Novel susceptibility locus at chromosome 6q16.3-22.31 in a family with GEFS+

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ABSTRACT

Background: Genetic epilepsy with febrile seizures plus (GEFS+) is a familial epilepsy syndrome with extremely variable expressivity. Mutations in 5 genes that raise susceptibility to GEFS+ have been discovered, but they account for only a small proportion of families.

Methods: We identified a 4-generation family containing 15 affected individuals with a range of phenotypes in the GEFS+ spectrum, including febrile seizures, febrile seizures plus, epilepsy, and severe epilepsy with developmental delay. We performed a genome-wide linkage analysis using microsatellite markers and then saturated the potential linkage region identified by this screen with more markers. We evaluated the evidence for linkage using both model-based and model-free (posterior probability of linkage [PPL]) analyses. We sequenced 16 candidate genes and screened for copy number abnormalities in the minimal genetic region.

Results: All 15 affected subjects and 1 obligate carrier shared a haplotype of markers at chromosome 6q16.3-22.31, an 18.1-megabase region flanked by markers D6S962 and D6S287. The maximum multipoint lod score in this region was 4.68. PPL analysis indicated an 89% probability of linkage. Sequencing of 16 candidate genes did not reveal a causative mutation. No deletions or duplications were identified.

Conclusions: We report a novel susceptibility locus for genetic epilepsy with febrile seizures plus at 6q16.3-22.31, in which there are no known genes associated with ion channels or neurotransmitter receptors. The identification of the responsible gene in this region is likely to lead to the discovery of novel mechanisms of febrile seizures and epilepsy. **Neurology® 2009;73:1264-1272**

GLOSSARY

CIDR = Center for Inherited Disease Research; **EFSCU** = Epilepsy Family Study of Columbia University; **FS** = febrile seizures; **FS+** = febrile seizures plus; **GBP** = gabapentin; **GEFS+** = genetic epilepsy with febrile seizures plus; **GTC** = generalized tonic-clonic seizure without aura or lateralizing postictal symptoms or signs; **Mb** = megabase; **MGR** = minimal genetic region; **PPL** = posterior probability of linkage; **SMEB** = borderline severe myoclonic epilepsy of infancy; **STRP** = short tandem repeat polymorphism; **UCSC** = University of California Santa Cruz.

Febrile seizures (FS) occur commonly in children, with an incidence of 2% to 5%.¹ Although FS aggregate in families and are believed to be strongly influenced by genetic susceptibility,²⁻⁵ the genes influencing risk for most cases are unknown. Genetic (formerly generalized) epilepsy with febrile seizures plus (GEFS+) is a familial epilepsy syndrome with extremely variable expressivity,⁶ with phenotypes including classic FS, febrile seizures plus (FS+; i.e., FS persisting beyond age 6 years or accompanied by afebrile generalized tonic-clonic seizures⁷⁻⁹), severe epileptic encephalopathy, and generalized or localization-related epilepsy.⁷ In GEFS+ families, mutations have been identified in 3 genes encoding sodium channel subunits (*SCN1B*, *SCN1A*, and *SCN2A*) and 2 genes encoding GABA receptor subunits (*GABRG2* and *GABRD*),¹⁰⁻¹⁴ but few GEFS+ families have mutations in

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Supported by NIH grants R01 NS020656 and R37 NS35129.

Disclosure: Author disclosures are provided at the end of the article.

Table 1 Maximum multipoint lod scores at GEFS+ and FEB loci

	Location	Maximum lod
GEFS+ genes		
<i>SCN1A</i> and <i>SCN2A</i>	2q24	−1.59
<i>SCN1B</i>	19q13	−4.36
<i>GABRG2</i> (<i>FEB8</i>)	5q31	−2.84
<i>GABRD</i>	1p36.3	−1.72
GEFS+ loci		
Reference 16	2p24	−7.71
<i>FEB3</i>	2q23-q24	−1.56
<i>FEB9</i>	3p	−1.06
Putative <i>FEB9</i> modifier	8p23-21	−1.23
<i>FEB4</i>	15q14-q15	−2.31
Reference 18	18p	−2.23
<i>FEB7</i>	21q22	−6.31
Familial FS loci		
<i>FEB1</i>	8q13-q21	−4.35
<i>FEB2</i>	19p13.3	−2.79
<i>FEB5</i>	6q22-24	0.216
<i>FEB6</i>	18p11.2	−1.97
Reference 26	3q26.2-26.33	−5.20

GEFS+ = genetic epilepsy with febrile seizures plus; FS = febrile seizures.

these genes.^{4,15} In addition, linkage studies have identified 7 genomic regions likely to harbor genes increasing risk for GEFS+ and 5 regions likely to harbor genes increasing risk for FS (table 1).¹⁶⁻²⁶ Two loci originally described as FS loci (*FEB3* and *FEB4*) were reported in pedigrees best classified as GEFS+ due to phenotypes beyond typical FS.^{20,21,27}

Here we describe a GEFS+ kindred from Central America with evidence for linkage to chromosome 6q16.3-22.31. This region has not been previously reported in association with FS, GEFS+, or other inherited epilepsies. Because there are no ion channel genes in this locus, identification of the causative gene may provide new insight into the pathogenesis of FS and inherited epilepsy.

METHODS Family ascertainment and phenotyping.

The family reported here was collected in the Epilepsy Family Study of Columbia University (EFSCU). Each subject was screened for seizures in a telephone interview, and those who screened positive were given a semistructured diagnostic interview, administered by a neurologist or general physician with specialized epilepsy training, to obtain information on seizure symptoms and etiologic factors. Whenever possible, medical

records were obtained from treating physicians. Individuals aged 13 years or older were interviewed directly; those aged 12 years or younger who were able to understand the questions were interviewed jointly with a parent or caregiver. If a subject was deceased or otherwise unavailable, we interviewed the relative likely to be most knowledgeable about the seizure history. Whenever the quality of information regarding seizure history was in question or the subject's own recall was insufficient, we interviewed additional family members to clarify the seizure history. Two experienced epileptologists (W.A.H. and T.A.P.) reviewed all assembled data on each subject to render a final diagnosis. To remove the possibility of bias, each subject was reviewed blindly with respect to the diagnoses of other family members. These methods have been validated^{28,29} and have been described in detail previously.^{30,31}

Collection of DNA. We collected blood samples via venipuncture from consenting family members. DNA was purified from blood leukocytes by the Gentra Systems purification kit and from Epstein-Barr virus-transformed cell lines by organic extraction from pelleted cells.

Microsatellite typing. The Center for Inherited Disease Research (CIDR) genotyped 390 short tandem repeat polymorphisms (STRPs, or microsatellite markers) spaced at an average of 9 cM throughout the genome. We evaluated the STRP data for consistency and genotyping errors with RELCHECK,³² PEDCHECK,³³ and SIMWALK2^{34,35} and recoded both alleles to "unknown" in any individual with a genotyping error that could not be resolved for a given marker. In the region defined by initial genome-wide analyses using the CIDR panel (chromosome 6q14-q23), we genotyped 29 additional STRPs from the Applied Biosystems (ABI) panel (ABI, Foster City, CA), using standard protocols to perform PCR reactions and electrophoresis of amplified markers on the ABI Prism 3100 Analyzer. We visually analyzed the lengths of the markers using ABI Prism Genescan and Genotyper 3.7 software.

Standard protocol approvals, registrations, and patient consents. The EFSCU was approved by the Columbia University Medical Center Institutional Review Board, and all participating subjects or their parents gave written informed consent.

Linkage analysis. The linkage analysis method involves evaluation of the evidence for cosegregation, within each family, of disease status with genetic marker alleles. The lod (logarithm of odds) score measures the strength of the association, with values above 0 indicating greater than chance cosegregation and values above 3.0 indicating significant genome-wide evidence for linkage. Initially, we screened for evidence of linkage across the genome using the CIDR markers in this family and many other EFSCU families. For the initial screen, we used a conservative model assuming autosomal dominant inheritance with 50% penetrance, no sporadics (i.e., zero penetrance in noncarriers of the susceptibility allele), and a 1% frequency of the susceptibility allele; this model was used to avoid inflation of the lod score. After obtaining preliminary evidence for linkage on chromosome 6q, we reanalyzed the data under a model more realistic for this family. Among 9 family members with affected children (obligate carriers under a dominant model), 8 were affected, suggesting that penetrance was approximately 90%. Hence, in all subsequent linkage analyses, we assumed autosomal dominant inheritance with 90% penetrance, no sporadics, and a 0.001 frequency of the susceptibility allele. We computed 2-point lod scores using FASTLINK³⁶ and multipoint lod scores using SIMWALK2 and assembled haplotypes using SIMWALK2.

Figure 1 Four-generation pedigree with GEFS+, with symbols denoting each individual's phenotype

The microsatellite marker names are listed at the left of the figure; the markers bordering the region with the haplotype shared among the affected individuals and obligate carrier are D6S962 and D6S287. GEFS+ = genetic epilepsy with febrile seizures plus; LRE = localization-related epilepsy.

We also performed 2-point and multipoint model-free linkage analysis using posterior probability of linkage (PPL) methods with KELVIN software.³⁷ This method evaluates the evidence for linkage without assuming values for unknown genetic model parameters (e.g., mode of inheritance, penetrance, allele frequency) or incurring the inflationary effects of maximizing over models.³⁸

We evaluated the linkage evidence specifically for the loci of the 5 previously identified GEFS+ genes, 7 GEFS+ loci, and 5 FS loci by calculating multipoint lod scores for each locus.

Selection and sequencing of candidate genes. Once the critical region of linkage was identified, we evaluated each gene in the region for reported protein function and levels of brain expression in the University of California Santa Cruz (UCSC) Genome Bioinformatics 2006 assembly (<http://genome.ucsc.edu/>). We designed forward and reverse oligonucleotide primers for all exons and flanking exon-intron junctions using Primer 3 software. We used standard PCR methods to amplify these regions in genomic DNA from 3 affected individuals (II:5, III:1, and III:5) and sequenced the amplified PCR products using automated fluorescent dye terminator methods (SeqWright, Houston, TX). Sequence analysis was performed using DNASTar software.

Evaluation of copy number variants in the critical region. We used a tiling array for chromosome 6 (Affymetrix, Santa Clara, CA), with 25-base pair oligonucleotide probes

placed approximately 35 base pairs apart, enriched in the regions of exons, to assess for heterozygous copy number changes. Genomic DNA from affected individuals II:5 and III:1 and unaffected individual I:2 was digested with restriction enzyme NlaIII and hybridized to the tiling array. Model-based analysis and a hidden Markov model were used to determine copy number, and evaluation for deletions and duplications was performed with Integrated Genome Browser software.

RESULTS Description of the pedigree. The pedigree shown in figure 1 contains 4 generations of individuals with FS, FS+, or epilepsy and shows a clear autosomal dominant pattern of inheritance. The family phenotype is highly consistent with GEFS+.

Fifteen individuals were designated as affected (table 2). None of them had a history of any illness or CNS insult likely to be related to epilepsy (e.g., severe head injury, stroke, brain tumor, brain surgery, brain infection), and all except III:1 (described below) were intellectually normal. Six individuals had FS: II:10, III:3, III:5, III:9, IV:1, and IV:3. All had onset of FS between ages 8 and 12 months, and reso-

Subject	Age, y	Phenotype	No. of febrile GTCs	Onset/offset of febrile GTCs	Afebrile seizure type and semiology	No. of afebrile seizures	Onset of afebrile seizures	EEG/MRI	Medication
I:1	51 (d)	Epilepsy	None	—	GTC	>4	45 y	Unk/Not done	PB, PHT
II:2	41	Epilepsy	None	—	GTC	15–20	2 y	Results unk for both	PB, PHT
II:4	49	Isolated unprovoked seizure	None	—	GTC	1	15 y	Unk/Not done	PB
II:5	48	FS and LRE	Unk	2 y/unk	Activity arrest, impaired speech, sensation of words repeating themselves, typically induced by reading; one episode followed by a generalized convulsion	>4	13 y	Normal/Normal by report	PB
II:10	43	FS	2	8 mo/unk	—	—	—	—	—
II:14	36	FS and epilepsy	10	1 y/5 y	GTC	2	24 y	Not done	PB
III:1	10	FS+ and severe epilepsy with developmental delay	20	1.5 y/7 y	GTC, complex partial, absence, myoclonic, and atonic seizures	Very frequent, weekly GTCs at times	3 y	EEG normal at 4 y, left or right posterior spike-wave complexes with generalization, bifrontal spike-wave, irregular generalized spike-wave from ages 6 to 13 y/MRI normal (7 y)	VPA, DZP, CBZ, PHT, LTG, GBP, TPM, FBM
III:2	26	Epilepsy	None	—	GTC	>12	11 y	Results unk/Not done	PHT
III:3	21	FS	1	8 mo	—	—	—	—	—
III:5	7	FS	4	1 y/unk	—	—	—	—	—
III:8	21	FS+	>20, convulsive SE with left-sided clonic onset (1 y)	5 mo/5 y	Aura described as nervousness, screaming or calling mother, hypersalivation; possible secondarily generalized seizures	>4 GTCs, >30 total	5 mo	Results unk/Not done	PB, PHT, DZP
III:9	21	FS	1	1 y	—	—	—	—	—
III:13	18	FS+	20	3 mo/7 y	GTC	3	4 y	Results unk/Not done	PHT, PB, CBZ
IV:1	5	FS	7	1 y/4 y	—	—	—	—	—
IV:3	3	FS	2	1 y/2 y	—	—	—	—	—

GEFS+ = genetic epilepsy with febrile seizures plus; GTC = generalized tonic-clonic seizure without aura or lateralizing postictal symptoms or signs; (d) = deceased; unk = unknown; PB = phenobarbital; PHT = phenytoin; FS = simple febrile seizures; LRE = localization-related epilepsy; FS+ = febrile seizures plus; VPA = valproate; DZP = diazepam; CBZ = carbamazepine; LTG = lamotrigine; GBP = gabapentin; TPM = topiramate; FBM = felbamate; SE = status epilepticus.

lution before age 4 years in those for whom that information was available; none had afebrile seizures.

Two subjects, II:5 and II:14, had FS and later epilepsy. Individual II:5 had afebrile seizures described as activity arrest with impairment of speech production, the perception that words that were read were repeating themselves, and fear. His seizures were typically induced by reading, and he had a single afebrile secondarily generalized convulsion. He was classified as FS and localization-related epilepsy. Individual II:14 had recurrent afebrile generalized tonic-clonic seizures (GTCs) with no aura or localizing symptoms reported at the onset and therefore was classified as FS and epilepsy with undetermined localization-related vs generalized onset.

Individuals III:8 and III:13 had FS+, in III:8 on the basis of FS accompanied by afebrile GTCs and in III:13 because FS persisted until age 7 years. Individual III:8 also had afebrile convulsive seizures with semiology consistent with focal onset, described as nervousness (screaming, hyperactivity, or calling her mother) and hypersalivation at the onset.

Subject III:1 had onset of FS at 18 months and had a total of 20 FS. He had afebrile GTCs sometimes as often as 4 per month between ages 3 and 6 years, myoclonic jerks reported at age 9 years, brief staring episodes diagnosed clinically as absence seizures, and complex partial seizures described as paroxysmal periods of altered responsiveness lasting up to 3 minutes between ages 5 and 10 years. Developmental milestones were normal in the first year of life, but he went on to have marked cognitive and behavioral difficulties. MRI of the brain was normal at age 7 years. EEG was normal at age 4 years; later EEGs, performed between ages 6 and 13 years, showed left- or right-posterior spike-wave complexes with generalization, bifrontal spike-wave complexes, and irregular generalized spike-wave complexes. This individual's history satisfied criteria for FS+ and was also suggestive of borderline severe myoclonic epilepsy of infancy (SMEB) because of the mixture of seizure types and developmental delay after onset of recurrent FS in a previously normal child. However, the clinical picture also differed from SMEB in several aspects: onset after 1 year, normal EEG at age 4 years, and lack of evidence of status epilepticus. Thus we designated this person as FS+ and severe epilepsy with developmental delay.

Individuals I:1, II:2, and III:2 had epilepsy comprised of recurrent afebrile GTCs with no aura or localizing symptoms at the onset, therefore of undetermined onset. Individual II:4 had only an isolated unprovoked seizure but also had a child affected with epilepsy. In our analyses, we considered individual II:4 to be affected.

Linkage analyses and identification of a critical region. Table 1 lists the maximum multipoint lod scores for markers in the CIDR panel located closest to the known GEFS+ genes (*SCN1B*, *SCN1A*, *SCN2A*, *GABRG2*, and *GABRD*) and at the GEFS+ and familial FS loci. There was no evidence for linkage to any of these previously delineated loci, and most could effectively be excluded (lod < -2.0).

In the initial genome-wide microsatellite marker screen using the CIDR data, the maximum 2-point lod score was 3.21 for D6S474 (chromosome 6q21), and the multipoint lod score was 3.66 in the same region. This was the only region associated with a lod score greater than 2.05. Reanalysis of all available marker data on chromosome 6, including the 29 newly typed markers, yielded a maximum 2-point and multipoint lod score of 4.68 at D6S1706 (figure 2A) assuming 90% penetrance. In the model-free analysis, the maximum PPL for the 2-point and multipoint methods was 0.89 at marker D6S1706 (figure 2B), indicating an 89% posterior probability of linkage, consistent with the results of the model-based linkage analysis.

Haplotype analysis using SIMWALK2 indicated that all affected individuals share a haplotype of alleles at 7 markers between D6S1021 and D6S304 (figure 1). The haplotype is delimited by 2 key recombination events: the first between markers D6S962 and D6S1021 in individual III:5 and his father II:7 (an obligate carrier), and the second between markers D6S304 and D6S287 in individual III:13. These data place the minimal genetic region (MGR) between markers D6S962 and D6S287 (i.e., the markers flanking the haplotype), an 18.1-megabase (Mb) region at 6q16.3-22.31 that spans from 107.15 to 119.55 cM on the deCODE map.

Two unaffected subjects, III:6 and III:12, also have recombinant haplotypes in this region (figure 1). Subject III:6 carries the portion of the disease haplotype centromeric to D6S1698, and subject III:12 carries the disease haplotype centromeric to D6S941. The evidence from subject III:6 suggests that the gene is likely to be telomeric to D6S941, which would reduce the MGR slightly, to the region between D6S941 and D6S287. Because of incomplete penetrance, the evidence from unaffected individuals is less definitive than that from affected individuals; thus, we have conservatively defined the MGR based on the data from affected individuals only. However, assuming 90% penetrance, it is unlikely that both III:6 and III:12 are nonpenetrant carriers; hence the disease locus is likely to be telomeric to D6S941.

Mutation screening of candidate genes in the critical region. In the region delimited by microsatellite markers D6S962 and D6S287, we found 137 entries

Figure 2 Results of linkage analysis using model-based and PPL methods

(A) Two-point and multipoint lod scores from linkage analysis of microsatellite marker data using an autosomal dominant model with 90% penetrance, no sporadics, and 0.001 frequency of the susceptibility allele. The horizontal axis depicts the chromosome 6 microsatellite markers. The peak lod score is 4.68 at D6S1706. (B) Two-point and multipoint posterior probability of linkage (PPL) values for each microsatellite marker. The horizontal axis depicts the chromosome 6 microsatellite markers. The peak PPL score is 0.89 at D6S1706.

for genes, complementary DNAs, and messenger RNAs in the UCSC Genome Bioinformatics 2006 genome assembly. Of these, 19 were duplicate or overlapping entries, leaving 118 unique entries. We considered 42 of these entries to be poor candidates because they represented either hypothetical proteins or poorly characterized entries. We considered the following 16 genes to be very good candidates based on protein function (e.g., membrane and transmembrane proteins, solute carrier proteins, proteins involved in intracellular trafficking) and/or reported

high levels of brain expression: *MAN1A1*, *NUS1*, *GOPC*, *DCBLD1*, *PIST*, *GPR6*, *GPRC6A*, *KPNA5*, *FAM26E*, *DSE*, *HDAC2*, *SLC16A10*, *SLC22A16*, *SLC35F1*, *SNX3*, and *NR2E1*.

Sequencing did not reveal any sequence changes that were predicted to alter the amino acid sequence or splicing in the exons or exon-intron junctions of these 16 candidate genes. We did observe some synonymous sequence variants, including previously reported single nucleotide polymorphisms.

Copy number assessment. When we used an oligonucleotide array of chromosome 6 to screen for deletions or duplications, we did not observe any copy number abnormalities in either of the 2 affected subjects or 1 unaffected subject screened (data available on request).

DISCUSSION In a 4-generation family with GEFS+, we identified an 18-Mb (12.4-cM) critical region of linkage at 6q16.3-22.31. The absence of any genes related to ion channel function in this region suggests that epilepsy in this family is due to a novel mechanism of seizure susceptibility and epileptogenesis. We sequenced 16 genes in this region that encode solute carriers, G protein-coupled receptors, and other proteins that we predicted might be related to an epilepsy phenotype, and did not find a causative mutation. Because GEFS+ is an autosomal dominant syndrome, a heterozygous deletion or duplication of a whole exon in the region 6q16.3-22.1 could be present but not detectable by direct sequencing. However, for each gene in the region, we excluded exonic deletions and duplications using a dense oligonucleotide tiling array.

Three of the 4 affected individuals without reported FS were in the older generations of the family. A recent report suggests that underreporting of FS is likely, even when parents are interviewed.³⁹ Thus, some of these subjects may have had FS, although this would not have changed their designation as affected. Also, some of the family members designated as unaffected may have had unreported FS and may therefore have been misclassified. The relatively high incidence of sporadic FS in the general population raises the possibility that 1 or more of the individuals with FS were phenocopies. However, if this were the case, we would not have expected to find a haplotype shared by all individuals classified as affected in the family.

The region we have identified from 6q16.3-22.31 is distinct from the previously described loci for FS or GEFS+. The nearest previously described locus is *FEB5*, spanning the region of 6q22.33-23.2 between microsatellite markers D6S1620 and D6S975.²³ As depicted schematically in figure 3, these regions are

Figure 3 Schematic view of chromosome 6

Schematic view of chromosome 6, demonstrating that our genetic epilepsy with febrile seizures plus locus, bounded by microsatellite markers D6S962 and D6S287 and highlighted in blue, is distinct from the *FEBS* locus, bounded by D6S1620 and D6S975 and highlighted in red.²³ Mb = megabase.

separated by 10.4 Mb. Furthermore, the phenotype described in the 3 families with linkage to the *FEBS* locus consists of simple FS with only 1 individual with later epilepsy.²³ Thus, we have no evidence to suggest that the relative proximity of our locus to *FEBS* represents more than coincidence.

The nearly complete correspondence between the haplotype and disease (or obligate carrier) status, coupled with linkage evidence consistent across model-based and model-free analysis methods, strongly suggests that this family has a mutation in a gene with a major effect on risk for GEFS+ in this region of chromosome 6. There are no genes in the 6q16.3-22.31 region encoding sodium channels, glutamate or γ -aminobutyric acid receptors, or other classes of proteins that have been thus far associated with GEFS+ or other inherited epilepsies. Although we have sequenced 16 candidate genes, we have not excluded the presence of a mutation in the other genes in the region or addressed the possibility of a mutation in an intron or other noncoding element in one of the genes sequenced. With the advent of more efficient and comprehensive technologies for sequencing and evaluation of copy number variation, a more precise understanding of the genetic etiology of GEFS+ in this pedigree should be possible in the future. For now, our data not only illustrate the heterogeneity of the etiologies of GEFS+ but also suggest that there are as yet undiscovered mechanisms underlying GEFS+ and epilepsy in general.

AUTHOR CONTRIBUTIONS

Statistical analyses were performed by Annapurna Poduri, Derek Gordon, Yuanjia Wang, Ayse Ulgen, and Robert Sean Hill.

ACKNOWLEDGMENT

The authors thank the family members for participating in this study; Christine Austin, Danielle Gleason, and Princess Christina Dejesus for technical work in the laboratory of Dr. Walsh; and Dr. Edward Fox at the Dana Farber Cancer Institute for facilitating the assessment of copy number variants in the region of chromosome 6. Genotyping services were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract from the NIH to The Johns Hopkins University, Contract Number HHSN268200782096c.

DISCLOSURE

Dr. Poduri was supported by the Children's Hospital Boston/Harvard Medical School Eleanor and Miles Shore fellowship. Dr. Wang receives research support from the NIH [R03 AG031113 (PI), R01 DA026305 (Coinvestigator), R01 MH045436 (Coinvestigator), R01 MH073915 (Coinvestigator), R01 MH082736 (Coinvestigator), R01 MH070741 (Coinvestigator), and R01 MH083795 (Coinvestigator)]; and has received a Diversity Research Fellowship from Columbia University. Dr. Gordon serves as Senior Editor of *Human Heredity*. Dr. Barral-Rodriguez and Ms. Chitsazzadeh report no disclosures. Dr. Barker-Cummings was funded by the NIH [R01 NS020656 and R01 NS043472]. Dr. Ulgen was a consultant at the American University of Paris; and received research support from the Institut National de la Sante et Recherche Medicale (Postdoctoral Fellow) and the Fondation Recherche Medicale [ACE20061209064 (Postdoctoral Fellow)]. Dr. Hill was supported as a research associate by the NIH [NIMH R01 MH083565 and NINDS R01 NS35129] and by the Simons Foundation. Dr. Risch has received speaker honoraria from the American Association of Cancer Research, The Jackson Laboratory, and the American Society of Nephrology; and receives research support from the NIH [R01 MH080957 (Coinvestigator), R01 MH067005 (Coinvestigator), R01 GM073059 (Coinvestigator), P50 NS023724 (Coinvestigator), R01 NS053998 (Coinvestigator), R01 HL87647 (Coinvestigator), R01 HL086718 (Coinvestigator), R01 MH075007 (Consultant), and R01 MH085543 (Co-PI)], the Wayne and Gladys Valley Foundation, the Ellison Medical Foundation, and the Robert Wood Johnson Foundation (to the Kaiser Permanente Research Program on Genes, Environment and Health, of which he is codirector). Dr. Hauser has served as a consultant to Pfizer Inc., Valeant Pharmaceuticals International, Schwartz Biomedical, LLC, and Lundbeck, Inc. (Ovation Pharmaceuticals, Inc.); serves on the editorial boards of *Acta Neurologica Scandinavica*, *Neuroepidemiology*, and *Epilepsy Research*; and has received research support from the CDC [AAMC MM-1002-07/07 (PI)], the NIH/NINDS [5 T32 NS07153 (PI), R01 NS020656 (Coinvestigator), R01 NS036319 (Coinvestigator), and R01 NS043472 (Coinvestigator)], and the Hotchkiss Neurological Institute. Dr. Pedley serves on the National Advisory Neurological Disorders and Stroke Council (NINDS/NIH); received funding for travel from the American Academy of Neurology Board of Directors (Committee Chair) and the American Neurological Association; and receives royalties from publishing *Epilepsy: A Comprehensive Textbook*, 2nd edition (Lippincott Williams & Wilkins/Wolters Kluwer, 2008) and *UpToDate* (electronic medical textbook, Neurology) (Wolters Kluwer). Dr. Walsh serves as a journal editor for *Neuron*, *Science*, *Encyclopedia of Neuroscience*, and *Neural Development*; has served on the scientific advisory board of the McKnight Endowment, the Allen Institute for Brain Science, and the John Merck Fund; has received speaker honoraria for non-industry-sponsored lectures; serves as a consultant to the Autism Consortium, the Anne and Paul Marcus Foundation, and Constellation Pharmaceuticals; and receives research support from the NIH [NINDS 2 R01 NS032457-08 (PI), NIMH R01-MH083565 (PI), and NINDS R01 NS35129 (PI)], the Howard Hughes Medical Institute, and the Nancy Lurie Marks Family/Simons Foundation. Dr. Ottman serves on the scientific advisory board for and holds stock options in Trigeminal Solutions, Inc; has received funding for travel from the International League Against Epilepsy, Fondazione Ettore Majorana E Centro, and the Latin American Network of Human Genetics; has received speaker honoraria for non-industry-sponsored lectures; serves as a

consultant to Ortho-McNeil Janssen Scientific Affairs, LLC; and received research support from the NIH [R01 NS020565 (PI), R01 NS043472 (PI), R01 NS036319 (PI), R01 NS036630 (Coinvestigator), R01 NS039422 (Coinvestigator), and R01 NS053998 (Coinvestigator)].

Received January 13, 2009. Accepted in final form July 16, 2009.

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Editor's Note to Authors and Readers: Levels of Evidence coming to *Neurology*®

Effective January 15, 2009, authors submitting Articles or Clinical/Scientific Notes to *Neurology*® that report on clinical therapeutic studies must state the study type, the primary research question(s), and the classification of level of evidence assigned to each question based on the classification scheme requirements shown below (left). While the authors will initially assign a level of evidence, the final level will be adjudicated by an independent team prior to publication. Ultimately, these levels can be translated into classes of recommendations for clinical care, as shown below (right). For more information, please access the articles and the editorial on the use of classification of levels of evidence published in *Neurology*.^{1–3}

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Classification scheme requirements for therapeutic questions

Class I. A randomized, controlled clinical trial of the intervention of interest with masked or objective outcome assessment, in a representative population. Relevant baseline characteristics are presented and substantially equivalent among treatment groups or there is appropriate statistical adjustment for differences.

Class II. A randomized, controlled clinical trial of the intervention of interest in a representative population with masked or objective outcome assessment that lacks one criterion a-e in Class I or a prospective matched cohort study with masked or objective outcome assessment in a representative population that meets b-e in Class I. Relevant baseline characteristics are presented and substantially equivalent among treatment groups or there is appropriate statistical adjustment for differences.

Class III. All other controlled trials (including well-defined natural history controls or patients serving as their own controls) in a representative population, where outcome is independently assessed, or independently derived by objective outcome measurements.

Class IV. Studies not meeting Class I, II, or III criteria including consensus or expert opinion.

AAN classification of recommendations

A = Established as effective, ineffective, or harmful (or established as useful/predictive or not useful/predictive) for the given condition in the specified population. (Level A rating requires at least two consistent Class I studies.)

B = Probably effective, ineffective, or harmful (or probably useful/predictive or not useful/predictive) for the given condition in the specified population. (Level B rating requires at least one Class I study or two consistent Class II studies.)

C = Possibly effective, ineffective, or harmful (or possibly useful/predictive or not useful/predictive) for the given condition in the specified population. (Level C rating requires at least one Class II study or two consistent Class III studies.)

U = Data inadequate or conflicting; given current knowledge, treatment (test, predictor) is unproven.