuN large (greater than 10 micron diameter) neurones in layer I	Calbindin positive neurogliaform cells in cortex.
1	**14**	**2162**	++
2	**15**	**882**	+++
3	**15**	**1111**	+++
4	**7**	**270**	+
5	**7**	**232**	+
6	**10**	**3333**	++
7	**9**	**2807**	+
8		**1500**	
9	**17**	**1500**	+++
10	**14**	**4705**	++
11		**1400**	
12	**8**	**300**	+
13	**8**	**256**	+
14	**20**	**1300**	++++
15	**10**	**700**	++
16	**8**	**400**	+
17	**10**	**120**	++
18	**7**	**1315**	+
19	**20**	**100**	++++
20	**14**	**0**	++
21	**14**	**2000**	++
22	**14**	**800**	++
23	**27**	**1578**	++++
24		**0**	
25	**17**	**1842**	+++
26	**16**	**2250**	+++
27	**27**	**3023**	++++
28	**15**	**1764**	+++
29	**17**		+++
30	**11**	**500**	++
31	**16**	**3555**	+++

Appendix 7 : Values for interneuronal distance in white matter neurones in temporal lobectomies

Case	Interneuronal Distance (microns)	White matter Neuronal Density (NeuN)/mm3	
1	**143**	**2016**	
2	**145**	**1997**	
3	**93**	**3219**	
4	**127**	**2078**	
5	**177**	**1212**	
6	**124**	**2418**	
7	**96**	**2330**	
8	**133**	**2174**	
9	**112**	**1340**	
10	**136**	**1487**	
11	**118**	**2077**	
12	**100**	**2243**	
13	**141**	**1974**	
14	**164**	**1938**	
15	**96**	**3246**	
16	**131**	**2076**	
17	**129**	**1611**	
18	**110**	**3282**	
19	**79**	**3448**	
20	**121**	**2144**	
21	**97**	**2649**	
22	**173**	**1741**	
23	**123**	**1624**	
24	-	-	
25	**155**	**1461**	
26	**140**	**1689**	
27	**180**	**3047**	
28	**155**	**1630**	
29	**135**	**1959**	
30	**133**	**2141**	
31	**141**	**2676**	
Min Max Mean SD	**79** **180** **130** **25.6**	**1212** **3448** **2164** **604**	

In case 24 artefacts in section prevented analysis

Appendix 8 :Values for cortical neuronal densities, white matter astrocytic densities in temporal lobectomies

Case	White matter astrocytic densities / mm3 (GFAP)	Middle temporal gyrus neuronal densities / mm3	Mean white matter Neuronal densities /mm3 (ND in the posterior temporal lobe)
1		**38181**	**2016 (2258)**
2	**7625**	**47768**	**1997 (2966)**
3	**6748**	**51666**	**3291 (3916)**
4	**4370**	**35161**	**2078 (1413)**
5	**10549**	**46506**	**1212 (1541)**
6	**13244**	**45365**	**2418 (2426)**
7		**42187**	**2330 (2584)**
8		**57741**	**2174 (2943)**
9		**43928**	**1340 (1388)**
10		**47037 (39200)**	**1487 (855)**
11	**9107**	**39625**	**2077 (2600)**
12	**4981**	**45500**	**2243 (2180)**
13	**5928**	**53750 (35172)**	**1974 (1601)**
14		**39277**	**1938 (2511)**
15	**5121**	**46086**	**3246 (2837)**
16	**6958**	**35700**	**2076 (2111)**
17	**6248**		**1611 (2464)**
18	**8703**	**41071**	**3282 (2537)**
19		**39059**	**3448 (3984)**
20			**2144 (1741)**
21			**2649 (2290)**
22			**1741 (2060)**
23		**30228**	**1624 (2000)**
24		**36369**	
25	**13959**	**30422**	**1461 (1760)**
26		**34180**	**1689 (1793)**
27	**9071**	**46441**	**3047 (2108)**
28	**9980**	**41853 (31156)**	**1630 (1719)**
29	**11492**	**28716**	**1959 (3806)**
30	**14221**	**37946**	**2141 (1849)**
31	**12628**	**39015**	**2676 (2169)**
Min	**4370**	**28716**	
Max	**14221**	**53750**	
Mean	**8940**	**40207**	
SD	**3193**	**6777**	

In the column middle temporal gyrus neuronal densities the figure in brackets shows the neuronal density in a region of obvious neuronal loss elsewhere in the specimen ; marked reduction in cell density was always observed in these regions.

Appendix 9 : Values for reelin and calbinin positive Cajal-Retzius cells in temporal lobectomies

CASE	Reelin cells in layer I /mm	Calbindin positive cells In layer I
1	0.9	1.6
2	0.75	
3	0.50	1.5
4	0.8	0.7
5	0.7	0.7
6	0.78	1.5
7	0.47	1.0
8	0.26	
9	0.13	1.7
10	0.2	1.7
11	0.3	0.7
12	0.08	
13	1.09	0.8
14	0.86	1.6
15	0.4	
16	0.34	2.0
17	0.25	
18	0.4	0.8
19	0.42	2.7
20	0.13	
21	0.16	
22	0.1	
23	0.31	
24	0.36	
25	0.2	

Case number	Microdysgenesis	Focal cortical dysplasia	Control cases
1	3.30	1.5	0.7
2	1.8	1.05	1.5
3	2.05	2.18	1.2
4	2	4.6	0.6
5	2.3	1.7	0.75
6	2.7	2.0	0.94
7	6.1	1.4	1.5
8	3.8	Nr	1.0
9	3.1	Nr	1.0
10	2.4	Nr	0.7
11	2.04	Nr	Nr
12	5	Nr	Nr
mean	**3.04**	**2.06**	**.98**
SD	**1.3**	**1.18**	**0.32**

Appendix 10 : Data on the values of ratios between NPY fibre length in cortical layer II to IV in microdysgenesis, focal cortical dysplasia and control cases.