Characterization of nodular neuronal heterotopia in children

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Summary
Neuronal heterotopia are seen in various pathologies and are associated with intractable epilepsy. We examined brain tissue from four children with subcortical or periventricular nodular heterotopia of different aetiologies: one with severe epilepsy following focal brain trauma at 17 weeks gestation, one with hemimegalencephaly and intractable epilepsy, one with focal cortical dysplasia and intractable epilepsy, and one dysmorphic term infant with associated hydrocephalus and polymicrogyria. The connectivity of nodules was investigated using histological and carbocyanine dye (DiI) tracing techniques. DiI crystal placement adjacent to heterotopic nodules revealed numerous DiI-labelled fibres within a 2–3 mm radius of the crystals. Although we observed labelled fibres closely surrounding nodules, the majority did not penetrate them. Placement of DiI crystals within nodules also identified a limited number of projections out of the nodules and in one case there was evidence for connectivity between adjacent nodules. The cellular and neurochemical composition of nodules was also examined using immunohistochemistry for calretinin and neuropeptide Y (NPY), which are normally expressed in GABAergic cortical interneurons. Within heterotopic nodules from all cases, numerous calretinin-positive neurons were identified, along with a few cell bodies and many processes positive for NPY. Calretinin-positive neurons within nodules were less morphologically complex than those in the cortex, which may reflect incomplete differentiation into an inhibitory neuronal phenotype. There were also abnormal clusters of calretinin-positive cells in the overlying cortical plate, indicating that the migratory defect which produces heterotopic nodules also affects development of the cortex itself. Thus, heterotopic nodules consisting of multiple neuronal cell types are associated with malformation in the overlying cortical plate, and have limited connectivity with other brain regions. This abnormal development of connectivity may affect neuronal maturation and consequently the balance of excitation and inhibition in neuronal circuits, leading to their epileptogenic potential.

Keywords: epilepsy; heterotopia; calretinin; neuropeptide Y; cortex

Abbreviations: DiI = 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate; GABA = γ-aminobutyric acid; LBCV = luxol blue/cresyl violet; NPY = neuropeptide Y

Introduction
Recent MRI studies have shown that developmental brain abnormalities of structure, or malformations of cortical development, are an important cause of refractory epilepsy in adults (Li et al., 1995); their prevalence in children with refractory epilepsy is less well studied but is increasingly recognized. Normal development of the cerebral cortex occurs through a co-ordinated programme of cellular events, including cell division, migration, selective cell death and
differentiation. Neurons and glia are produced via cell proliferation in the ventricular and subventricular zones, followed by sequential migration and layer-specific differentiation to form the laminated cortical plate. The disruption of these processes, especially cell migration, may lead to a variety of symptomatic malformations (Ferrer and Catala, 1991; Palmini et al., 1992; Lee et al., 1994; Flint and Kriegstein, 1995; Kuzniecky, 1995; Barkovich and Kuzniecky, 1996; Dobyns et al., 1996).

A major manifestation of developmental malformations is epilepsy (Palmini et al., 1991; Barkovich and Kjos, 1992; Farrell et al., 1992; Raymond et al., 1994; Mattia et al., 1995; Battaglia et al., 1996). The pathogenesis of epilepsy associated with developmental abnormalities in general is poorly understood, though there is extensive evidence for the intrinsic epileptogenicity of such lesions (Francione et al., 1994; Palmini et al., 1995). Histological studies of such cases indicate many abnormalities at various levels, including changes in cell structure and composition (Taylor et al., 1971; Manz et al., 1979; Duong et al., 1994), neuronal distribution (Meencke and Janz, 1984) and cortical composition and organization (Ferrer et al., 1992). Recent studies have concentrated on immunohistochemical findings in focal cortical dysplasia. In all three cases studied by Spreafico et al. (1998), an increased number of giant (excitatory) pyramidal neurons, a decrease in inhibitory γ-aminobutyric acid (GABA)ergic neurons and abnormal baskets of parvalbumin-positive terminals around presumed excitatory neurons were found in dysgenetic regions. Ying et al. (1998) demonstrated alterations in glutamate receptor subtype in dysplastic neurons, suggesting that they are hyperexcitable. Neither connectivity nor the inhibitory components of neuronal networks were studied, although there is a suggestion from such work that a network imbalance of excitation and inhibition may be fundamental to epileptogenesis. Heterotopia, both subcortical and periventricular, are an important class of malformation and have aroused much interest recently because they are common, refractory to medical and surgical treatment, and may provide insight into the genetic control of human cerebral development (Huttenlocher et al., 1994; Eksioglu et al., 1996; Musumeci et al., 1997). In the case of heterotopic nodules, despite a few structural and functional studies (Francione et al., 1994; Dubeau et al., 1995; Eksioglu et al., 1996), the epileptogenesis is poorly understood.

An important element of the balance between excitation and inhibition is the GABAergic system. Identification of components of the GABAergic network has been facilitated by immunohistochemistry (Wolff et al., 1984; Hendry et al., 1989; Kisvarday et al., 1990; Hornung and De Tribolet, 1995).
Staining for calcium-binding protein (calretinin, parvalbumin) and neuropeptide Y (NPY) identifies putative GABAergic neurons. These markers may be differentially expressed in these neurons (Hornung et al., 1992; del Rio and DeFelipe, 1996, 1997; Sorvari et al., 1996). Several forms of modification of GABAergic neurons have been demonstrated in animal and human studies. Marco et al. (1996), for example, showed reduction of inhibitory circuitry with the loss of parvalbumin-immunoreactive chandelier cells in the human epileptogenic temporal neocortex, implicating an imbalance of excitation and inhibition in the mechanism of seizure generation. NPY, a signalling peptide with anticonvulsant activity (Baraban et al., 1997; Woldbye et al., 1997), is another useful marker for specific inhibitory neuronal cell populations in the cortex. We therefore chose to study the GABAergic composition of a variety of heterotopic neuronal nodules using antibodies specific for calretinin and NPY.

Circumstantial evidence suggests that heterotopic nodules may be connected to extranodular regions (Dubeau et al., 1995), allowing propagation of postulated intrinsic nodular epileptogenic activity. We therefore also attempted to examine the connectivity of nodules using carbocyanine dye staining techniques. Carbocyanine dyes are fluorescent lipophilic dyes. 

**Fig. 2** Examples demonstrating the gross morphology of the excised pathological tissues from all four cases: Case 1 (A), Case 2 (B), Case 3 (C) and Case 4 (D). In A and C the white box indicates the location of the regions used for subsequent immunohistochemistry and for carbocyanine dye tracing. In B the arrow indicates a subcortical nodule. Note the undulating boundary of the cortical plate and white matter. In D two periventricular nodules, which protrude into the ventricle, are indicated with arrows. Scale bar = 10 mm.
Fig. 3 LBCV staining of tissue sections from Case 3 (A and C) and Case 4 (B and D), showing cell densities in the cortex (A and B) and the nodule/white matter (C and D). In Case 3 (C) the fibres, which are also stained by this method, can be seen coursing around the nodule. In the neonatal Case 4 (D), where fibres lack the myelination required for fibre staining, the nodule is seen protruding into the ventricle and contains a high density of cells. Scale bar = 200 µm.

which travel along fixed cell membranes by passive diffusion (Godement et al., 1987). The placement of crystals of the carbocyanine dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) adjacent to the nodules in the fixed tissue facilitated DiI transport along processes which traversed the nodules and analysis of the extent of innervation into and out of the nodules. The question of how aberrant cortical development may lead to epileptic seizures has thus been addressed in the context of more specific questions regarding the nature of the cells which constitute the nodules and their relative connectivity. Though the overlying cortex may also be abnormal, this report concentrates on the nodules themselves.

Case reports

Case 1
Right hemispherectomy specimen from a 9-year-old male. Accidental cerebral injury at 17 weeks of gestation during amniocentesis. Seizure onset at 6 months with jerking of left arm and subsequent generalization. Further seizure types including episodes of convulsive and non-convulsive status. Cognitive decline over 3 years prior to surgery. The seizures were refractory to treatment with a wide variety of drugs, including carbamazepine, phenobarbitone, phenytoin and vigabatrin, the latter causing a violent behavioural disorder.

MRI scan (Fig. 1A) showed enlarged ventricles, a paucity of gyri in the right frontotemporal region with thickening of the cortex locally, loss of white matter and marked insular cortical attenuation. After hemispherectomy the patient was seizure-free at last follow-up, 1 year after surgery, with improved behaviour and no epileptiform activity on EEG.

The tissue obtained from the surgical treatment was immediately fixed in formalin. The gross morphology of the excised pathological tissue is illustrated (Fig. 2A). The hemisphere had a small defect in the insular cortex, where it was replaced by glial tissue. The adjacent cortex was very thick and irregular. There were periventricular heterotopic nodules of neurons beneath this abnormal cortex.

Case 2
Probable seizure onset at 7 months of gestation (mother noted episodic rhythmic movements in utero); forceps delivery due to large foetal head. Generalized twitching noted immediately after birth, uncontrolled for 24 h. Various seizure types, refractory to treatment. Developmental delay followed by regression for 2 years prior to surgery. Examination revealed left hemiparesis and hemianopia, head circumference 97th centile, weight 3rd centile, with a linear pigmented naevus over the right forehead. MRI at 10 years of age (Fig. 1B) showed enlargement of right hemisphere with absence of
detectable gyri. After right hemispherectomy there was developmental and behavioural improvement; at 12 months follow-up the patient was seizure-free.

The specimen, obtained by surgical treatment at 10 years of age and immediately fixed in formalin, showed dilatation of the lateral ventricle with reduction in volume of white matter, which showed widespread spongy multicystic change. Several small heterotopic neuronal nodules were seen close to the ventricular wall and subcortically. A representative area of resected tissue from Case 2 is shown in Fig. 2B.

Case 3
Left hemispherectomy specimen of a 6-year-old female with severe intractable epilepsy, starting at 8 weeks of age, following a normal birth and delivery. Marked behavioural difficulties, mild right hemiparesis. Seizures refractory to treatment; vigabatrin led to non-convulsive status epilepticus. MRI brain scan revealed abnormality in the left frontal lobe, with abnormal agric grey matter and absence of subcortical white matter. She was seizure-free at 6-month follow-up.

The tissue obtained from the surgical treatment was immediately fixed in formalin. Examination of the fixed specimen (Fig. 2C) revealed a large area of the frontal cortex to be thickened and irregular. There were numerous nodules of heterotopic grey matter occupying almost the entire white matter of the frontal lobe.

Case 4
Dysmorphic term infant with hydrocephalus and polymicrogyria, who died at 5 days of age. This male infant was born by emergency caesarean section for foetal distress. The baby was hydrocephalic with bilateral cleft lip and palate, born by emergency caesarean section for foetal distress. The brain, which was removed and formalin-fixed post-mortem, was found to be abnormal with grossly dilated lateral ventricles and an irregular cortical gyral pattern with multiple small gyri. Almost confluent nodules of heterotopic grey matter were seen in the subependymal zone (Fig. 2D). Histology confirmed virtually confluent subependymal neuronal heterotopia, polymicrogyria, agenesis of the corpus callosum and hydrocephalus. There was aqueduct forking with a cluster of narrow irregular ependymal channels in the midbrain. The inferior olives were malformed and appeared as thick C-shaped bands. There were thick bands of tissue containing groups of neurons on the inferolateral surfaces of the medulla in the site of the corpus pontobulbare. The cerebellum was normal. The central canal of the spinal cord was replaced by multiple small channels. The appearances were of a complex series of neuronal migration disorders involving cerebral and medullary migration pathways. Confluent subependymal heterotopia associated with polymicrogyria has been described in three other males associated with an X-chromosome (Xq28) mutation (Fink et al., 1997).

Control cases
Cortical tissues from a neonate and children aged 7 and 9 years who had died from causes not related to neurological diseases were used as a comparison for immunohistochemical staining.

Method
Histological analysis
As part of routine pathological diagnosis, representative blocks of tissue were taken and 10 μm paraffin-embedded tissue sections cut. Sections were dewaxed and hydrated through descending changes of alcohol solutions, stained with haematoxylin and eosin as well as luxol fast blue and cresyl violet (LBCV), dehydrated and mounted. Sections were examined and bright-field photomicrographs prepared using a light microscope (Leica, Wetzlar, Germany).

DiI analysis
Crystals of DiI (Molecular Probes, Eugene, Oreg., USA) were placed in the formalin-fixed tissue blocks adjacent to or within the heterotopic nodules, using a thin stainless steel wire under an operating microscope. Tissues were incubated at 37°C in 4% (w/v) paraformaldehyde in phosphate buffer (PB) for 6 months, or 3 months for Case 4 due to the younger age with less myelination, allowing faster DiI membrane transport. Tissues were then embedded in 5% (w/v) agar and 65–100 μm sections cut on a vibratome (General Scientific, Redhill, UK). Sections were counterstained with bisbenzimide [10 min in 2.5 μg/ml solution in PBS (phosphate-buffered saline); Riedal-De Haen AG, Seelze-Hannover, Germany] to reveal chromatin in cell nuclei and thus the major boundaries and layers in the sections. Sections were coverslipped under Hydromount (National Diagnostics). The extent of DiI transport along fibres was analysed by fluorescence microscopy (Leica). Colour photomicrographs were taken using Kodak Ektachrome colour reversal film (400 ASA).

Immunohistochemistry
Immunohistochemistry was performed using antibodies directed against calretinin (rabbit polyclonal; SWant, Bellinzona, Switzerland) and NPY (rabbit polyclonal, Sigma-Aldrich, Poole, UK). Immunoreactivity in the heterotopia cases was compared with that of age-matched control cortex from a patient with no known neurological condition. Sections of fixed tissue were cut either on a vibratome (as described for DiI analysis) or a freezing microtome. For freezing microtome sectioning, tissues were immersed in 30% sucrose in PB overnight. Sectioned tissues (50 μm) were rinsed in two changes of PBS. Sections were blocked for 30 min with 5% (v/v) normal goat serum, 0.3% Triton X-100 in PBS, and then incubated at 4°C overnight in primary antibody diluted (1 : 5000 for calretinin and NPY) in 1% (v/v) normal...
Fig. 4 Myelin staining in Case 1 reveals pathological organization of the white matter with the ectopic islands of nodules and their relative isolation. (A) Normal appearance of cortex and white matter taken from an adjacent region of the same section. (B) Photomicrograph taken from the region in which heterotopic nodules are clearly visible. Note the highly abnormal pattern of fibre stain delineating the abnormal folding and surrounding the periventricular nodules (arrows). (C) High-power view of the nodules at the ventricular surface. Note their relative isolation, with relatively few fibres penetrating them. Scale bar = 1 mm in A and B, 500 µm in C.
Fig. 5 Examples of DiI tracing of fibres from sites outside the heterotopic nodules. (A and B) Case 1 nodule in which bisbenzimide labelling (A, blue) reveals the boundaries of the nodules and the ventricular surface (A, lower right corner), and DiI labelling (B, orange) of the same field demonstrates the vast majority of labelled fibres coursing around but not within the nodules. A thin fibre bundle separates two adjacent nodules. The labelled crescent-shaped structures in the nodule are blood vessel artefacts. (C–E) A single carbocyanine dye crystal was placed 2–3 mm to the left of the nodule in Case 2. The position of crystal placement (C, orange circle) is shown relative to the nodule (C, dark blue circle) with the area of interest between the two highlighted (C, white box). Some fibres are seen extending across the margin of the nodule (D, small arrows) and have punctate termini close to cell bodies within the nodules, viewed by bisbenzimide labelling (E, blue cells). (F and G) In Case 4 there is a high density of cells within the nodule visualized with bisbenzimide (F) and few DiI-labelled fibres are present in the nodule (G). Scale bar = 200 mm in A and B, 1.3 μm in C, 100 μm in D, E, F and G.
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Assessed included cell size, process length and the number and drawn with the aid of the camera lucida attachment. The morphology in different regions. Neurons were viewed at

Fig. 6 DiI labelling of fibres from a single DiI crystal (orange) placed into the nodule and cells labelled for bisbenzimide (blue). (A and B) Case 4 with a large nodule close to the ventricular surface (A, arrows) very intensively labelled; few fibres are seen. There is relatively little labelling in the surrounding tissue, in contrast with crystal placements directly into the white matter (B, arrows). (C–E) In Case 1, a nodule revealed by bisbenzimide staining (C) was labelled by DiI (D) to reveal fibres extending outside the boundary (arrow) of the nodule (E, enlarged view of boxed region in D). (F–I) In Case 3, DiI crystal placement in one of a cluster of nodules (F, arrow) labelled fibres within the nodule (G) but also connected fibres in adjacent nodules (enlarged in H and I, arrows). Scale bar = 1.3 mm in A, B and F, 400 μm in C, D and G, 100 μm in E, 200 μm in H and I.

In Case 1 (Fig. 4), staining in the white matter revealed axons streaming around the nodules, without significant penetration. The normal gross appearance of other parts of the cortex and white matter taken from the same tissue section is illustrated (Fig. 4A). Histology of the malformed cortex showed cortical disorganization with malorientation of neurons, which were divided into irregular clusters by bands of glial cells and myelinated fibres. Neuronal and glial ectopias were seen in the leptomeninges. These appearances were considered to be consistent with traumatic brain puncture at 17 weeks gestation and interruption of neuronal migration and corticogenesis (Fig. 4B and C).

Similar patterns were observed in the other cases (Fig. 3 and data not shown). The resected cortex from Case 2 was thick with many small gyri. Histology showed a variety of histological abnormalities, including complete lack of normal lamination. In some areas polymicrogyria was seen and there were foci of meningeal glioneuronal heterotopia. Abnormal giant dysplastic neurons were seen throughout the cortex and in the white matter. Fibre connections were seen for nodules adjacent to the ventricular surface (Fig. 4C).

Histology from Case 3 showed the cortical malformation to have the characteristic features of polymicrogyria, with neurons forming a single band festooned beneath fused surface layers. The appearance was that of a focal cortical dysplasia with underlying neuronal heterotopia.

Camera lucida drawings of immunoreactive neurons present in the 50 μm experimental sections were used to assess cell morphology in different regions. Neurons were viewed at high magnification (×40 objective) using the light microscope and drawn with the aid of the camera lucida attachment. The features of neuronal cell morphology that were specifically assessed included cell size, process length and the number and extent of dendritic branching.

Results

Histological studies

Haematoxylin and eosin and LBCV histology was carried out to examine the structure of the nodules and overlying cortical plate. Heterotopic nodules in all cases consisted of well-defined masses of grey matter with randomly arranged large and small neurons (Fig. 3). The cellular composition of the nodules, distinct from the surrounding periventricular white matter, is clearly seen in Case 4 (Fig. 3D), in one region of the almost confluent subependymal neuronal heterotopia.

For control experiments, the primary antibody was replaced by 0.3% Triton X-100 in PBS and then reacted as above. These control sections showed no positive immunoreactivity for any of the antibodies used. This protocol was also adapted to stain paraffin wax sections (10 μm) of the same tissues. Slides were dewaxed in xylene and hydrated through descending changes of alcohol solutions, and immunohistochemistry was performed as described above, with PBS alone used as wash buffer. Bright-field photomicrographs were prepared by light microscopy.

Morphological analysis of immunoreactive neurons

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DiI tracing in and around the nodules

Neural connectivity between the nodules and their environment was investigated using the technique of carbocyanine dye tracing, which permitted analysis of the extent of fibre projection into and out of the nodules. DiI tracing of fibres from sites outside the heterotopic nodules combined with bisbenzimide labelling (Fig. 5A) revealed the boundaries of the nodules and the ventricular surface. In Case 1, the vast majority of DiI-labelled fibres coursed around but not within the nodules (Fig. 5B). A thin fibre bundle separated two adjacent nodules. This pattern of fibres skirting the nodule was also seen for Case 2 (Fig. 5C–E). At high magnification, some fibres in this region were seen to penetrate the nodule (Fig. 5D) and to have punctate terminals, possibly forming synapses, in the vicinity of cell bodies within the nodules (Fig. 5E). This pattern, in which a small fraction of labelled fibres penetrated the nodules, was also observed in Case 3 (data not shown). In Case 4 (Fig. 5F and
Fig. 7 Immunohistochemistry for calretinin (A–C) and NPY (D–F) in Case 1. (A) Calretinin-immunoreactive cells in normal cortex from a 7-year-old (control case). (B) Calretinin-immunoreactive cells in the cortex from Case 1. (C) A subcortical nodule stained for calretinin. (D) Small periventricular nodules stained for NPY. (E) High-power photomicrograph of NPY-immunoreactive cell bodies and processes in the cortex. (F) High-power photomicrograph of NPY-immunoreactive cell bodies and processes in a heterotopic nodule. Scale bar = 200 µm in A, B, C and D, 100 µm in E and F.

G), the high density of cells within the nodule appeared to present a barrier to the penetration of most fibres. The extent of connectivity between nodules and their cellular environment was also assessed by DiI labelling of fibres (orthogradely and retrogradely) by placement of a single DiI crystal into the nodule (Fig. 6). In Case 1, a bisbenzimide-
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Fig. 8 Immunohistochemistry for calretinin (A, B and D) and NPY (C and E) in Case 2. (A) Abnormal clusters of calretinin-positive neurons (arrows) are seen in the lower cortical plate as well as a heterotopic nodule (lower boxed region). (B) High-magnification photomicrograph of the upper boxed region indicated in A, showing clustered distribution of calretinin-positive neurons in the cortical plate. (C) Distribution of NPY-positive neurons in the cortical plate of an adjacent section. NPY is present in a smaller proportion of neurons than calretinin. (D) High-magnification photomicrograph of the lower boxed region (nodule) indicated in A, showing a high density of calretinin-positive neurons in the nodule, comparable to that in the cortical plate. (E) NPY immunoreactivity in a nodule from a section adjacent to D. Very few NPY-positive neurons were found within such nodules. Scale bar = 1 mm in A; 400 µm in B, 200 µm in C, D and E.

Immunohistochemical analysis of neurons in nodules and cortex

The expression of the neuronal markers calretinin and NPY, which are expressed by GABAergic interneurons, in the labelled nodule (Fig. 6C), also labelled by DiI (Fig. 6D), demonstrated some fibres extending outside the boundary of the nodule (Fig. 6E). Similar observations were made for Case 2 (data not shown). In Case 3, DiI placement in one nodule (Fig. 6F), labelled not only fibres within this nodule (Fig. 6G) but also fibres in adjacent nodules (Fig. 6H and I). This indicates that neuronal connections existed between these heterotopic nodules. In Case 4 (Fig. 6A and B) a large nodule close to the ventricular surface was extensively labelled, although few labelled fibres were seen to leave the region (Fig. 6A). There was relatively little labelling in the surrounding white matter, in contrast to the situation when the DiI crystal was placed directly into a similar position in the normal white matter (Fig. 6B). The majority of labelled cells of the nodule clearly did not have extensive projections beyond the nodule boundaries, whereas similar crystal placements into the neighbouring white matter resulted in substantial labelling over a 2–4 mm radius. In summary, DiI crystal placement within nodules and in the adjacent normal white matter revealed a few labelled fibres which crossed the borders of the nodules, suggesting limited connectivity with neurons outside the nodules, including those located in other nearby nodules.

The expression of the neuronal markers calretinin and NPY, which are expressed by GABAergic interneurons, in the
resected tissues was examined using immunohistochemistry. The extended fixation of these tissues in formalin rendered the detection of the inhibitory neurotransmitter GABA difficult, but allowed excellent antibody detection of the large calretinin and NPY molecules. Calretinin and NPY immunoreactivity was observed both in the cortex and in heterotopic nodules from each of the cases. Calretinin-immunoreactive neuronal cell bodies and processes were seen in cortex from Case 1 (Fig. 7B), although abnormal clustering of these neurons was apparent, compared with age-matched control cortex (Fig. 7A). The density of calretinin-positive cells in the cortical plate (Fig. 8B) was similar to that observed in the nodule (Fig. 8D), although the extent of stained neuronal processes in the nodule was reduced. In Case 2, NPY was present in a smaller proportion of neurons in the cortical plate (Fig. 8C) than was calretinin (Fig. 8B). There were also very few NPY-positive cell bodies present within the nodule (Fig. 8E) relative to calretinin-positive cell bodies (Fig. 8D). Nevertheless, the presence of extensive NPY-positive processes in Case 2 nodules was comparable to that observed in Case 1 (Fig. 7F).

Comparison of immunohistochemistry in the cortical plate (Fig. 9A and C) and a subcortical nodule (Fig. 9B and D) from Case 2 revealed some differences in neuronal morphology. Of particular interest is the observation that neurons in the nodule (Fig. 9B) did not appear as morphologically mature as those in the overlying cortical plate (Fig. 9A).

In the cortical plate, a cluster of NPY-positive neurons appeared against a background of punctate NPY staining. While there was only one NPY-positive neuronal cell body

![Fig. 9 Comparison of high-power magnification micrographs showing immunohistochemical distributions of calretinin- and NPY-positive neurons in the cortex (CTX; A and C) and a subcortical nodule (NOD; B and D) from Case 2. (A and B) Calretinin immunoreactivity in neurons from overlying cortical plate (A) and a subcortical nodule (B). While the density of the calretinin-positive neurons is similar in the nodules and cortical plate, the neurons in the nodules have less extensive processes, suggesting a reduced level of maturation relative to the cortical plate. (C and D) NPY immunoreactivity in an adjacent section containing cortical plate (C) and a subcortical nodule (D). In the cortical plate, a cluster of NPY-positive neurons is shown against a background of punctate NPY immunoreactivity. While there is only one NPY-positive neuronal cell body at the edge of this nodule, the nodule itself (D, lower left) is demarcated by strong punctate NPY immunoreactivity which is in contrast to the white matter (D, upper right). Scale bar = 100 µm.](https://academic.oup.com/brain/article-abstract/122/2/219/357445)
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Fig. 10 Immunohistochemical analysis of calretinin (A–D) and NPY (E) immunoreactivity in Case 3. The high density of calretinin-positive neurons within the nodules was consistently found. (A) Calretinin-immunoreactive neurons in the cortex. (B) Calretinin-immunoreactive neurons in nodules. The immunopositive processes rarely extend outside the boundary of the nodule. (C) Calretinin-immunoreactive neurons. (D) Calretinin-immunoreactive neurons in a nodule. (E) NPY-immunoreactive neurons from a periventricular nodule. Scale bar = 400 µm in A and B, 100 µm in C, D and E.

at the edge of this nodule, the nodule (Fig. 9D) also had characteristic punctate NPY immunoreactivity, which is in contrast to the white matter (right side of Fig. 9D). Labelling of processes was confined to the nodule and did not extend far into the neighbouring white matter.

Analysis of calretinin and NPY immunoreactivity in Case 3 (Fig. 10) revealed distributions of immunoreactive neurons comparable to those seen for Cases 1 and 2. While calretinin immunoreactivity highlighted the abnormal gyri of the cortex (Fig. 10A), multiple periventricular nodules in close proximity were also calretinin-positive (Fig. 10B). The differences in morphology between calretinin-positive neurons in the cortex (Fig. 10C) and nodule (Fig. 10D) were not as pronounced as in Case 1 (Fig. 7) or Case 2 (Fig. 9). A few NPY-positive neurons were found within the nodules of Case 3 (Fig. 10E).

The contrast of immunoreactivity and neuronal cell morphologies in the cortex and nodules was also observed for Case 4 (Fig. 14 and data not shown). The high density of calretinin-positive neurons within the nodules was thus found consistently across cases (Figs 7–10).

Morphological differences between neurons present in nodules and in the overlying cortical plate were assessed from camera lucida drawings of immunoreactive neurons. Using the morphological criteria of cell size, process length...
Fig. 11 Camera lucida drawings from Case 1 of all calretinin-immunoreactive neurons present in a periventricular nodule (CR NOD; inset with arrow) and from all cortical cells of a region in the overlying cortex indicated with a box (CR CTX). Calretinin-positive cells appear less morphologically complex in the nodule than in overlying cortex. Scale bar in inset = 1 mm; 100 μm for cells.
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and the number of process branches, the calretinin-positive neurons in the cortex were clearly more mature than those within the nodule, as shown in Case 1 (Fig. 11). This relative immaturity was also seen when comparing NPY-positive neurons in the cortex and nodule (Fig. 12). Interestingly, even the occasional white matter NPY neurons appeared more mature than the nodular NPY cells (Fig. 12).

This difference in length and complexity of processes was also seen in Cases 2 and 3 (Figs 9A and C and 13), when comparing calretinin-positive neurons of the cortex (Fig. 13, CR CTX) and the subcortical nodule (Fig. 13, CR NOD). The difference in neuronal morphology was still present, although less pronounced, when comparing NPY-positive neurons from the cortex (Fig. 13, NPY CTX) and nodule (Fig. 13, NPY NOD).

The observations made in Cases 1–3 were similar to those made in Case 4, although, since this case was neonatal, there was a somewhat different pattern of expression of the developmentally regulated proteins calretinin and NPY. Immunoreactive neurons in the cortex of Case 4 did not have the same level of maturity as those in the cortex of the older cases, which was not unexpected. Camera lucida drawings of immunoreactive cells in Case 4 are shown (Fig. 14). These results also demonstrate a disruption of the developmental maturation of neurons within the nodules, which appeared to have remained extremely immature with minimal differentiation.

In summary, immunohistochemical analysis of tissues from these cases of heterotopia reveals differences in neuronal cell morphologies between the cortex and the underlying nodules. Of great interest is the observed reduction in the morphological maturation of interneurons in the nodules relative to that in the cortex.

Discussion

It is widely accepted that cortical malformations can be intrinsically epileptogenic. There is clinical evidence for this, ranging from their association with epilepsy in imaging studies (Li et al., 1995; Raymond et al., 1995), results of
Fig. 13 Camera lucida drawings of calretinin- and NPY-immunoreactive neurons present in nodules and the overlying cortical plate from Case 2. Individual neurons were drawn by camera lucida from the regions indicated with boxes in Fig. 8A. Representative calretinin-positive neurons are illustrated from the cortex (CR CTX) and a nodule (CR NOD). There are clear differences in the extent of processes and average soma size. The cells positive for calretinin in the nodule appear smaller and have fewer and less extensive processes. This difference is less apparent with respect to NPY-positive neurons from the cortex (NPY CTX) and a nodule (NPY NOD). Scale bar for all cells = 100 µm.
Fig. 14 Camera lucida drawings of calretinin immunoreactive neurons in Case 4 from the cortical plate (CR CTX) and nodule (CR NOD). The regions in the representative tissue section from which neurons were drawn are boxed. Scale bar in inset = 1 mm; 100 µm for cells.
direct electrical recording from abnormal tissue (Dubeau et al., 1995) and outcome after surgical resection (Palmini et al., 1995). In vitro studies of resected tissue and modelled lesions support these findings. For heterotopia in particular, there is much indirect evidence for their epileptogenicity. Heterotopic nodules contain neurons which are metabolically active (Hatazawa et al., 1996; Marsh et al., 1996) and may be connected to extranodular targets that are appropriate, given the neurons’ likely birth dates (Jensen and Killackey, 1984). Periventricular nodules are the most common dysgenetic findings in refractory epilepsy in adults (Raymond et al., 1984); both subcortical and periventricular nodules have been reported very rarely in adult patients in the absence of epilepsy or a family history of epilepsy. This circumstantial evidence is supported by the finding that surgical resection leaving periventricular nodular tissue behind does not render patients seizure-free (Li et al., 1997), and by direct electrical recordings from heterotopic nodules demonstrating independent ictal activity in two cases (Francione et al., 1994; Dubeau et al., 1995). Such reports are rare, since most patients do not undergo presurgical evaluation in view of the recognized poor seizure prognosis after surgery. The cellular basis of this epileptogenicity, however, is not well understood. At the cellular level, we have shown a number of abnormalities in heterotopic nodules and the overlying cortex that may provide a preliminary structural basis for understanding their intrinsic epileptogenicity.

GABAergic neurons and an imbalance of excitatory and inhibitory mechanisms in epilepsy

There are clinical (Marco et al., 1996; Spreafico et al., 1998) and animal model (Buhl et al., 1996) studies which implicate disorders of GABAergic interneurons in epilepsy. Despite the varying macroscopic appearances from the four cases, we found broadly similar cellular composition within the nodules. Nodules all contained GABAergic neuronal populations, as judged by staining for calretinin and NPY. The GABAergic neurons appeared immature, consistent with the results of a proton magnetic resonance spectroscopy study which suggested increased cellular activity or persistence of immature neurons in a case of grey matter heterotopia (Marsh et al., 1996). During development, immature/early GABAergic cells are known to be excitatory and later become inhibitory, via modulation of GABA_A receptor function (reviewed by Ben-Ari et al., 1997). Slowed or arrested development within the nodules, as shown by the morphological immaturity of the cells, might prevent this functional maturation of GABAergic synapses. Excitatory pyramidal neurons, immunopositive for the microtubule associated protein MAP2 have been identified within nodules in these cases (Mirkovic et al., 1998). Hence, the nodules themselves might have an overall excitatory bias. The refractory nature of epilepsy in Cases 1–3 could be due to immaturity in the nodular neurotransmitter phenotypes (reviewed by Ben-Ari et al., 1997) affecting the molecular targets of pharmaceutical intervention.

Aberrant activity of inhibitory GABAergic neurons in adults may be associated with epilepsy. It is thought that this is part of the epileptogenic mechanism in abnormal corticothalamic activity in primary generalized epilepsy (Duncan and Panayiotopoulos, 1995). Vigabatrin is an antiepileptic drug that inhibits GABA-transaminase and thus increases brain GABA levels (Petroff et al., 1996). The immaturity of GABAergic neurons we have observed in nodules in these cases indicates that vigabatrin would actually increase excitation, through the action of GABA as an excitatory transmitter in immature neuronal networks. In this context the deterioration in clinical condition brought about by treatment with vigabatrin in Case 1 is easily understood and mimics the aggravation of seizure activity in primary generalized epilepsy that vigabatrin can cause.

Nodular connectivity and pathogenic implications

Our examination of the connectivity of nodules showed that the nodules did have connections, although sparse, with extranodular regions. Some of this connectivity may have been with other adjacent nodules, as shown explicitly in one case, but the possibility of connection with the cortex cannot be excluded as DiI labelling can only mark axons for a short distance. The relatively few NPY-positive neuronal cell bodies within the nodules and the presence of intranodular punctate staining for NPY (which is known to be localized within axons, dendrites and synapses as well as cell bodies) suggests that some of the nodular NPY arose from the processes of extranodular neuronal cell bodies, which would be consistent with the fibre staining data. The small number of intranodular NPY-positive neuronal cell bodies is also of interest in the light of the anticonvulsant activity of NPY (Baraban et al., 1997; Woldbye et al., 1997). Thus, it is likely that the nodules have reciprocal connections, however sparse, with other parts of the hemisphere, including the cortex. This would allow postulated focal discharges arising in the nodules to spread to the cortex, which is known to be an excellent amplifier.

Cortical abnormalities associated with neuronal heterotopia

Although not the main focus of this study, our observations that there were abnormal clusters of neurons within the cortical plate itself raises the possibility that epileptic activity may also be due to abnormal neuronal distribution and connectivity in the cortical plate. During brain development the defect which leads to the formation of nodules may also induce abnormalities of connection or cell type or number in the more normal-appearing, adjacent neocortex. It is possible that these abnormalities could be responsible for, or contribute to, the epilepsy. In addition to these findings, quantitative MRI analysis suggests that patients with apparently focal subcortical heterotopia (Sisodiya et al., 1995) and male patients with periventricular heterotopia (unpublished results) also have
other, visually occult, structural abnormalities of the neocortex, raising the possibility of dual pathology.

In summary, we have shown that the abnormal immaturity of GABA networks within nodules could produce an excess of excitatory over inhibitory neuronal circuitry, thus inducing pathological activity. We have also identified connections between neurons in different nodules and shown that intranodular neurons are connected to other regions of the brain, possibly including the cortex itself. Thus, intrinsic nodular epileptogenicity might be communicated to the cortex. The cortex is a highly effective amplifier of synchronized activity and therefore few connections are required to set off cascades of activity, including epileptiform discharge. Therefore we have shown that the nodular tissue structure and composition might be structurally capable of acting as epileptogenic substrates, and that abnormal activity may be transmitted by connections from the nodules. In addition, abnormalities of cellular composition, as well as cytoarchitecture and interneuronal connectivity, of the overlying cortex have also been demonstrated. These changes may allow the cortex either to act independently as an epileptogenic substrate, providing further histological confirmation of the dual pathology concept, or to amplify and propagate the epileptiform activity generated within the nodules themselves.

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References
Hornung JP, De Tribolet N Chemical organization of the human


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