Original article

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Heterotopic reelin in human nodular heterotopia: a neuropathological study

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ABSTRACT – *Aim*. The extracellular matrix glycoprotein reelin plays a crucial role in the control of neuronal migration and during development is expressed by Cajal-Retzius cells in the marginal zone. The purpose of this study was to investigate the possible involvement of reelin in the pathogenesis of human nodular heterotopia, a malformation of cortical development frequently associated with focal drug-resistant epilepsy. *Methods*. Five patients presenting with subcortical nodular heterotopia and referred for epilepsy surgery, after a comprehensive presurgical investigation, were considered. The surgical specimens were studied by combining immunohistochemistry, double immunofluorescence, and *in situ* hybridisation procedures. *Results*. The selected cases were characterised by the presence of multiple nodules presenting in the core cell-free zones, reminiscent of the cortical molecular layer. In all cases, small reelin-positive cells, without typical Cajal-Retzius cell features, were distributed inside the nodules and localised in these cell body-sparse regions. *Conclusion*. The presented data corroborate the hypothesis that reelin might be involved in human heterotopic nodular formation.

Key words: reelin, nodular heterotopia, human epilepsy, immunocytochemistry

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Reelin is an extracellular matrix glycoprotein secreted by early-generated Cajal-Retzius (CjR) cells in the marginal zone (MZ), underneath the pial surface. Its absence causes an inversion of the cortical layers in the *reeler* mutant mouse (Curran and D'Arcangelo, 1998), granular cell dispersion both in experimental and human temporal lobe epilepsy (Haas and Frotscher, 2010), and lissencephaly with cerebellar hypoplasia in an autosomal recessive human disorder (Zaki *et al*., 2007). These findings suggest that reelin is involved in the control of normal migration and laminar arrangement during brain development. Heterotopic reelin, expressed in the developing mouse cortex, induces migrating neurons to form subcortical neuronal aggregates according to an "inside-out" mechanism, whereby late-born neurons migrate past early-born neurons (Kubo *et al*., 2010). These results highlight the

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ability of reelin to form cellular aggregates around reelin-rich regions and suggest a possible role of this protein in the pathogenesis of nodular heterotopia, a human malformation of cortical development characterised by nodules of grey matter in abnormal locations presenting a rudimentary laminar organisation (Garbelli *et al*., 2009). We present an immunohistochemical study, performed on surgical specimens from 5 patients with subcortical nodular heterotopia (SCH), demonstrating the presence of reelin-rich regions in the core of nodular formations.

Methods

Patients

Five of 16 surgical specimens from patients with periventricular nodular heterotopia (PNH; 12 with subcortical heterotopia [SCH] and 4 with subependymal heterotopia [SEH]), who received surgery at the C Munari Epilepsy Surgery Centre for intractable partial epilepsy from May 1996 to October 2008, were retrospectively selected for this study. The cases were selected according to the following neuropathological criteria: the presence of one or more cell-free zones within the nodular heterotopia based on original slides and the availability of vibratome sections suitable for the procedures listed below.

The 5 considered patients underwent surgery only after comprehensive evaluation that, in addition to MRI, comprised neurological examination and history taking in order to establish age at onset, type and frequency of seizures, and comprehensive video-EEG examination with at least one ictal recording to relate ictal EEG events to clinical manifestations. In all the patients, the EEG and MRI data were insufficient to unambiguously locate the epileptogenic zone (EZ) and thus presurgical stereo-EEG (SEEG, with intracerebral electrodes) was carried out.

Tissue procedures

After surgical resection, the specimens were dissected into two adjacent blocks and processed for: 1) neuropathological assessment; and 2) immunohistochemistry (IHC) including immunofluorescence and *in situ* hybridisation (ISH). Tissue from the first block, processed for routine neuropathological analysis, was immediately fixed in 10% formalin and paraffin-embedded; sections (7 μm thick) were stained with thionin, haematoxylin-eosin, Kluver-Barrera, and Bielschowsky, and IHC was performed using anti-glial fibrillary acid protein (GFAP) and anti-non-phosphorylated neurofilament (SMI 311) antibodies. The second block was fixed in 4% paraformaldehyde and 50 μm serial vibratome sections (Leica, Heidelberg, Germany) were processed for: 1) immunostaining with immunoperoxidase using antibodies against neuronal nuclear protein (NeuN), SMI 311, microtubule-associated proteins (MAP2), GFAP, reelin (CR-50), and the calcium-binding proteins parvalbumin (PV), calbindin (CB), and calretinin (CR); 2) double immunofluorescence combining anti-NeuN/CB, anti-NeuN/GFAP, and anti-NeuN/CR-50 antibodies; and 3) ISH using *GAD 65/67* probes combined with immunofluorescence with CR-50 antibody. We used the same methodology as that employed in a previous study (Garbelli *et al*., 2009).

Results

The principal characteristics of the 5 patients are shown in *table 1*. All the patients were suffering from drug-resistant epilepsy, showing a posterior (temporo-parieto-occipital) onset of discharges, on the basis of anatomo-clinical and video-EEG evaluation. MRI revealed unilateral multiple subcortical nodular formations in four patients, located in the temporo-occipital regions, on the right side, with a transmantle organisation. In only 1 patient (Patient 4), the malformation was prevalent on the left side, with nodules on the posterior part of the ventricle and a transmantle extension to the overlying cortex. On the right side, only nodules were visible. Video-EEG recordings demonstrated monolateral onset of seizures, also present in the patient with bilateral malformation. SEEG was considered mandatory considering the clinical symptomatology and the large extension of the malformation. After the correct identification of the location and extension of the EZ, surgery was performed only in the temporal lobe in one patient. In the other 4 patients, a multilobar resection was necessary; temporo-occipital in 2 patients and temporo-parieto-occipital in the other 2 patients. The resection of the malformation was incomplete and not necessary in all the patients. An outcome of Engel class I was achieved in 4 patients and class III in 1 patient (patient with bilateral malformation).

In 3 cases, the histological analysis revealed multiple nodules in subcortical white matter extending from the ventricular wall to the cortex and in 2 cases heterotopic nodules invading cortical grey matter. The overlying cortices were characterised by the presence of laminar disorganisation and discrete gliosis, thus diagnosed as type I focal cortical dysplasia (FCD). In sections processed for NeuN, several nodules of variable size were present in the white (*figure 1A*) and grey matter. Within the nodules, cell-free zones,

Table 1. Main clinical characteristics.

Patient	Sex	Age at surgery (years)	Age at seizure onset (years)	Monthly seizure frequency	Side/site of surgery	MRI	SEEG	Neuropathological diagnosis of cortex	Outcome Engel class	Duration of postoperative follow-up (years)
1	F	20	11	2	Right/ TO	SCH	yes	FCD 1A	I	11
2	F	32	13	60	Right/ TO	SCH	yes	FCD 1A	I	7
3	M	30	16	10	Right/ T	SCH	yes	FCD 1A	I	7
4	F	21	1	30	Left/ TOP	Bilat SCH	yes	FCD 1A	III	6
5	M	35	23	10	Right/ TOP	SCH	yes	FCD 1B	I	4

F: female; M: male; FCD: focal cortical dysplasia; O: occipital; P: parietal; SCH: subcortical and transamantle heterotopia; Bilat: bilateral; T: temporal.

with a maximum diameter of 500 μm, were evident and surrounded by aggregates of densely packed neurons (*figures 1B and 1C*). These cellular aggregates were mainly composed of radially-arranged small and medium-sized pyramidal cells, as highlighted by SMI 311 and MAP2 immunostaining (*figure 1I*). Within these aggregates, CB- (*figure 1C*) and CR- (*data not shown*) positive interneurons, intermingled with pyramidal neurons, were also present, while PV-positive interneurons were scattered within the nodules without any apparent laminar organisation.

In cell-free zones, GFAP-positive glial elements, reminiscent of the radial pattern of superficial gliosis, were observed (*figure 1D*). Scattered reelin-positive cells were present within the core of the nodular formations and distributed inside the cell-free zones (*figures 1E, 1F, and 1J*). Double-labelling experiments combining *in situ* hybridisation with *GAD 65/67* probes and immunohistochemistry for reelin revealed that these cells were reelin-positive interneurons and not typical CjR cells (*figures 1G and 1H*). In the overlying cortex, reelin immunoreactivity was only seen in layer I (*figures 2A and 2B*). The majority of these cells were positive for *GAD 65/67* probes (*figure 2C*), while some of them, with morphological characteristics of typical CjR cells, were GAD-negative (*figures 2D, 2E, and 2F*), in line with their presumed glutamatergic phenotype.

Discussion

Nodular heterotopias are currently considered to be the result of defective neuronal migration in early development. In bilateral subependymal PNH, *filamin I* mutation is reported in 50% of patients whereas no mutation has been demonstrated in SCH. Neuropathological studies performed on autoptic or surgical tissues have demonstrated that the nodules, despite the associated broad clinical and imaging data, have similar morphological characteristics in all cases (Thom *et al*., 2004; Tassi *et al*., 2005). Moreover, by examining the expression pattern of several layer-specific markers, a rudimentary laminar arrangement of the neurons inside the nodules has been demonstrated (Garbelli *et al*., 2009). To explain these features, the authors suggested that displaced reelin-secreting cells during early stages of cortical development may attract migrating neurons to heterotopic positions. This hypothesis was supported by experimental evidence that the heterotopic expression of reelin is able to induce cell aggregates, exhibiting an "inside-out" cell arrangement (Kubo *et al*., 2010), similar to those observed in human nodular heterotopia.

In the present paper, we report an immunohistochemical study performed on 5 surgical specimens presenting multiple heterotopic nodular formations, characterised by several cell-free zones within the nodules, surrounded by dense aggregates of neurons. In specimens from patients not included in this study, we failed to detect cell-free zones. However, such cell-free zones may have been overlooked during neuropathological inspection as a result of their small size or difficulty in identifying these areas due to the extent of the specimen.

Our results, which add to previous observations (Thom *et al*., 2004), show the presence of small

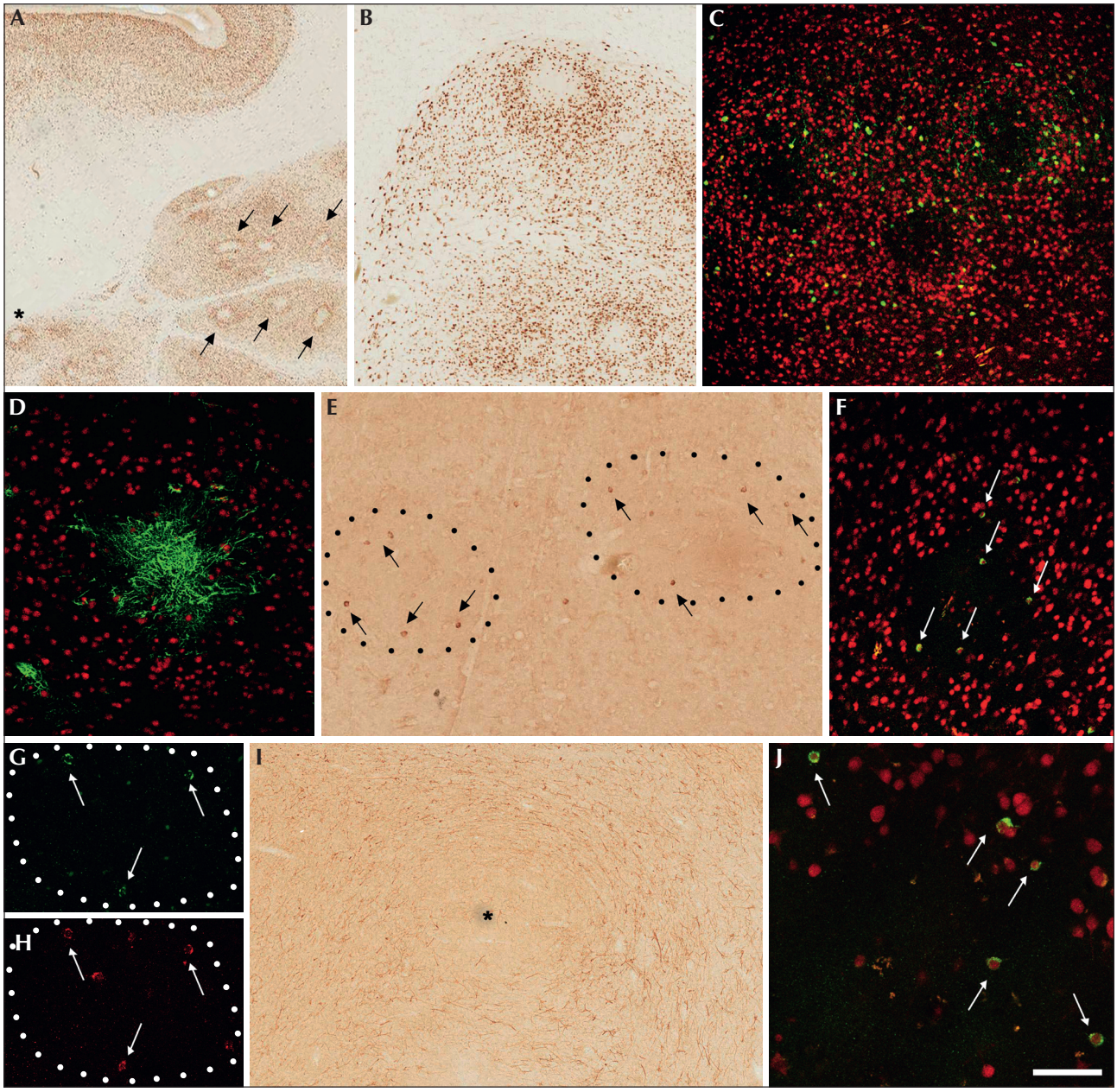

Figure 1. Reelin expression in nodular heterotopia (A) NeuN-immunostained section showing the presence of several nodules with cell-free zones (arrows); the asterisk corresponds to a cell-free zone shown in (B) at higher magnification (C, D) Double immunofluorescence images combining NeuN (red) and CB (green) labelling showing CB-positive cells in the cell aggregates (C), and NeuN (red) and GFAP (green) labelling showing glial elements reminiscent of superficial gliosis in the cell-free zone (D) Reelin-positive cells are concentrated in the cell-free zone (E) which is clearly indicated in double immunofluorescence images (F, J) obtained by combining NeuN (red) and CR-50 (green) labelling (G, H) Double-labelling experiments combining IHC with labelled reelin (green) and ISH with >GAD 65/67 probes> (red) show that reelin-positive cells co-express GABA (I) SMI 311 immunostaining showing a cell-free zone (asterisk) surrounded by aggregates of densely-packed neurons Images A-H are from Patient 1 and I and J from Patient 5. Bars: A: 1.3 mm; B: 302 μm; C: 242 μm; D-F: 130 μm; G-H: 90 μm, I: 370 μm; J: 70 μm.

reelin-positive cells, double-labelled with *GAD 65/67* probes, within the cell-free zone, whereas typical large CjR cells were not detected. In layer I of the overlying cortex, small, reelin-*GAD 65/67* double-labelled cells were present, intermingled with few, large, typical CjR GAD-negative cells.

It is widely recognised that reelin is essential for ordered neuronal migration and normal arrangement of cortical layering. In early development, reelin is selectively synthesized and secreted by specialised neurons, termed CjR cells, confined to the MZ. During the period of maximal migration of the cortex, in addition

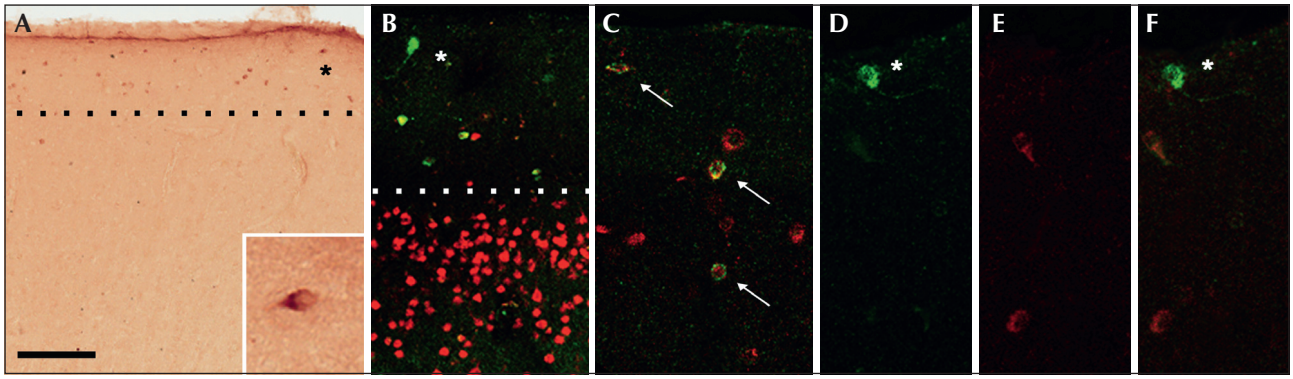

Figure 2. Reelin expression in the cortex (A, B) IHC and double-immunolabelling with anti-NeuN (red) and CR-50 (green) antibodies, showing reelin-positive cells confined to cortical layer I; note that some (asterisks) immediately beneath the pial surface resemble typical CjR cells (see also inset in A) (C-F) Double-labelling combining IHC with labelled reelin (green) and ISH with >GAD 65/67 probes (red) shows that the majority of reelin-positive cells co-express GAD (C; arrows), whereas morphologically identifiable CjR cells are GAD-negative (D-F; asterisks) Dashed lines indicate the boundary between layers I and II. Bars: A: 165 μm (36 μm for inset); B: 143 μm; C-F: 50.

to the early-generated CjR cells, other reelin-positive neurons with different morphological and neurochemical profiles are continuously delivered into the preplate and MZ through both radial and tangential migration (Meyer and Goffinet, 1998). In particular, at six weeks of gestation, before the emergence of the cortical plate, reelin-positive cells which do not display the typical morphology of CjR cells have been observed in the human preplate and, at the same developmental stage, neurons with similar distribution and morphology have been shown to express GABA. The possibility that these early reelin-positive cells co-express GABA has not been directly investigated in humans but has been demonstrated in an early preplate cell population in the rat cerebral cortex (Zecevic and Milosevic, 1997; Meyer *et al*., 1999).

By combining these developmental data and the present results, we can hypothesize that during early stages of cortical development some of the early reelin-secreting cells that are misplaced within the subplate after the emergence of the cortical plate attract migrating neurons to a heterotopic position to form the nodular heterotopia, highlighting the possible role of reelin protein as a key factor in the pathogenesis of nodular heterotopias. □

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