Markers of Cellular Proliferation Are Expressed in Cortical Tubers

Allana Lee, BA,[1] Michelle Maldonado, BA,[1]
Marianna Baybis, MS, Christopher A. Walsh, MD, PhD,[2]
Bernd Scheithauer, MD,[3] Raymond Yeung, MD,[4]
Jack Parent, MD,[5] Howard L. Weiner, MD,[6]
and Peter B. Crino, MD, PhD[1]

p34cdc2, collapsin response mediator protein 4 (CRMP4), doublecortin (DCX), HuD, and NeuN expression was assessed in tuber (n = 16) and subependymal giant cell astrocytoma (SEGA; n = 6) specimens in tuberous sclerosis complex to define the developmental phenotype and lineage of giant cells (CGs) in these lesions. Many GCs exhibited HuD and NeuN immunolabeling suggesting a differentiated neural phenotype. Giant cells in tubers, SEGAs and subependymal nodules in the Eker rat model of TSC expressed CRMP4 and DCX. Tubers and SEGAs exhibit a heterogeneous profile of differentiation and may share a common cellular lineage. Tubers may contain a subpopulation of newly generated cells.

Ann Neurol 2003;53:668–673

Tubers are epileptogenic cortical malformations in tuberous sclerosis complex (TSC).[1,2] Tubers contain abnormal cells known as dysmorphic neurons (DNs) and giant cells (GCs) that express proteins normally detected in progenitor cells within the embryonic ventricular zone (VZ) or subventricular zone (SVZ) such as nestin, vimentin, and polysialylated neural cell adhesion molecule.[3,4] Persistent expression of these proteins in tubers may imply incomplete cellular differentiation or that there is a population of newly generated cells in tubers. GCs rarely express proliferating cell nuclear antigen (PCNA) and Ki-67, markers of cell division, suggesting ongoing cell proliferation in cortical tubers.[3] Neurogenesis occurs in the adult rat SVZ olfactory bulb pathway, rat and human hippocampal dentate gyrus, and potentially, cortex,[5,6] and accelerated proliferation of dentate granule cells has been demonstrated in rat epilepsy models in response to recurrent seizures.[7]

We assayed the expression of protein markers that demarcate distinct brain developmental epochs including neural proliferation (p34cdc2, CRMP4),[8,9] migration (DCX, LIS-1),[10] and differentiation (HuD, NeuN)[11,12] in tubers and subependymal giant cell astrocytomas (SEGAs) from TSC patients as well as human control cortex and subependymal nodules (SENs) in the Eker rat model of TSC[13] as a strategy to define the phenotypic maturity of DNs and GCs. We propose that tubers and SEGAs derive from the SVZ, and that tubers are dynamic lesions containing a subpopulation of newly generated cells that are the progeny of SVZ progenitors.

Patients and Methods

Tissue Specimens

Tubers (n = 10) resected from TSC patients[14] at the Children's Hospital of Philadelphia, the Mayo Clinic, and New York University for the treatment of intractable epilepsy were analyzed (mean patient age, 14 years; range, 1–20 years; four female and six male patients; eight frontotemporal, two parietal). Three patients had *TSC2* mutations and one had a *TSC1* mutation. SEGAs were removed from six TSC patients (distinct from patients in whom tubers were removed) for evolving hydrocephalus. Specimens had the typical histological appearance of tubers or SEGAs.[15]

Perituberal cortex with normal cytoarchitecture from three TSC patients obtained postmortem (mean age, 17 years; two female and one male patient), temporal neocortical specimens with intact cytoarchitecture obtained intraoperatively from epilepsy patients with no history of TSC (n = 4; two male and two female patients; mean age, 22 years), and postmortem neocortex obtained from four patients who died of nonneurological causes (mean age, 14 years; one male and three female patients; average postmortem interval, <14 hours) were assessed as control specimens. Human tissues were studied in accordance with the University of Pennsylvania Institutional review board.

The Eker rat strain (n = 4 adult female rats) exhibits a spontaneous *Tsc2* mutation and single SENs in the walls of the lateral ventricles.[13] Animal experiments were performed in accordance with protocols approved by the Institutional Animal Care Committee, University of Washington.

Immunohistochemistry and Western Analysis

Paraformaldehyde-fixed, paraffin-embedded tissue blocks were sectioned at 8μm and probed with CRMP4 (1:1,000; courtesy Dr S. Hockfield, Yale University), DCX (doublecortin; 1:500; courtesy C. Walsh), HuD (1:250; Santa Cruz Biotechnology, Santa Cruz, CA), LIS-1 (1:100; Santa Cruz), NeuN (1:500; Chemicon, Temecula, CA), or p34cdc2 antibodies (1:500; Santa Cruz) overnight at 4°C.

From the [1]PENN Epilepsy Center and Department of Neurology, University of Pennsylvania Medical Center, Philadelphia, PA ; [2]Division of Neurogenetics, Beth Israel Deaconess Medical Center, Boston, MA ; [3]Division of Anatomic Pathology, Mayo Clinic, Rochester MN; [4]Department of Surgery, University of Washington, Seattle, WA; [5]Department of Neurology, University of Michigan Medical Center, Ann Arbor, MI; [6] Division of Pediatric Neurosurgery, New York University Medical Center, New York, NY

Received Aug 21, 2002, and in revised form Jan 16 and Feb 4, 2003. Accepted for publication Feb 4, 2003.

Address correspondence to Dr Crino, Department of Neurology, University of Pennsylvania, 3 West Gates Bldg., 3400 Spruce St., Philadelphia, PA 19104. E-mail: crinop@mail.med.upenn.edu

Immunolabeling was visualized using the avidin-biotin conjugation method (Vector Laboratories; Burlingame, CA). For Western analysis, frozen tissue samples (flash-frozen at −80°C) were homogenized and centrifuged cell lysates were electrophoresed on 7.5% sodium dodecyl sulfate polyacrylamide gel and transferred onto nitrocellulose membrane. Western blots were probed with primary antibody (CRMP4, 1:10,000; DCX, 1:10,000; HuD, 1:5,000) overnight at 4°C and then with horseradish peroxidase–conjugated goat anti–rabbit secondary antibody (1:10,000 dilution; Amersham, Piscataway, NJ) and visualized by enzyme chemiluminescence.

mRNA Expression

Arrays containing CRMP4, DCX, and HuD cDNAs (cDNA encoding NeuN is not available) were probed with amplified ^{32}P-CTP–radiolabeled aRNA generated from whole tuber or control sections as described previously.[16] aRNA-cDNA hybridization was determined by phosphorimaging. Differences in relative abundance were determined using a Bonferroni adjusted one-way analysis of variance ($p < 0.05$ was significant).

Results

CRMP4, DCX , and HuD mRNA Expression

CRMP4 and DCX mRNAs were low abundance in the control specimens but were increased (CRMP4 [fourfold] and DCX [fivefold]) in tubers compared with control sections ($p < 0.05$; Fig 1). HuD mRNA abundance did not differ between tuber and control specimens. There was no difference in CRMP4, DCX, and HuD mRNA expression between the three control specimen types (not shown).

p34cdc2, CRMP4, DCX , LIS-1, HuD, and NeuN Protein Expression

HuD protein levels did not differ between tubers and control cortex. Increased CRMP4 and DCX protein levels were detected in the tubers (see Fig 1). Two additional CRMP4 bands detected in the perituberal cortex, and tubers samples may reflect distinct CRMP4 isoforms (R. Elliott, personal communication). Levels of CRMP4 and DCX protein were slightly increased in

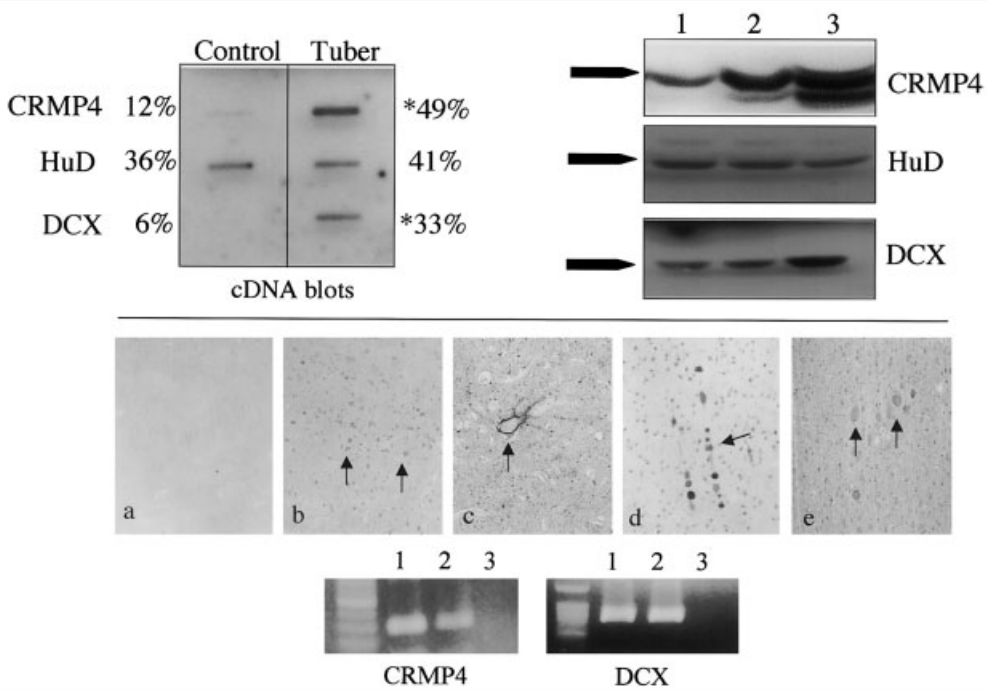

*Fig 1. (top) Representative cDNA blot and Western analysis of CRMP4, DCX, and HuD. (left) mRNA expression in control (n = 4) and tuber (n = 10) specimens. Numbers depict mean hybridization intensity represented as a percentage of total blot hybridization intensity. Note equivalent HuD mRNA in control and tuber samples but increased CRMP4 and DCX mRNA abundance in tubers (*p < 0.05). (right) Western analysis of CRMP4 (64kD), DCX (40kD), and HuD (approximately 40kD) in representative control cortex (lane* 1*), perituberal cortex (lane* 2*), and tuber (lane* 3*) samples* (arrows). *Note increase in CRMP4 and DCX protein levels in tubers compared with control cortex. (middle) (a) CRMP4 in control cortex; (b) CRMP4 in tuber; (c) high magnification of CRMP4-labeled dysmorphic neuron; (d) chain of DCX-labeled GCs in white matter; (e) LIS-1–labeled GCs. (bottom) Detection of CRMP4 (220bp) and DCX (301bp) mRNAs by reverse transcription polymerase chain reaction in single cell types (CRMP-4 primers: 5′-CCAACT GCCCCCTTTATGTCACC-3′; 5′-GATAGCCGTGAAGTTGTCCTTC-3′; DCX:5′-CTGTC-CTTGGATGACTCGGACTC-3′; 5′-CATCCCTGGAATGCTGCCCCAAA-3′). Lane* 1 *contains reverse transcription polymerase chain reaction amplicons from a CRMP4 immunolabeled GC, lane* 2 *is from DCX-immunolabeled GC, and lane* 3 *is from a HuD-labeled control neuron. CRMP4 and DCX are detected in lanes* 1 *and* 2 *but not in lane* 3.

perituberal cortex specimens compared with control cortex (because of probable inclusion of a small contaminant of tuber cortex during gross dissection of the frozen block) but were less than in tubers.

There was no apparent difference in marker expression between the *TSC1* case compared with the three *TSC2* cases. p34cdc2 immunoreactivity was not observed in any of the tissue specimens (not shown). CRMP4 labeling was confined to a small population of cells in control cortex scattered across all cell layers that likely reflect oligodendrocytes. CRMP4-immunolabeled cells were observed, often in clusters, throughout the extent of the tuber and especially in the white matter. CRMP4 staining was within the somatic cytoplasm and near the cell membrane in DNs and GCs (see Fig 1). None of the GCs or DNs exhibited immunoreactivity for myelin/oligodendrocyte specific protein, a marker for oligodendrocytes (not shown).

DCX-immunoreactive cell clusters and chains were observed in the subcortical white matter extending to the pial surface (see Fig 1). However, some GCs and DNs were DCX stained, whereas other directly adjacent GCs or DNs were not labeled. DCX labeling in GCs was corroborated by LIS-1 immunolabeling. Detection of CRMP4 and DCX mRNAs in single microdissected CRMP4 or DCX but not HuD-immunoreactive cells ($n = 5$ each) by reverse transcription polymerase chain reaction corroborated the immunolabeling profiles (see Fig 1).

Neuronal HuD and NeuN immunoreactivity was noted in neurons throughout all laminae in all control specimens (Fig 2). In tubers, most DNs and GCs were

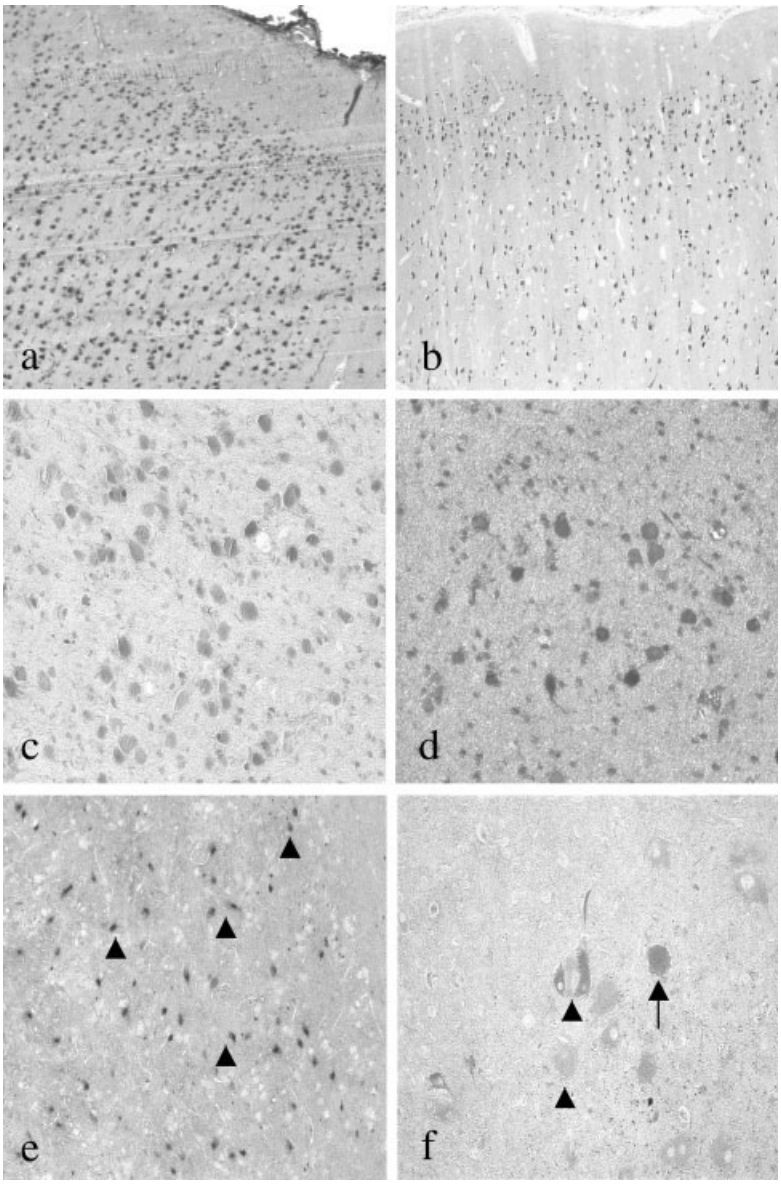

Fig 2. Expression of NeuN (a) and HuD (b) in control cortex. NeuN (c) and HuD (d) immunolabeling in tubers. (e) There are multiple NeuN-immunolabeled cells in tubers (arrowheads) *that are directly adjacent to large giant cells* (white spaces) *that do not exhibit NeuN staining. (f) Heterogenous HuD immunostaining pattern in dysmorphic neurons and giant cells. Some cells are robustly labeled* (arrow)*, whereas others exhibit minimal immunoreactivity* (arrowheads) *even when they are directly adjacent.*

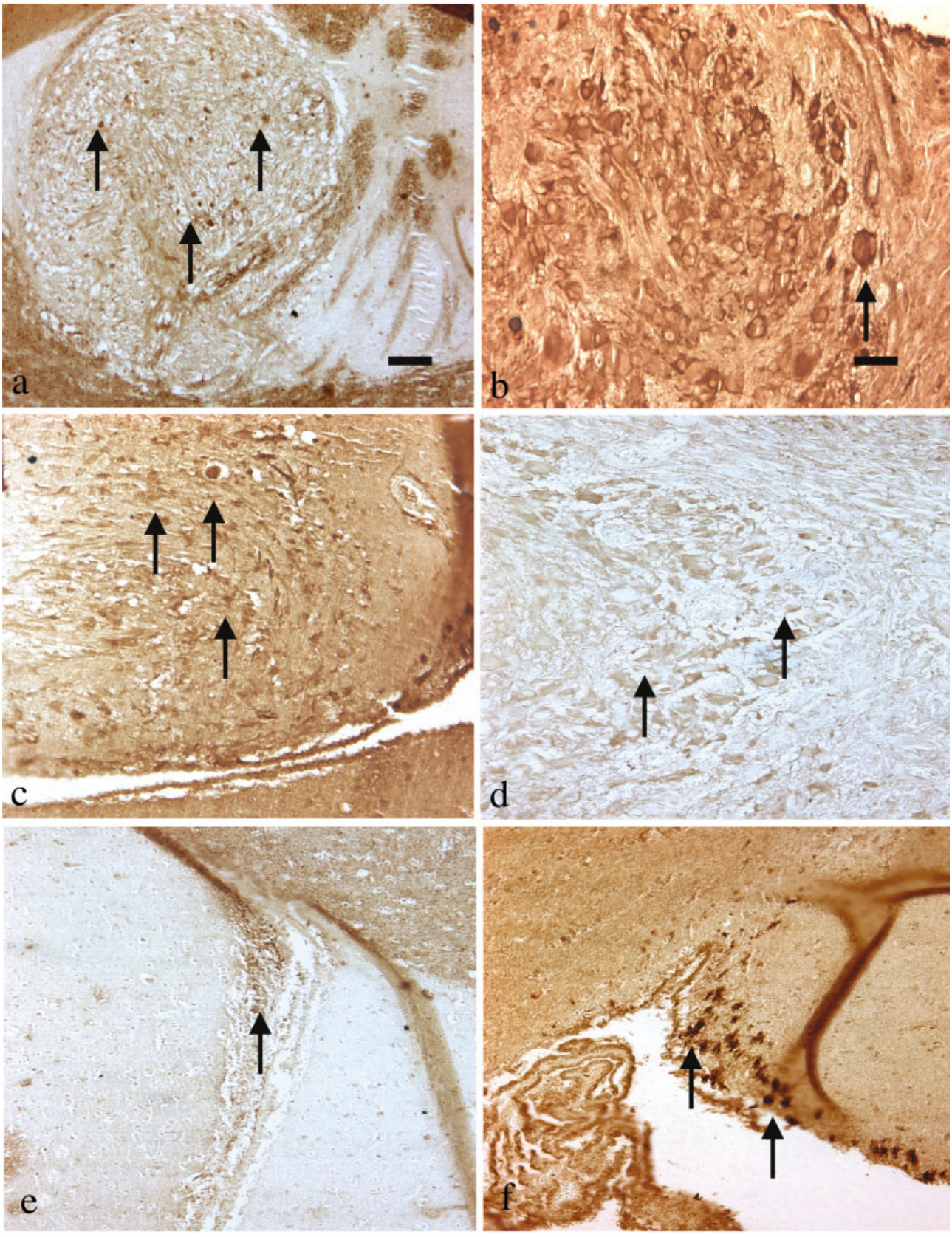

Fig 3. Expression of CRMP4 (A) and doublecortin (C) in select cells (arrows) *within subependymal nodules of the Eker rat strain. Expression of CRMP4 (B) and DCX (D) in human subependymal giant cell astrocytomas* (arrows). *Many large giant cells are CRMP4 (B) and DCX labeled in subependymal giant cell astrocytomas (D). (E) CRMP4 and (F) doublecortin expression in the rat subventricular zone* (arrows).

NeuN and HuD immunostained (see Fig 2). However, NeuN and HuD immunolabeling of GCs was heterogeneous in that some GCs were unstained, whereas directly adjacent GCs exhibited NeuN or HuD immunoreactivity.

CRMP4 and DCX in Human Subependymal Giant Cell Astrocytomas and Eker Rat Subependymal Nodules

CRMP4 and DCX labeling was observed in the SVZ of control rats. In the Eker rats, rare CRMP4 and DCX-labeled neurons were noted in the pyriform and entorhinal cortex. CRMP4 and DCX-immunoreactive cells were observed in the SENs within all four Eker rats (Fig 3). Numerous CRMP4 and DCX-immunolabeled cells were identified in all six SEGA specimens. Most cell types were similar to the GCs identified in tubers (see Fig 3).

Discussion

We show that DNs and GCs express cell markers reflecting distinct neurodevelopmental epochs. Select DNs and GCs expressed HuD and NeuN consistent with a differentiated neuronal phenotype. CRMP4 and DCX expression was observed in SENs, tubers, and SEGAs, suggesting that these lesions share a common origin during development and may be derived from the SVZ. CRMP4, DCX, and LIS-1 expression may identify newly generated cells that actively migrate from the SVZ or from SENs/SEGAs into tubers where they then differentiate and express NeuN or HuD.

Neurogenesis and cell migration in the mature mammalian brain have been demonstrated by the expression of markers including CRMP4, DCX, LIS-1, PCNA, and BrdU incorporation.[3,5,6,10,17] Cell lineage or developmental phenotype designation with immunohistochemical markers has inherent limitations. For example, expression of a particular marker does not necessarily define cell lineage or differentiation, and expression of any one particular marker does not definitively prove the identity of a specific cell type. However, we have surmounted these limitations by assaying multiple markers to corroborate specific developmental epochs such as DCX and LIS-1 or HuD and NeuN.[9–12]

Recurrent seizures in rodents are associated with ectopic migratory pathways of DCX-labeled neurons into the forebrain.[17] White matter abnormalities adjacent to tubers[18] may highlight the pathway of migrating neurons, and, in fact, many of the DCX or CRMP4-immunoreactive cells were in the subcortical white matter, possibly en route to tubers. Tuber and SEN number are positively correlated,[18,19] and, although there is no current evidence to suggest that tubers can enlarge, the variable magnetic resonance imaging characteristics of tubers suggest that these lesions may be dynamic.[19]

SENs in the Eker rat exhibit loss of *Tsc2* heterozygosity, whereas the overlying cortex of this animal strain is *Tsc2* heterozygous and expresses functional tuberin. CRMP4 and DCX expression in SENs but not cortex supports a highly specific upregulation of CRMP4 and DCX in response to loss of tuberin and ongoing cell proliferation. Alternative explanations include failure to transcriptionally silence CRMP4 or DCX or abnormalities in cytoskeletal assembly within these cell types.[11] Conversely, a population of dormant progenitors[20] may exist within tubers that yield new cells expressing CRMP4 and DCX.

We propose that tubers, SENs, and SEGAs are pathogenically linked and derive from progenitor cells in the VZ and SVZ. Thus, therapeutic interventions targeted to prevent the formation of these lesions may be directed at the SVZ. Although we cannot at this time extend our results to all tubers, we propose that tubers highly associated with epilepsy may be dynamic rather than static lesions.

This work was supported by the Tuberous Sclerosis Alliance/Center Without Walls (NS045021, NS39938, P.B.C.) and the Esther A. and Joseph Klingenstein Fund.

We thank the Brain and Tissue Bank for Developmental Disorders, University of Maryland, and the TSC patients and families who permitted the use of brain tissue for analysis. Mutational analysis was courtesy of D. Kwiatkowsky and E. Henske.

References

1. Crino PB, Henske EP. New developments in the neurobiology of the tuberous sclerosis complex. Neurology 1999;53: 1384–1391.
2. Koh S, Jayakar P, Dunoyer C, et al. Epilepsy surgery in children with tuberous sclerosis complex: presurgical evaluation and outcome. Epilepsia 2000;41:1206–1213.
3. Crino PB, Dichter MA, Trojanowski JQ, Eberwine J. Embryonic neuronal markers in tuberous sclerosis: single cell molecular pathology. Proc Nat Acad Sci USA 1996;93:14152–14157.
4. Arai Y, Edwards V, Becker LE. A comparison of cell phenotypes in hemimegalencephaly and tuberous sclerosis. Acta Neuropathol (Berl) 1999;98:407–413.
5. Eriksson PS, Perfilieva E, Bjork-Eriksson T, et al. Neurogenesis in the adult human hippocampus. Nat Med 1998;4: 1313–1317.
6. Gould E, Reeves AJ, Graziano MS, Gross CG. Neurogenesis in the neocortex of adult primates. Science 1999;286:548–552.
7. Parent JM, Yu TW, Leibowitz RT, et al. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 1997;17:3727–3738.
8. Lew J, Qi Z, Huang QQ, et al. Structure, function, and regulation of neuronal Cdc2-like protein kinase. Neurobiol Aging 1995;16:263–268.
9. Quinn CC, Gray GE, Hockfield S. A family of proteins implicated in axon guidance and outgrowth. J Neurobiol 1999;41: 158–164.

10. Meyer G, Perez-Garcia CG, Gleeson JG. Selective expression of Doublecortin and LIS1 in developing human cortex suggests unique modes of neuronal movement. Cereb Cortex 2002;12: 1225–1236.
11. Szabo A, Dalmau J, Manley G, et al. HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. Cell 1991;67: 325–333.
12. Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. Development 1992;116: 201–211.
13. Yeung RS, Katsetos CD, Klein-Szanto A. Subependymal astrocytic hamartomas in the Eker rat model of tuberous sclerosis. Am J Pathol 1997;151:1477–1486.
14. Roach ES, Gomez MR, Northrup H. Tuberous sclerosis complex consensus conference: revised clinical diagnostic criteria. J Child Neurol 1998;13:624–628.
15. Scheithauer BW, Reagan TJ. Neuropathology in the tuberous sclerosis complex. In: Gomez MR, Sampson JR, Whittemore VH, eds. Tuberous sclerosis complex. 3rd ed. New York: Oxford University Press, 1999:101–121.
16. Kacharmina JE, Crino PB, Eberwine J. Preparation of cDNA from single cells and subcellular regions. Methods Enzymol 1999;303:3–18.
17. Parent JM, Valentin VV, Lowenstein DH. Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. J Neurosci 2002;22:3174–3188.
18. Griffiths PD, Bolton P, Verity C. White matter abnormalities in tuberous sclerosis complex. Acta Radiol 1998;39:482–486.
19. Baron Y, Barkovich AJ. MR imaging of tuberous sclerosis in neonates and young infants. Am J Neuroradiol 1999;20: 907–916.
20. Palmer TD, Ray J, Gage FH. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci 1995;6:474–486.

New T2 Lesions Enable an Earlier Diagnosis of Multiple Sclerosis in Clinically Isolated Syndromes

Catherine M. Dalton, MRCPI,[1] Peter A. Brex, MRCP,[1] Katherine A. Miszkiel, FRCR,[2] Kryshani Fernando, MRCP,[1] David G. MacManus, MSc,[1] Gordon T. Plant, FRCP,[3] Alan J. Thompson, FRCP,[1] and David H. Miller, FRCP[1]

In clinically isolated syndromes, the new McDonald criteria for multiple sclerosis diagnosis require new gadolinium-enhancing lesions for dissemination in time at a 3-month follow-up magnetic resonance imaging scan. In a cohort of 56 patients, these criteria were specific (95%) but less sensitive (58%) for clinically definite multiple sclerosis at 3 years. If new T2 lesions were allowed as an alternative for dissemination in time, sensitivity increased (74%) with maintained specificity (92%), enabling an accurate diagnosis of multiple sclerosis in more patients.

Ann Neurol 2003;53:673–676

New diagnostic criteria developed by an international panel on multiple sclerosis (MS)[1] allow magnetic resonance imaging (MRI) evidence of dissemination in time[2] and space[3,4] that enable a diagnosis of MS to be made in patients with clinically isolated syndromes. This contrasts with previous criteria that have required clinical evidence for dissemination in time and space to diagnose clinically definite MS.[5,6]

We previously reported application of the new (McDonald) criteria to a cohort of clinically isolated syndrome patients followed up clinically and with MRI within 3 months of symptom onset, and again 3 months, 1 year, and 3 years later.[7] If a first MRI is performed less than 3 months after the onset of the clinical event, a second scan performed 3 months or more after the clinical event must show a new gadolinium-enhancing lesion to fulfill the MRI criteria for dissemi-

From the [1]NMR Research Unit, Institute of Neurology; [2]Lysholm Department of Neuroradiology, National Hospital for Neurology and Neurosurgery; and [3]Moorfields Eye Hospital, London, United Kingdom.

Received Oct 1, 2002, and in revised form Dec 30. Accepted for publication Feb 5, 2003.

Published online Apr 21, 2003, in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.10580

Address correspondence to Dr Miller, NMR Research Unit, Department of Neuroinflammation, Institute of Neurology, University College London, Queen Square, London WC1N 3BG, United Kingdom. E-mail: d.miller@ion.ucl.ac.uk

