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ORIGINAL PAPER

The application of cortical layer markers in the evaluation of cortical dysplasias in epilepsy

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Abstract The diagnostic criteria for focal cortical dysplasia type I (FCD I) remain to be well and consistently defined. Cortical layer-specific markers (CLM) provide a potential tool for the objective assessment of any dyslamination. We studied expression patterns of recognised CLM using immunohistochemistry for N200, ER81, Otx1, Map1b (subsets of V/VI projection neurones), Pax6, Tbr1, Tbr2 (differentially expressed in cortical neurones from intermediate progenitor cells), Cux 1 (outer cortical layers) and MASH1 (ventricular zone progenitors). Dysplasia subtypes included FCD I and II, dysplasias adjacent to hippocampal sclerosis (HS) or dysembryoplastic neuroepithelial tumours (DNTs); all were compared to neonatal and adult controls. Laminar expression patterns in normal cortex were observed with Tbr1, Map1b, N200 and Otx1. FCDI cases in younger patients were characterised by abnormal expression in layer II for Tbr1 and Otx1. FCDII showed distinct labelling of balloon cells (Pax6, ER81 and Otx1) and dysmorphic neurones (Tbr 1, N200 and Map1b) supporting origins from radial glia and intermediate progenitor cells, respectively. In temporal lobe sclerosis cases with dysplasia adjacent to HS, Tbr1 and Map1b highlighted abnormal orientation of neurones in layer II. Dyslamination was not confirmed in the perilesional cortex of DNT with CLM. Finally, immature cell types (Otx1, Pax6 and Tbr2) were noted in varied pathologies. One possibility is activation of progenitor cell populations which could contribute to the pathophysiology of these lesions.

Keywords Cortical layer markers · Dysplasia · Epilepsy

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Introduction

Following the revision to the classification of focal cortical dysplasias (FCD) in epilepsy in 2004 [41], there has been improved consistency in the clinical, radiological and pathological correlative data for the more severe types of dysplasia (FCD II) characterised by abnormal cytomorphology [38]. The criteria for milder dysplasias (FCD I), characterised mainly by abnormalities in cortical lamination, are less well defined [7] and more open to subjective interpretation with poor reproducibility shown between observers [12]. Varied neocortical laminar abnormalities are also reported adjacent to hippocampal sclerosis (HS)

[51] and glioneuronal tumours including dysembryoplastic neuroepithelial tumours (DNT) [17, 50]. These dysplasias are also less well defined, including their distinction from isolated FCD.

Cortical layer-specific markers (CLM) are a potential diagnostic tool for the evaluation of mild dysplasias in epilepsy [26]. CLM include transcription factors, cytoskeletal proteins and calcium-binding proteins which show neuronal subtype and/or layer-specific expression patterns reflecting ontogeny and cortical maturity. Largely studied in the rodent cortex [26], there are few studies evaluating these markers in human epilepsy tissues [24, 32]. CLM expression is likely to vary between species, the stage of cortical maturation, between cortical regions and with neuronal activity, including seizures; these factors have to be considered in their evaluation in human pathologies [26].

Our aim was to assess a panel of CLM in the discrimination of commonly encountered dysplasia subtypes in epilepsy surgical tissue. We selected a panel shown by previous studies of cortical development and maturation demonstrated to reflect layer or neuronal lineage specificity [26, 36]. Any abnormalities in their distribution might enable a more accurate classification of these disorders.

Materials and methods

Case selection and histopathological features

The study was approved by the respective ethics committees of the participating centres where informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. All surgical cases underwent therapeutic resection for refractory epilepsy and the pathological tissue was surplus to diagnostic requirement. In all cases we selected larger resection specimens so that adjacent normal cortex was more likely to be included. We aimed to include cases involving the temporal neocortex for all pathology groups where possible. The details of cases, including main histological features, are outlined in Table 1. Within groups similar dysplasia pathology types were studied, including cases from previous published series [28, 51]; 14 cases of FCD IA (henceforth referred to as FCDI), 9 cases of FCD II B (henceforth referred to as FCD II), 8 cases of dysplasia adjacent to DNT, 5 cases of dysplasia associated with HS and temporal lobe sclerosis (TLS) in addition to 14 control cases including epilepsy controls (no dysplasia). We did not include FCD type IB or IIA cases in the present study.

Table 1 Details of the cases and controls studied

Group	Number of cases	Age range	Characteristic features of dysplasia	Localisation of tissue block[a]	Nature of tissue
Control groups					
Paediatric	8	1 day–17 years	No dysplasia	Temporal (5) Frontal (2) Occipital (1)	PM
Adult	2	33–41 years	No dysplasia	Temporal (2)	S
Epilepsy	4	6–49 years	Hippocampal sclerosis only	Temporal (4)	S
Dysplasia groups					
FCD type I [28]	14	2–28 years	Columnar cortical architecture Excess of white matter neurones	Temporal[a] (9) Frontal (4) Occipital (1)	S
FCD type II	9	1–52 years	Balloon cells Dyslamination Dysmorphic neurones	Temporal (4) Frontal (5)	S
Dysplasia adjacent to HS (TLS) [51]	5	27–47 years	Clustering of neurons in layer II and abnormal orientation. Neuronal loss from layer II and III.	Temporal (5)	S
Dysplasia adjacent to DNT	8	22–43 years	Hypercellularity in layer I (7/8) Dyslamination Intracortical hamartias (5/8)	Temporal (5) Frontal (2) Unspecified site (1)	S

S surgical case, *PM* postmortem, *FCD* focal cortical dysplasia, *DNT* dysembryoplastic neuroepithelial tumour, *TLS* temporal lobe sclerosis

[a] In some of the FCD type I cases the pathology was multifocal, although only the temporal lobe from each case has been included in the present study

Table 2 Details of cortical layer marker panel for immunohistochemistry

Antibody	Source	Antibody dilution	Rationale for inclusion	References
Tbr1 (*T-box-brain-gene*)	Gift form Dr. R. Hevner[a]	Polyclonal 1:4,000	Expressed in cortical neurones derived from IPC cell lineages IPC have a role in cytoarchitectural regional organisation	[30, 43]
Tbr2 (*T-box-brain-gene*)	Gift from Dr. R. Hevner[a]	Polyclonal 1:1,500	Expressed in IPCs or basal progenitors in the SVZ	[20, 27]
Pax6 (*Paired box 6*)	Santa Cruz Biotechnology, Inc., CA, USA	Polyclonal 1:100	Expressed in apical progenitors in the VZ (radial glial stem cells) Largely confined to the VZ and SVZ from 10 to 17 weeks gestation	[5]
Map1b (*Microtubule associated protein type 1b*)	Abcam, Cambridge, UK	Monocloncal 1:2,500	Earliest MAP expressed during development Identifies a subset of layer V neurones in foetal neocortex with expression retained in adult neocortex	[46, 26, 14]
N200 (*Neurofilament heavy chain*)	Sigma–Aldrich, Saint Louis, Missouri, USA	Polyclonal 1:3,000	Expressed in projection neurones in layer II, III, V and VI. Highlights selective non-cortical projecting populations within layer V neurones	[35]
Otx1 (Orthodenticle homolog 1)	Abcam, Cambridge, UK	Polyclonal 1:100	Expressed in a subset of layer V/VI projection neurones	[27, 35]
ER81 (*ETS transcription factor*)	Abcam, Cambridge, UK	Polyclonal 1:6,000	Highlights a subset of layer V projection pyramidal neurones in rodents and primates as well as in the developing human neocortex	[5]
Cux1 (*Homeobox cut gene 1*);	Abcam, Cambridge, UK	Monoclonal 1:2,000	Localises to the superficial cortex, layer II–IV in rodent cortex	[2, 39]
MASH1 (*Mammalian achaete-scute homolog-1, or Ascl1*)	Abcam, Cambridge, UK	Polyclonal 1:200	A helix–loop–helix transcription factor expressed in basal progenitors in the VZ, promoting progenitor cell maturation	[8, 32]

IPC intermediate migrating progenitor cell, *VZ* ventricular zone, *SVZ* subventricular zone

[a] Dr R Hevner's Laboratory, Seattle Children's Hospital Research Institute, Seattle, WA, USA

Immunohistochemistry

In all cases a single representative formalin-fixed, paraffin-embedded tissue block from each case was selected and further sections cut at 7 μm thickness. Sections were dewaxed and rehydrated, followed by blockage of endogenous peroxidase activity by 15 min incubation in 0.9% hydrogen peroxide in de-ionised water. A panel of 9 CLM antibodies was selected (as detailed in Table 2) in addition to NeuN (1:100, Chemicon). After washing, the sections were microwaved 12 or 15 min in antigen retrieval solution, then cooled for 20 min. Staining was visualised with diaminobenzidine (DAB) and NovaRed for N200 antibody. Sections where DAB was applied were then enhanced in copper sulphate, and counterstained with haematoxylin. Between each step, sections were washed in phosphate buffer saline with 0.05% Tween 20 (PBS+T). Negative controls were treated identically except that the primary antibody was omitted. For some of the antibodies with developmental regulation, e.g. Pax6, Tbr2, sections of foetal brain including the VZ and SVZ (20–36 gestational weeks) were used as a positive control. The laminar staining pattern with each CLM in all cases was assessed through comparison to the corresponding NeuN-stained section (Fig. 1a).

Immunofluorescence

Cases were selected for double labelling immunofluorescence using combinations of NeuN, GFAP, CD68 CD34, calretinin with CLM, following the initial observations with immunohistochemistry. 7 μm sections were dewaxed and rehydrated and washed in water. Endogenous peroxidase was quenched with 3% hydrogen peroxide and de-ionised water. Sections were microwaved in antigen retrieval buffer. Protein blocking was carried out with normal horse serum followed by incubation of primary antibodies overnight at 4°C. Sections were washed and

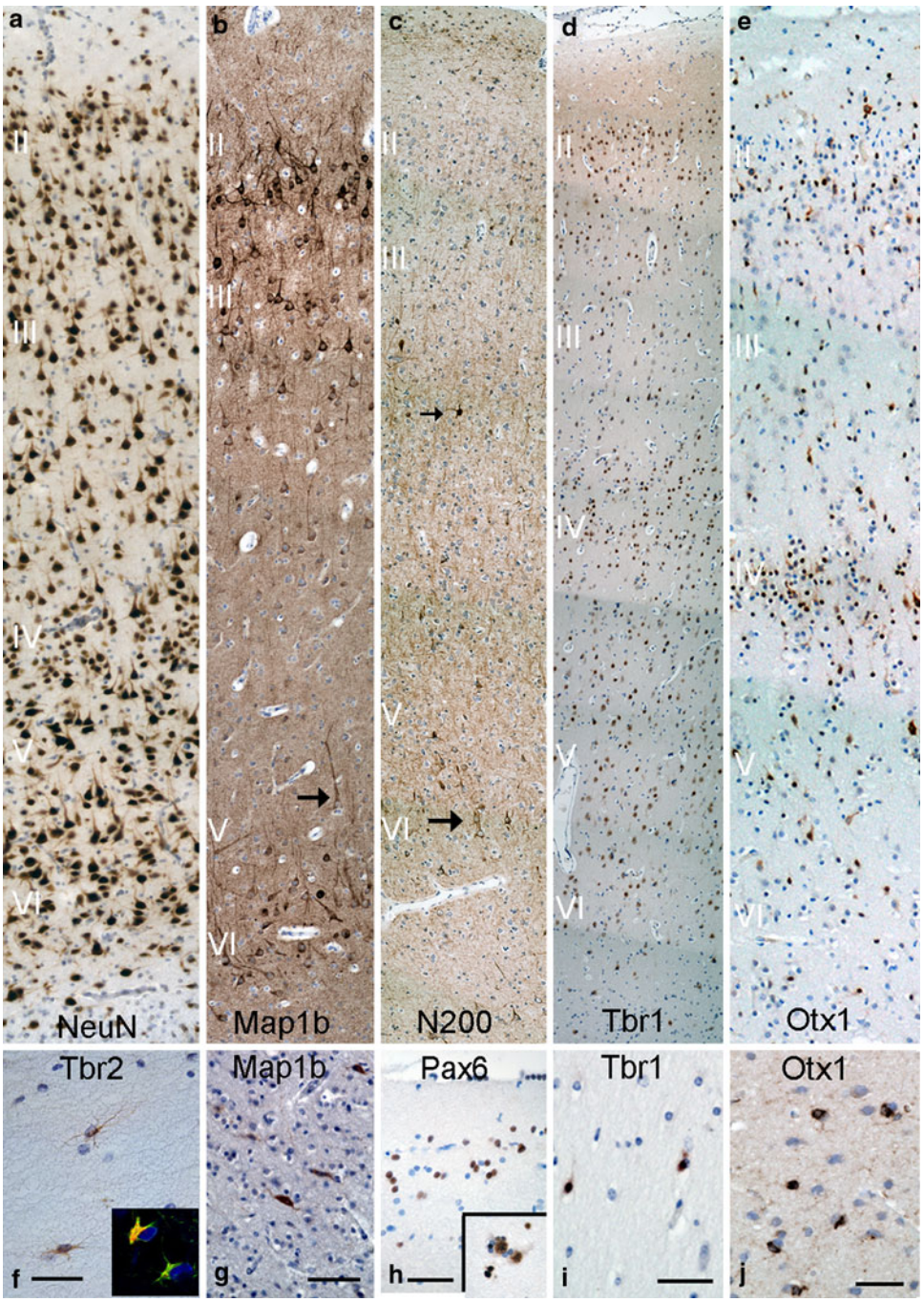

Fig. 1 Controls. **a** Adult control: *NeuN* labelling in temporal lobe demonstrating distinct cortical laminae. **b** Adult control: *Map1b* showing cytoplasmic labelling in layers II, III, V (*arrowed*) and VI pyramidal cells with an absence of labelling in mid layers. **c** Adult control: *N200* with labelling of isolated pyramidal cells mainly in layer V and VI and to a lesser extent the upper cortical layers (*arrows*). **d** Adult control: *Tbr1* with nuclear labelling of a proportion of neurones in all cortical layers particularly IV, V and VI. **e** Paediatric control (day 1): *Otx1* with frequent cells visible throughout the cortex but more prominent in layers II and IV. **f** *Tbr2* frequent cytoplasmic labelling of multipolar cells through the white matter were observed in neonatal period. (*inset* double labelling for Tbr2 and GFAP showing co-localisation in some white matter cells GFAP *green*, Tbr2 *red*). **g** *Map1b* staining of the same case as in **f** labelled only occasional cells in cortical layer V at 1 day in contrast to the hippocampus which showed numerous Map1b-positive cells. **h** *Pax6*: nuclear labelling of small cells in the periventricular white matter in neonate (*inset* ER81, nuclear labelling of pyramidal cells and a subset of satellite cells). *i* Tbr1 staining at 2 years confirming nuclear labelling in proportion of layer V neurones. **j** *Otx1* layer II in adult epilepsy control showing frequent cytoplasmic labelling of small, cells with immature morphology. *Bars* **f**, **g**, **i** 30 μm; **h**, **j** 45 μm, **a**–**e** cortical panoramas representing layers I–VI, taken at original magnification (×10 objective) and rescaled to align layers

incubated with secondary anti-rabbit ImmPRESS (Vector Laboratories) for polyclonal antibodies followed by Cy3, or fluorescein tyramide signal amplification (PerkinElmer Life and Analytical Sciences, Boston, MA). Sections then were washed and quenched in 1% hydrogen peroxide in PBS for 20 min in order to prevent any deposited tyramide combining with the second tyramide signal that followed. The sections were incubated with anti-mouse ImmPRESS (Vector Laboratories) for monoclonal antibodies followed by CY3 or fluorescein tyramide with dilutions for monoclonal GFAP (Dako, 1:30), polyclonal GFAP (Dako; 1:300), and polyclonal Calretinin (1:2,000; Swant, Bellinzona, Switzerland). Alexa Fluor chicken anti-rabbit 594, (1:100) and goat anti-mouse 488, (1:70) (Molecular Probe; Eugene, OR, USA) was used. After each step, sections were washed in PBS. Sections were mounted on Vectashield with DAPI (Vector Laboratories) and visualised with a Zeiss LSM 510 Meta or Leica confocal laser microscope.

Results

Controls

CLM expression for control cases is summarized in Table 3 (Fig. 1). The most distinct lamina-specific expression patterns were noted for Map1b (layers V–VI and II), N200 (layers V–VI) and Tbr1 (layers IV–VI) (Fig. 1b–d). For three CLM (N200, Map1b, Tbr1) progressively greater intensity and frequency of cortical neuronal staining was noted in older children and adults compared to infant controls (Fig. 1b, g). In a further three CLM (Otx1, Tbr2, Cux1) the expression reduced with cortical maturation (Fig. 1e, f) and in three (Mash1, ER81, Pax6) no obvious difference was noted between age groups (Fig. 1h and inset). Immature cell types, some with bipolar or multipolar processes were noted with Tbr2, Pax6, Otx1 particularly in paediatric cases (Fig. 1e, f). In epilepsy controls without dysplasia, enhanced labelling with Otx1 and Tbr2 was noted compared to non-epilepsy controls (Table 3; Fig. 1j).

Cortical dysplasias

The patterns of CLM expression specific to each dysplasia type are summarized below (and in Table 4). All markers were studied for each pathology but only detailed where they deviated from control findings.

FCD I

A striking finding in six cases (between 2 and 6 years of age) was intense nuclear labelling of small cells at the

Table 3 Summary of the main laminar staining patterns with antibody panel in control tissues

Layer	Paediatric control (no epilepsy)	Adult control (no epilepsy)	Epilepsy (no MCD) (where different from non-epilepsy controls)
I	Tbr2 (M[a])	ER81 (A)	Otx1 (I)
	ER81 (A)	Cux 1 (N)	Tbr2 (M)
	Cux 1 (I, M)	Pax6 (I)	
	MASH1 (P)		
II	Otx 1 (I)	Tbr1 (A)	Otx 1 (I, S)
	Cux 1 (N)	Map1b (P)	Tbr2 ± (I, M)
	ER81 (A)	N200 ± (P)	
		ER81 (A)	
		Cux1 (N)	
III	ER81 (A)	Tbr1 (P)	Otx 1 (I, S)
	Map1b (P)	Map1b (P)	
		N200 ± (P)	
		ER81 (A)	
		Cux1 (N)	
IV	Map1b ± (N)	Tbr1 (N)	Otx 1 (I, S)
	Otx1 (I)	ER81 (A)	
	Cux 1 (N)	Cux1 (N)	
	ER81 (A)		
V	Tbr1 ± (P)	Tbr1 (P)	Otx 1 (I, S)
	Map1b ± (P)	Map1b (P)	
	N200 ± (P)	N200 (P)	
	ER81 (A)	ER81 (A)	
	MASH1 ± (P)	Cux1 (N)	
VI	Tbr1 (P)	Tbr1 (P)	Otx 1 (I, S)
	Map1b ± (P)	Map1b (P)	
	N200 ± (P)	N200 (P)	
	ER81 (A)	ER81 (A, S)	
	Otx 1 (I)	Cux1 (N)	
	MASH1 ± (P)		
White matter	Tbr1 (I)	Tbr1 (A)	Otx 1 (I, S)
	Tbr2 (M[a])	N200 (P)	
	Otx1 (I)	Map1b ± (A)	
	ER81 (A)	ER81 (A)	
	Cux 1 (I)	Pax6 (I)	
	MASH1 ± (P)	Cux 1 (N)	
	Pax6 (I)	MASH1 ± (P)	

For the epilepsy control group markers are listed only where they deviated from other control groups. Markers underlined indicate more intense and frequent labelling of cells in the corresponding lamina

MCD malformation of cortical development, ± indicates less frequent cells were seen to label, *P* pyramidal cell, *N* non-pyramidal cell, *A* all types of mature neuronal cells, i.e. pyramidal and non-pyramidal, *M* small, multipolar cell with staining of cytoplasmic processes, including bipolar cells, *I* immature small cell of indeterminate lineage (small nucleus with little cytoplasm), *S* perineuronal satellite cells

[a] Co-expression with GFAP shown in controls in some populations

Table 4 Summary of main positive findings with cortical layer markers in dysplasia types in epilepsy surgical pathologies

Pathology group	Cell types/pathology feature	Expression of cortical layer markers	Cortical layer	Hypothesis of origin based on immunophenotype
FCD I	Immature cells	Tbr1, Pax6, Otx1	Layer I/II interface	Immature layer II cells
	Columnar alignment	Highlighted by Map1b	Layers II–IV mainly	Abnormal maturation (mid-cortical layers)
FCD II	Balloon cells	Pax6, Otx1, ER81, Cux1 $\gg$ Tbr2	White matter and layer I mainly	Derived from radial glia/IPC transitional cell type
	Dysmorphic neurones	Map1b > Tbr1 N200 > Otx1 ER81, MASH1	All cortical layers, except layer I	Resemble mature cortical neurones; derived from IPC
TLS	Neuronal clustering and mal-orientation	Map1b, Tbr1, > Otx1, Tbr2, Pax6	Layer II (superficial border)	Selective re-organisation of mature layer II neurones
DNT	Neurones in glioneuronal element	Map1b N200 Tbr1	Layers II, III, IV and VI V, VI > II, III IV–VI	Entrapped normal cortical neurones
	Adjacent cortex layer I hypercellularity	ER81, Cux1 > Otx1, Pax6 > Tbr2, N200.	Layer I	Similar to tumour OLC: origins from residual layer I progenitor cells ('secondary germinal matrix') rather than IPCs.

DNT dysembryoplastic neuroepithelial tumour, *TLS* temporal lobe sclerosis, *OLC* oligodendrocyte-like cell, *IPC* intermediate progenitor cells

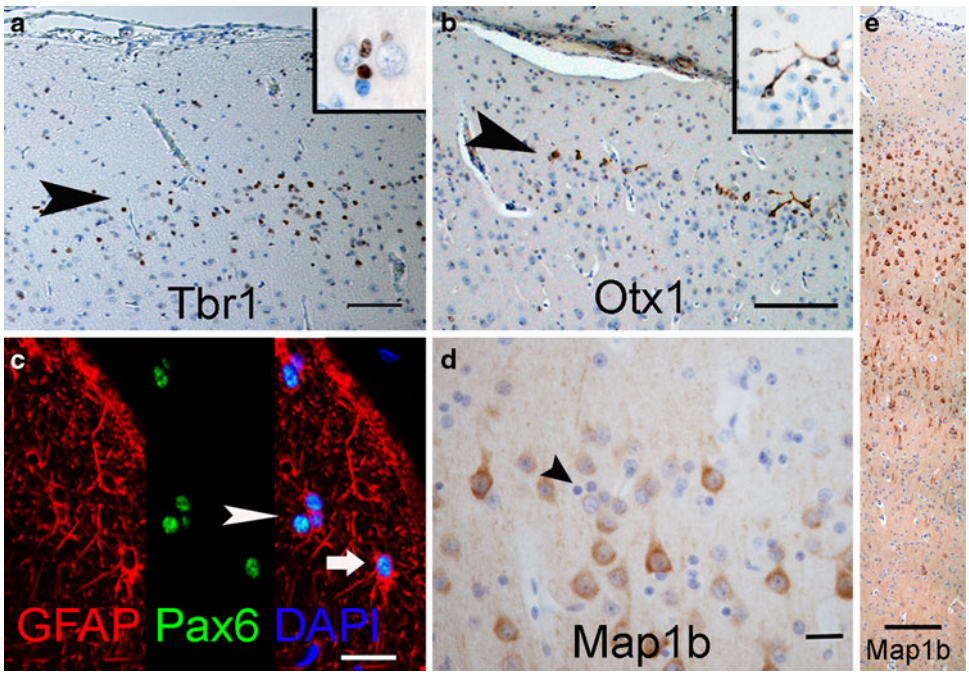

Fig. 2 Focal cortical dysplasia (type Ia). **a** *Tbr1* nuclear labelling of small, immature appearing cells localised to the interface of layer I and II (*arrowhead*); similar cells are shown at higher magnification from another FCDI case in the *inset*. **b** *Otx1* labelling of cells at the interface of layer I and II with extension of cytoplasmic processes into layer I (shown at higher magnification in *inset*). **c** Double labelling with *Pax6* and *GFAP* in layer I confirmed co-localisation in a proportion of cells. **d** *Map1b* the small immature cells in layer II are negative (*arrowed*) compared to the positive mature pyramidal cells. **e** *Map1b* in another case confirming expansion of labelling in upper compared to lower cortical layers. *Bars* in **a**, **b** 30 μm, **c** 20 μm, **d** 10 μm, **e** 500 μm

interface of layer I and II with Tbr1 (Fig. 2a). Similar cells also labelled with Otx1 (Fig. 2b). Occasional cells with multipolar processes were noted with Tbr2, scattered in both the upper cortical layers and white matter. These cells were not clearly identified on conventional stained sections and cells of a similar immunophenotype were not seen in older FCDI cases (6–28 years). Enhanced labelling of small cells at the interface of layer I and II was also noted

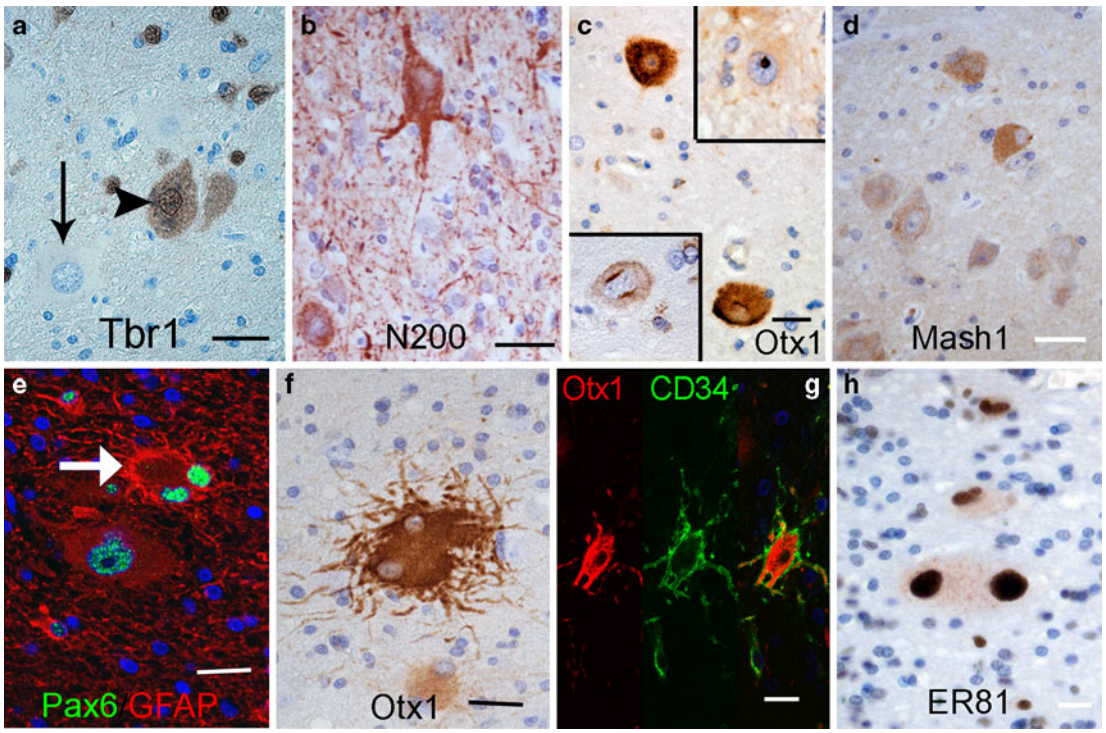

Fig. 3 Focal cortical dysplasia type IIB. **a** *Tbr1* moderate labelling of the cytoplasm of dysplastic neurones was observed (*arrowhead*) compared to negative labelling of other dysplastic cells in proximity (*arrow*) and strong intensity of interspersed normal neuronal nuclei. **b** *N200* positive labelling of dysmorphic neurones. **c** *Otx1* dysmorphic neurones showing diffuse cytoplasmic positivity and occasional intranuclear inclusions (*top-right inset*) and perinuclear labelling (*bottom-left inset*). **d** *Mash1* weak cytoplasmic labelling of dysmorphic neurones is observed in this case. **e** *Pax6* co-localisation with GFAP in balloon cells including a multinucleated balloon cell (*arrowed*). **f** *Otx1* balloon cell showing strong cytoplasmic positivity and multipolar processes. **g** *Otx1* co-localisation with CD34 in a balloon cell. **h** *ER81* strong nuclear staining of balloon cells is observed. *Bars* **a**–**d**, **h** 20 μm; **e**, **f** 10 μm; **g** 30 μm

with Pax6 (Fig. 2c); confocal imaging confirmed co-localisation of a proportion with GFAP (Fig. 2c) but not NeuN. In eight cases, labelling of pyramidal neurons for Map1b in layers II–III (Fig. 2d) was greater than in deeper cortical layers, giving the impression of expansion of the superficial cortical layers (Fig. 2e) compared to controls. The Map1b-positive pyramidal neurons remained normally orientated. In regions with enhanced columnar architecture, this organisational pattern was enhanced on Map1b staining with sheaths of perpendicular dendrites separating columns of neurons in the mid-cortical layers.

FCD II

Dysmorphic neurones (DN) and balloon cells (BC) showed distinct CLM expression patterns regardless of laminar location. DN were present through all cortical layers except layer I and showed variable immunopositivity with Tbr1 (Fig. 3a) but more frequently with Map1b and N200 (Fig. 3b). Cytoplasmic, perinuclear or occasional intranuclear labelling patterns for Otx1 in DN was observed (Fig. 3c). Weak to strong cytoplasmic labelling of DN for MASH1 was observed (Fig. 3d), although many were not labelled.

BC, including those located in the white matter and layer I, showed variability of staining with Tbr2 both within and between cases. Strong nuclear labelling of BC was noted for Pax6 in four cases, with co-localisation with GFAP but not NeuN (Fig. 3e). Labelling of the cytoplasm of BC for Otx1 was striking in the majority of cases, highlighting multipolar cellular processes (Fig. 4f). There was co-expression of Otx1 with GFAP and CD34 (Fig. 3g) in a proportion of BC. BC in the white matter or layer I also showed intense nuclear positivity for ER81 (Fig. 3h) in contrast to DN which were more weakly labelled. The majority of BC were Cux1 positive compared to a near absence of labelling of DN with Cux1. In addition, intracortical cytoplasmic labelling of multipolar cells and small immature intracortical cells was noted with Tbr2 and Otx1, as in epilepsy controls.

Dysplasia adjacent to hippocampal sclerosis [temporal lobe sclerosis (TLS)]

These cases all showed neuronal loss in the supragranular cortex and re-organisational dysplasia of neurons in layer II as previously described [51]. With CLM residual clusters

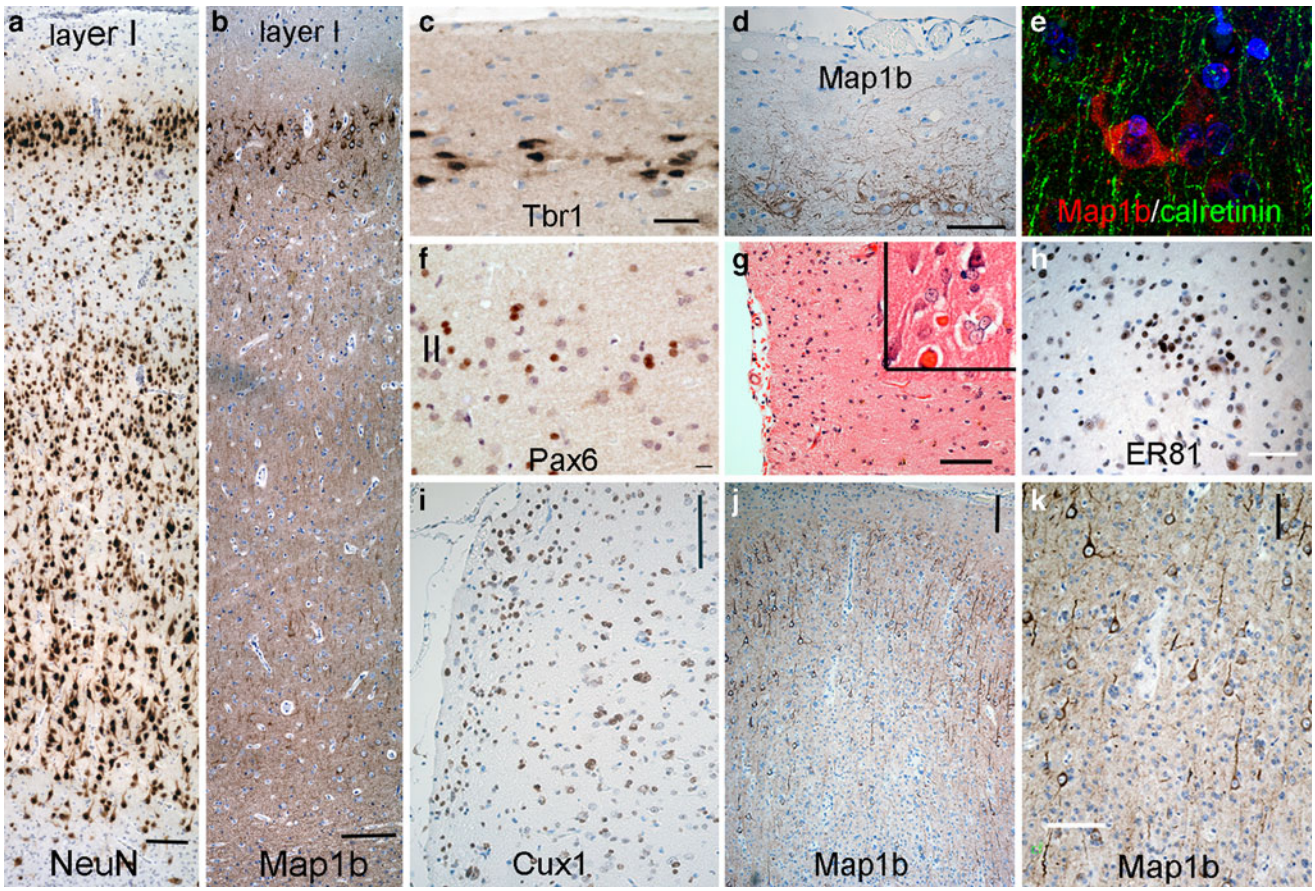

Fig. 4 Temporal lobe sclerosis (TLS) and hippocampal sclerosis with dysplasia and layer marker patterns within dysembryoplastic neuroepithelial tumour (DNT) and the dysplasia in the adjacent cortex. **a** *NeuN* TLS case illustrating neuronal loss from layers II and III and an impression of hypercellularity and disorganisation in layer II. **b** *Map1b* intense labelling of neurones in upper cortical layers (layer II) is appreciated compared to deeper cortical layers in TLS case. **c** *Tbr1* intense nuclear labelling of remaining, mature appearing neurones in outer layer II was appreciated in some cases; a more horizontal alignment of these neurones was noted in some regions in TLS. **d** Map1b highlighted the abnormal orientation and clustering or aggregation of neurones and entanglement of processes in layer II in TLS. **e** Double labelling of Map1b and calretinin in layer II confirmed that the orientation of the calretinin radial processes was normal with only selective misalignment or horizontal orientation of the Map1b positive layer II cells in TLS. **f** *Pax6* frequent nuclear labelling of small immature cells in the layer I/II was observed in TLS. **g** *DNT* H&E staining of the perilesional cortex demonstrating hypercellularity in layer I. *Inset* a cluster of immature cells with vesicular nuclei between more mature neurones in the adjacent cortex which form a small satellite nodule or hamartia (these were CD34 positive). **h** *ER81* hamartia or satellite clusters of oligodendroglial like cells (OLC) were strongly positive in DNT. **i** *Cux1* the layer I hypercellularity adjacent to the DNT was highlighted with Cux1 with the majority of cells showing nuclear positivity. **j** *Map1b* DNT tumour nodule in cortex highlighting the pyramidal neurones suspended within the nodule that retain Map1b positivity, normal morphology, orientation and laminar specific distribution. **k** *Map1b* higher magnification of case shown in **j**. *Bars* **a**, **b** 500 μm; **b**, **j** 20 μm; **d**, **e** 50 μm; **f** 10 μm; **g**, **h**, **k** 40 μm

of neurons in layer II in many regions showed intense labelling with Tbr1 (Fig. 4a, c) and Map1b (Fig. 4b, d) highlighting their abnormal, horizontal orientation. Double labelling for calretinin, which labels a subset of fusiform inhibitory interneurones that predominate in upper cortical layers, and Map1b showed that there appeared to be selective mal-alignment of the Map1b-positive cells (Fig. 4e). In addition, labelling of scattered, small immature cells was observed in the upper cortical layers with Tbr2 (layers II, III), Pax6 (layers I, II) (Fig. 4f), Cux1 and ER81 (layer I) and Otx1 (layers I–VI). Co-localisation between Cux1 and GFAP was observed in some cells.

Dysplasia adjacent to DNT

In the perilesional cortex of DNT with apparent dyslamination in conventional stained sections (Table 1), CLM patterns were as in controls, including labelling of white matter neurons. Therefore, abnormal lamination was not confirmed. ER81 and Cux1 highlighted regions with increased cellularity in layer I adjacent to the tumour (Fig. 4g, i), in addition to the small intracortical hamartia-like clusters of immature cells (Fig. 4g, h). No significant expression of Tbr1, Pax6, Map1b or MASH1 by these cells was noted. Small numbers of immature and multipolar cells

were noted with Tbr2 (layers I, II), N200 and Otx1. Double labelling with Otx1 and GFAP showed no co-localisation. Within the tumour itself, oligodendrocyte-like cells were ER81, and to lesser extent Cux1, positive but negative for other markers; the astroglia component was negative. The floating pyramidal neurons within the glioneuronal element of the tumour or within cellular nodules showed layer-appropriate expression of Tbr1, Map1b (Fig. 4j, k) or N200 in continuity with the adjacent cortex; this supported their representing entrapped neurons.

Discussion

It is acknowledged that the diagnostic criteria FCD I are less well defined than FCD II [7, 41]. FCD I lacks the distinctive and abnormal cell types that characterise FCD II and usually they are not detected with in vivo MRI. Their histological diagnosis is currently based on standard stains, such as cresyl violet, for assessment of dyslamination and likely includes heterogeneous pathologies. With lack of specific markers there may be subjective interpretive bias which probably explains the variation in diagnosing these lesions between epilepsy centres and pathologists [12, 31, 38]. Consistently applied pathological criteria for FCD I are desirable for reliable clinico-pathological and radiological correlations to emerge, which can guide appropriate surgical management and outcome prediction in these patients.

CLM offer a potential for objective assessment of developmental or acquired dyslamination and heterotopic positioning of neurones. CLM have been more extensively studied in experimental models than in humans. CLM are developmentally, temporally and regionally regulated and specific to different neuronal migratory pathways [26, 32, 36]. CLM expression, including cytoskeletal proteins and transcription factors, are therefore likely to reflect both the intrinsic properties of the cell as well as extrinsic factors specific to locality [25]. Thus, any abnormal cellular expression patterns in dysplasias, as well as diagnostic value, might in theory provide additional information regarding the timing of the insult and cell lineages involved.

In our selected CLM panel, layer specificity was shown for Otx1, N200, Tbr1 and Map1b in keeping with previous data [26, 46]. CLM have been most extensively studied for layer V neurons [26, 35 for review] where diverse neuronal subtypes are identified. ER81 selectively identifies layer V pyramidal neurons in rodent [37, 60] and primate cortex [57] with low levels of expression reported in layers II/III and VI [57, 60]. Although little studied in human brain [26], in our cases ER81 showed no layer specificity, being expressed in a proportion of pyramidal and non-pryamidal neurons (and perineuronal satellite cells) through all layers. Cux1, a putative marker of upper cortical layer neurones in experimental models [39], showed pan-cortical staining, with preferential labelling of layers II and IV and non-pyramidal cell types in keeping with recent data demonstrating expression in subsets of cortical interneurones through all cortical layers [15]. One explanation for these differences may be technical reasons, for example, different methodologies (including antibody source) and different fixation and processing methods between experimental and human tissues. However, it is also possible that CLM expression patterns in experimental models are not always replicated in human tissues.

Within dysplasia groups, relatively consistent abnormalities were identified with our CLM panel. The FCD I series, with pathology as previously described [28], demonstrated distinct populations of immature cells at the interface of layers I and II with Otx1, Tbr1, and Pax6, primarily in the younger patients. The localization and morphology of these cells was reminiscent of those we recently described in this pathology with doublecortin immunohistochemistry [49]. Similar cells were not identified in controls or other dysplasia types. In FCD I, expansion of the distribution of Map1b-positive mature-appearing neurons in outer cortex was noted, compared to control patterns [14]. The persistent mini-columnar architecture seen on conventional stains was highlighted [28]. These features all suggest a delay in maturation and organisation of upper cortical layers in this FCD I subtype.

FCD II is a well characterised malformation encountered in epilepsy surgical pathology with hallmark features of DN and BC. The CLM patterns we observed were distinct from FCD I supporting the view that this is a different biological entity. Although shared pathways for aberrant neuronal–glial fate specification for the abnormal cell types in FCD II have been suggested [21], distinct immunoprofiles for DN and BC cells were shown with our broader panel of antibodies. This could support origin from different migratory cell subsets during development. DN in all layers, bar layer I, commonly expressed N200, Map1b and Tbr1, albeit Tbr1 expression was weaker compared to normal pyramidal cells. Map1b expression has been previously recognised in dysmorphic neurons in FCD [14]. Otx1 and Mash1 expression were less often observed in dysmorphic neurones, as also noted in a previous study of FCDIIB [32]. No labelling of DN with Tbr2 and Cux1 was seen. The immunophenotype of DN therefore most closely resembles that of mature cortical pyramidal projection neurons [56], derived from intermediate progenitor cells (IPCs), rather than from radial glial cell progenitors as previously suggested [32]. IPCs are present throughout corticogenesis, with developmentally regulated expression of Pax6, Tbr2 and Tbr1 and produce neurons for all cortical layers [30, 43]. It is proposed IPCs play a role in

determination of laminar patterns, cortical thickness and regional cyto-architectonics. Local disturbance of IPC populations during the stages of cortical expansion could conceivably be implicated in the causation of FCD IIB.

BC in FCDII showed CLM expression patterns distinct from DN. Cytoplasmic positivity with Otx1 and Tbr2 and nuclear labelling for Pax6, ER81 and Cux1 was shown. The multipolar morphology of these abnormal cell types has previously been demonstrated with immunohistochemistry for GFAP-delta [34] CD34 [22], calbindin [52] and doublecortin [49]. Increased Otx1 mRNA [6] and Pax6 expression [32] but variable Map1b expression [14, 21] has previously been reported in BC, in keeping with our observations. The striking Pax6/GFAP co-expression we observed in BC supports their origin from radial glial cells as previously proposed [10, 32]. A proliferative capacity of BC has also been demonstrated [55], although many appear to be arrested in G1 phase of the cell cycle [54]. Pax6 regulates exit from the cell cycle, controlling cortical cell number [45]. It is conceivable therefore that the observed sustained Pax6 expression in balloon cells also plays a role in determining this cell cycle arrest.

Varied abnormalities in the temporal lobe and pole adjacent to HS in patients with mesial temporal lobe epilepsy have been described, including mild dysplasias (previously called microdysgenesis) to FCD I [23, 53]. It is debated whether these represent independent malformative pathologies or re-organisational or dysmaturational changes linked with temporal lobe damage and HS [7]. One distinct pattern, observed in the context of temporal lobe sclerosis and HS, includes cytoarchitectural changes involving layer II. Neuronal clustering and mal-orientation may be extensive in layer II, although overlooked on conventional stains [51]. We have shown that Map1b and Tbr-1 highlights selective mal-orientation of neurones at the interface of layer I and II. Calretinin-immunopositive interneurones in this lamina, derived from tangential migration [59], remained normally orientated. This specific type of dysplasia, involving the last cortical layer to be formed, may therefore be due to a relatively late event or insult in the process of radial cortical migration, maturation and organisation.

Dysplasias in the vicinity of DNTs are reported as an added component in one-third [29] to 86% of cases [40]. The descriptions are principally cortical dyslamination [16, 18, 19, 29] corresponding to FCD I [41]. FCDII in association with DNT has been less often reported [47, 50]. The perilesional cortex of DNT may be relevant to epileptogenesis [3, 4, 33] and the completeness of surgical resection could potentially influence seizure outcome following surgery [40, 47, 50]. Correct classification of this dysplasia type and discrimination from tumour infiltration zone is therefore desirable for accurate clinico-pathological correlations.

Common features observed in conventional stains in our DNT cases in the perilesional region included layer I hypercellularity, impressions of dyslamination and satellite clusters of immature cells (intracortical *hamartias*). CLM studies, however, showed a normal laminar pattern in the disrupted adjacent cortex. Furthermore, the pyramidal cells within the glioneuronal elements and nodules of the tumour displayed layer-appropriate expression, maturity and orientation with Map1b, N200 and Tbr1 suggesting that they are normal cortical neurones entrapped by the tumour. The layer I hypercellularity and hamartias were highlighted by ER81 and to a lesser extent Cux1, with cell phenotype similar to tumour oligodendrocyte-like cells. These peritumoural changes could represent either tumour extension or precursor lesions from which the tumour arises, as originally proposed [17], for example remnants of the secondary germinal layers or marginal zone [13, 61]. Cortical changes adjacent to DNT are therefore distinct from other FCD types. For perceived dyslamination on H&E, CLM may aid in the distinction of true dysplasia from peritumoural changes which will require verification through larger series. The mature neuronal component within the glio-neuronal element are probably residual entrapped cortical neurones.

In control cases we confirmed variation in expression patterns between neonatal and adult cortex for some CLM, particularly Otx1 which showed strong labelling in cases less than 1 year old. Otx1 does not influence early determination of neuronal cell type and laminar fate but has later roles in maturation and organisation of superficial neurons as well as deeper cortical layers including of GABAergic neurones [1, 42, 44, 48, 58]. We noted increased expression of Otx 1 in small immature cells in epilepsy cases, including those without dysplasia. CLM also highlighted small, immature cell populations, for example with Pax6 and Tbr2 in epilepsy cases, a proportion co-expressing GFAP. These immature cell types may represent activation of residual progenitor cell populations or a delay in normal rates of cell maturation. Recent studies have confirmed a physiological contribution of immature neurones to seizures in FCD lesions [11]. Residual populations of multipotential cells in the maturing cortex may switch to either glio-genic or neuronal pathways (including GABAergic neurones) [25]. The regulation of this process and diversification of cell types may depend on developmental stage, local factors and the maturing environment [9, 25]. This process could conceivably be deregulated by seizures. Therefore, the further delineation of immature cell populations may be of relevance to our understanding of the pathophysiology of dysplasias.

In conclusion, this study highlights the potential application of CLM to the confirmation and categorization of distinct dysplasia types and differentiation from normal cortex. The spectrum of immature cell types highlighted and their contribution to the pathophysiology of these lesions deserves further study.

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