2. Methods

2.1. Mouse model

Animals:

Commercially available C57BL/6J (B6), DBA/2J (D2), and B6.D2-Mtv-7a/Ty (Mtv7) mice of both genders were purchased from the Jackson Laboratory (Bar Harbor, ME). Female B6 mice were then crossed with male D2 mice to produce F1 progeny. F1 intercrosses produced F2 progeny. Mice were weaned at about 4 weeks of age and group housed (three or four mice per cage) by gender. All mice were maintained on a 12-h light/12-h dark schedule with food and water freely available. Experiments were approved by Animal Care and Use Committees overseeing each participating laboratory.

Seizure testing:

Maximal electroshock seizure threshold (MEST) was determined as previously described (Ferraro et al 1998a). MEST determination involved a standard paradigm in which 8- to 12-week-old mice received one shock per day with a daily incremental increase in electrical current until a maximal seizure was induced. Each shock induced at least a partial clonic seizure. The following sequence of events characterizes a session in which a maximal seizure occurred: tonic forelimb flexion, tonic hindlimb flexion, tonic hindlimb extension, and hindlimb clonus. The maximal seizure event was scored positive upon observation of tonic hindlimb extension.

Genome scan:

We constructed a genotyping panel of 90 DNA markers from published maps (Dietrich et al 1996) and an online database (http://www.informatics.jax.org/). The

mean (+S.D.) distance between markers was 16.7 (+4.4) cM, with the largest gap estimated at 33 cM between markers *D10Mit28* and *D10Mit186*. Other regions of the genome where distances between markers were estimated to be greater than 22 cM include *D3Mit29–D3Mit200*, *D6Mit159–D6Mit263*, *D12Mit105–D12Mit114*, and *D16Mit130–D16Mit13*. We tried to use markers with alleles that differed by more than 8 bp in length between strains. For such markers, we analyzed PCR amplicons by agarose gel electrophoresis with ethidium bromide staining (Ferraro et al 1998b). After electrophoresis, two independent scorers recorded genotypes from Polaroid 667 (3000 ISO) black and white prints and entered them into a database for subsequent error checking.

Congenic strains and fine mapping:

In order to further narrow down the seizure susceptibility locus on distal mouse chromosome 1 (Szs1), we used reciprocal, interval-specific congenic strains (Ferraro et al 2004). Briefly, we started with a B6xD2 intercross, followed by an F1 backcross to parental strains. N1 generation breeding was conducted in a crossed-gender fashion with both F1 males and females mated to parental strains. Heterozygosity at introgressed DNA markers was used to select mice for subsequent rounds of breeding. In total, 10 backcross generations were produced in a reciprocal fashion over the course of approximately 3 years. N10F1 mice were tested for seizure sensitivity at 8–12 weeks of age in a blind fashion with regard to genotype. Phenotypes and genotypes for these animals were correlated subsequently.

Mouse candidate gene analysis:

We compared the coding region sequences of Szs1 candidate genes between B6 and D2 mice using RT-PCR, amplification from genomic DNA and database searching. We could identify 12 brain-expressed genes with SNPs that predict a protein amino acid variation. Of these, the most compelling seizure susceptibility candidate is *Kcnj10*, which differed for aminoacid 262 (Serine D2, Threonine B6). A survey of the *Kcnj10* SNP among other inbred mouse strains revealed a significant

effect on seizure sensitivity such that most strains possessing a haplotype containing the B6 variant of *Kcnj10* have higher seizure thresholds than those strains possessing the D2 variant (Ferraro et al 2004).

Confirmation of QTL:

In order to further investigate the role of Szs1, we used a gene transfer strategy involving a bacterial artificial chromosome (BAC) DNA construct that contains several candidate genes harbored in tandem within the critical interval (including *Kcnj9* and *Kcnj10*). Fertilized oocytes from the seizure-susceptible congenic strain B6.D2-*Mtv7a*/Ty-27d were injected with BAC RPCI-23 157J4 DNA and three independent lines of BAC-transgenic mice were generated. Mice were then tested for MEST. Expression and mRNA analysis was performed and correlated with phenotypic effects.

2.2. Human Candidate gene analyses

Subjects:

All patient samples were collected using proper informed consent and protocols approved by Institutional Review Boards at each clinical site. Patients carried a diagnosis of either IGE or TLE. Clinical inclusion criteria for patients collected in Germany have been published previously (Sander et al 2000). Detailed inclusion criteria for the US sample are described elsewhere (Buono et al 2004).

Sequence analyses of ATP1A2 and KCNJ10:

Derived from the mouse model, the homologous human ion channel genes ATP1A2 and KCNJ10 were considered high ranking candidate genes. Intron–exon boundaries were systematically screened for mutations in 30 individuals with epilepsy and 30

controls using single strand conformation polymorphism (SSCP) analysis as described (Berrettini et al 1998). All SNPs were confirmed by subcloning and sequencing PCR amplicons from at least three separate PCR reactions from different individuals.

Association studies of ATP1A2:

ATP1A2 in TLE: Sequencing analysis of exon 2 surrounding intronic regions of the ATP1A2 gene (NCBI Access # J05096) revealed a 4 base pair insertion. We genotyped the ins-6704/TTCC polymorphism in 56 TLE patients and 56 controls using radiolabled PCR products and denaturing polyacrylamide sequencing gels. Gels were scored independently by two readers, and data were analyzed using Chi-square distribution statistics.

ATP1A2 in IGE: Genotypes were assessed in 152 idiopathic generalized epilepsy (IGE) patients of German ancestry and 111 healthy German controls for seven polymorphisms. The intron 1 ins-6704/TTCC and intron 22 variations were genotyped using SSCP analysis. The exon 9 SNP introduced a *Dde*I restriction site allowing genotyping by standard agarose gel electrophoresis and ethidium bromide staining of the restriction fragment length polymorphism (RFLP). Additional SNPs for genotyping were chosen based on availability of *Applied Biosystems* assays-on-demand, location in the gene and allele frequencies. Genotyping of four additional intronic SNPs across the ATP1A2 gene was performed using the *Applied Biosystems* "Assays-ondemand" SNP genotyping assay as per manufacturers protocol (rs1016732, rs1407130, rs6686067, rs2070702). Genotypes and allele frequencies were compared between groups using Chi-square contingency analysis. A two-tailed type I error rate of 5% was chosen for the analysis.

Association analysis of *KCNJ10*:

KCNJ10 in IGE and TLE: Sequencing identified the common missense variation Arg271Cys in the KCNJ10 gene. Genotypes were obtained for 407 epilepsy patients and 284 controls using RFLP, pyrosequenging and SSCP. Genotypes and allele

frequencies were compared between groups using chi-square contingency analysis. A two-tailed type I error rate of 5% was chosen for the analysis.

Confirmation of *KCNJ10*:

KCNJ10 replication study: In an attempt to replicate our initial finding, 563 IGE patients and 660 controls were genotyped for the Arg271Cys variation in KCNJ10 gene using ABI Assays-on-Demand service (rs1130183). Allele and genotype frequencies, χ2-tests, odds ratios (OR) for individuals carrying the Cys271 allele ($OR_{Cys271+}$) together with the 95% confidence interval (95% CI) and the test for Hardy–Weinberg equilibrium were calculated. With regard to the `a priori hypothesis that the Cys271 allele confers seizure resistance, a one-sided type I error rate of P = 5% was chosen for the analyses. Power analysis for the replication study indicated that sample sizes of 446 IGE subjects and 660 controls provide a statistical likelihood of 88% for a one-side type-I error rate of P = 0.05 to detect a seizure resistance effect with an attributable relative risk of 0.52 (Lenzen et al 2005).