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Development of a New Genetic Model for Absence Epilepsy: Spike-Wave Seizures in C3H/He and Backcross Mice

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To characterize the genetic basis of spike-wave discharges (SWDs) detected by electroencephalography (EEG) in C3H/He mice, substrains of C3H mice were evaluated by EEG and sensitivity to ethosuximide. Crosses with the SWD-negative strain C57BL/6J were performed to map the underlying gene(s). C3H/He substrains exhibited a modest incidence (average of 19 SWDs per hour) of 7–8 Hz SWDs when at rest, compared with the C3HeB/Fe subline (four SWDs per hour). In the mapping backcross, however, many mice showed a very high incidence (50–220 SWDs per hour) throughout the recording period. SWDs were first detected at 3.5 weeks of age, were associated with behavioral arrest, were suppressed by ethosuximide, and were strongest in the cerebral cortex and thalamus. The major C3H determinant of SWDs, *spkw1* (spike-wave 1), mapped to chromosome (Chr 9), and together with a C57BL/6J determinant on Chr 8, *spkw2*, accounted for more than one-half of the phenotypic variation in the backcross mice. The modest SWD incidence in C3H/He mice and the high incidence in backcrosses implies that SWD could be a confounding variable for other behaviors. Because C3H/He mice have no other brain abnormalities, they are an attractive alternative for studying idiopathic absence epilepsy.

Key words: **absence epilepsy; spike-wave discharge; inbred strain; genetic; C3H; corticothalamic**

Introduction

Absence (or petit-mal) epilepsy is characterized by a brief arrest of normal behavior associated with abnormal spike-wave discharges (SWDs) as seen by electroencephalogram (EEG). Absence epilepsy has a strong genetic component, but its genetic basis is not well understood. However, several mutant and inbred strains of rodents show SWDs. Genetic absence epilepsy rats from Strasbourg (GAERS) and WAG rats have polygenic SWDs, but the underlying genes are not known (Gauguier et al., 2004; Rudolf et al., 2004). Several mouse spontaneous mutations exhibit SWDs as monogenic phenotypes, and the genes for these mutations are known: "tottering" (*Cacna1a*^{tg}) (Fletcher et al., 1996), "lethargic" (*Cacnb4*^{lh}) (Burgess et al., 1997), "ducky" (*Cacna2d2*^{du}) (Barclay et al., 2001), "stargazer," (*Cacng2*^{stg}) (Letts et al., 1998), "mocha" (*Ap3d1*^{mh2J}) (Kantheti et al., 1998), "slow-wave epilepsy" (*Slc9a1*^{swe}) (Cox et al., 1997), and "coloboma" (*Cm*, *Snap25*) (Zhang et al., 2004). In addition, the *Hcn2* knock-out mouse (mutant I_h channel) also has SWDs (Ludwig et al., 2003). Interestingly, four of these mutations occur in subunits of the high-threshold voltage-dependent calcium channel (VDCC). Although the functional relationship between these and the T-type channels known to be involved in absence seizures is unknown, the relationship between tonic firing (associated with P/Q type currents) and burst firing (T-type currents) in the circuits has been suggested as a link (Kim et al., 2001; Song et al., 2004). However, all known mouse strains with SWDs also have severe motor abnormalities not typically associated with absence epilepsy in humans.

Common inbred mouse strains, such as C57BL/6 (B6), BALB/c, and C3H are often used as wild type, but this does not mean that they are normal. Being inbred and subjected to many generations of selection, inbred strains often have phenotypic abnormalities, some of which may model human disease. Many mouse strains (including C3H) carry a mutation in the *Pde6b* gene that causes severe retinal generation (Pittler and Baehr, 1991); others (such as I/Ln) carry mutations such as piebald with defects in the development of the neural crest; these are just two of many examples (Bogue, 2003). In the field of epilepsy, several mouse strains (e.g., EL, SWXL-4, PL) exhibit tonic–clonic and generalized convulsions with routine handling (Rise et al., 1991; Frankel et al., 1994; Kitami et al., 2004). In some strains, it has been shown that convulsions are explained by the inheritance of susceptibility alleles from nonepileptic parental strains (Frankel et al., 1994). These seizure disorders are inherited as polygenic traits, with each mapped locus only accounting for a fraction of the phenotypic variance, resulting in slow progress in gene identification in such models.

Although inbred mouse strains have not been described as having absence epilepsy phenotypes per se, in the 1970s an electroencephalographic phenomenon known as a "brief spindle episode" (BSE) was described in a few strains (Ryan and Sharpless, 1979). Those researchers discussed the possibility that the BSEs emanated from thalamocortical circuits, as do SWDs, but stopped short of describing them as absence models. BSEs have since been mentioned in a few studies as an outcome of alcohol withdrawal in some mouse strains (Veatch and Becker, 2002).

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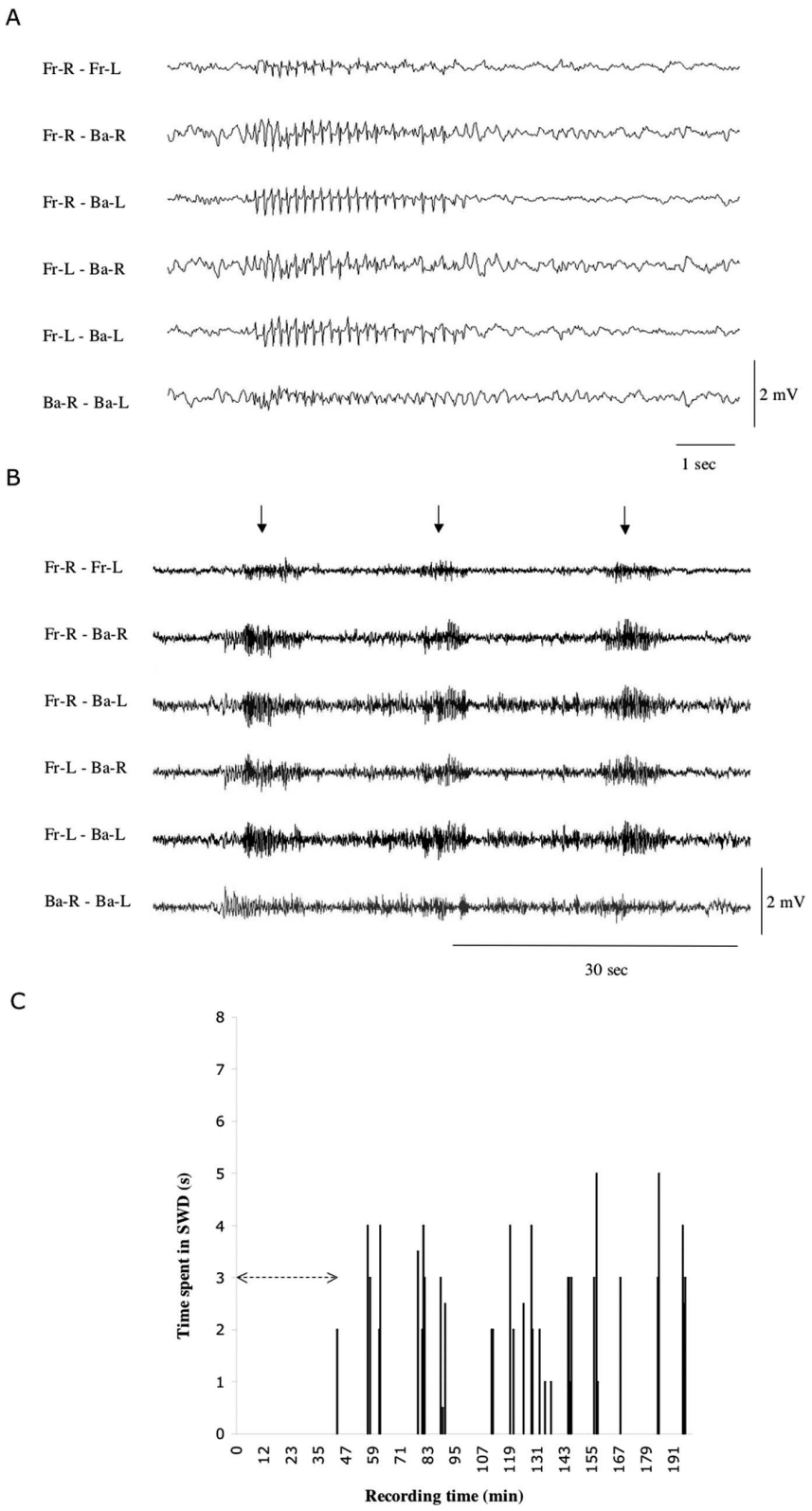

Figure 1. Representative SWDs of C3H/HeJ mice. ***A***, High resolution. ***B***, Low resolution. Arrows denote the SWD bursts described in Results. ***C***, Onset and incidence of SWDs in a typical recording session (the double-headed arrow highlights a typical lag). Fr, Front; Ba, back; R, right; L, left.

Here we describe SWDs in a common mouse strain, C3H/He, with no other brain abnormalities. We find that these SWDs have pharmacological and behavioral properties that model absence epilepsy, that they are modified by genetic background, and that a single major C3H-derived gene on chromosome 9 (Chr 9), in combination with a modifier gene on Chr 8, accounts for the majority of SWD incidences.

Materials and Methods

Mice. All mice used in this study were obtained originally from The Jackson Laboratory (Bar Harbor, ME). C3H/HeJ mice used for depth recordings were housed at the University of Pennsylvania in an Association for Assessment of Laboratory Animal Care accredited facility with a 12 h light/dark cycle and *ad libitum* access to food and water; all other animals were housed in the Research Animal Facility at The Jackson Laboratory. All animal procedures were approved by the respective institutional animal care and use committee.

Surface or cortical EEG recordings. Mice that were between 3.5 and 10 weeks of age were tested for spontaneous SWD activity. Mice were anesthetized with tribromoethanol (400 mg/kg, i.p.) and placed in a stereotaxic holder fitted with a mouse incisor bar. Burr holes were drilled (posterior to bregma and 1 mm lateral to midline) on both sides of the skull. Either of two procedures was used to measure EEG activity. Initial studies were done using two Teflon-coated bipolar electrodes implanted at 0.1–0.5 mm below the dura. Screws were placed at the periphery of the skull to anchor the dental cap. Later studies (including genetic mapping) were done using four silver electrodes, soldered onto a microconnector, that were slid between the skull and the dura, two on each side of the cortex, and then a dental cap was applied. After the mice recovered from surgery and a 24 h resting period, EEG recordings were taken over a 3 d period, for a maximum of 3 h each day, using a Grass EEG Model 12 Neurodata Acquisition System and PolyviewPro software program (Grass Instruments, West Warwick, RI). The parameters for detecting SWDs have been described previously (Hosford et al., 1995).

Localized EEG recordings. To assess the brain regions involved in the generation of SWDs in C3H/HeJ mice, local bipolar electrodes were placed in the hippocampus [anteroposterior (AP), −2.5; lateral (Lat), −3; depth, −4], thalamus (AP, −2.5; Lat, −1; depth, −4), and parietal cortex (AP, −2.5; Lat, −2; depth, −2) relative to bregma using the stereotaxic coordinate system. A total of 14 mice were used for this portion of the study. Among these, three mice had cortical bipolar recordings, four mice had hippocampal bipolar recordings, and seven had thalamic bipolar recordings. The surgical procedure was performed under isoflurane anesthesia. The mice were 10–11 weeks of age during recording. Two hours of resting EEG activity was recorded 7 d after electrode implantation using Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Power spectral analysis with fast Fourier transforms was performed on the first five SWDs and five areas of interictal (non-SWD) activity for theta activity (7.5–9 Hz). A repeated-measures ANOVA was used to find main effects of SWDs and electrode placement. Significant interactions between SWDs and placement were

followed by planned comparisons to determine regional changes in power with SWDs.

Genome scan, genetic analysis. For genetic mapping, genomic DNA from tail tips was prepared and sent to Kbiosciences (Hertfordshire, UK) for genome-wide single nucleotide polymorphism (SNP) analysis, using a marker approximately every 15 centimorgans in the genome. Additional simple sequence length polymorphism markers were typed as needed, in certain regions. Genotype data were tabulated, genetic marker maps were constructed, and genome scans were done for linkage of the trait SWDs per hour using MapManager QTX version b20 (www.mapmanager.org). Genome-wide significance thresholds for both main effect and pairwise scans were performed using the permutation test feature of MapManager, for 1000 permutations. Multivariate analysis was done using JMP software (SAS Institute, Cary, NC).

Results

SWDs in C3H/He sublines

In 2002, during EEG recordings done for the purpose of measuring auditory evoked potentials from several mouse strains, we noticed that C3H/He mice exhibited a high frequency of events that looked strikingly like the SWDs seen in stargazer mice (high-amplitude, synchronous, bilateral spike-waves lasting for several seconds in the absence of auditory stimuli). To assess the brain activity of C3H/HeJ mice systematically, EEG recording was done using either depth, cortical (subdural), or epidural surface electrodes. In initial studies at the University of Pennsylvania, 80 male adult mice were implanted with unipolar intrahippocampal electrodes referenced to the ipsilateral frontal sinus; this electrode configuration reflects whole-brain EEG activity. SWDs with a frequency of ~6–8 Hz and an amplitude between ~800 and 2300 μV were noted in every mouse before and during auditory evoked potential tasks 7 d after electrode implantation. In follow-up studies at The Jackson Laboratory, both male and female adult C3H/HeJ mice were implanted with four unipolar surface electrodes, placed over each quadrant of the cerebral cortex, and differential recordings were made between poles. We observed high-amplitude, bilateral, synchronous SWDs frequently during a given recording session (Fig. 1*A*). Most C3H/HeJ mice tested exhibited at least some SWD activity, defined as an episode lasting >0.5 s, greater than twice the amplitude of baseline, and observed in two or more channels. The burst frequency of the SWDs was ~7–8 Hz. The average duration was typically 2.5 s, and the EEG appeared fairly normal before and after each episode. Although most of the study was done in adult mice, similar SWDs were observed in C3H/HeJ mice as young as 3.5 weeks of age (data not shown).

Using the less-invasive surface electrodes, the SWD episodes in adults appeared predominantly ~30–40 min after the beginning of recording, after the subject became less active from exploring the box and was settling down to rest; it was not yet asleep and still engaged in intermittent activities such as grooming. Once settled, however, the SWD bursts often appeared in clusters (Fig. 1*B*,*C*).

To determine whether other substrains of C3H mice exhibit SWDs, we recorded from C3H/HeOuJ, C3H/HeSnJ, and C3HeB/FeJ mice, the three major sublines that exist at The Jackson Laboratory. Whereas both the HeOu and HeSn sublines showed frequent SWDs in the same manner as the HeJ subline, including the burst frequency, incidence, and tendency to show episodes during wake–rest transition, significantly fewer SWDs ($p = 0.004$; t test) were observed in mice from the eB/FeJ subline (see Fig. 3*A*). F_1 hybrids between HeJ and eB/FeJ showed the same low incidence of SWDs ($p = 0.07$) as eB/FeJ (see Fig. 3*A*). Together, these results suggest that a recessive mutation(s) causing the higher SWDs was not fixed in the C3HeB/FeJ subline when it diverged from the others and that additional variants associated with a low incidence of SWDs are shared among all C3H strains.

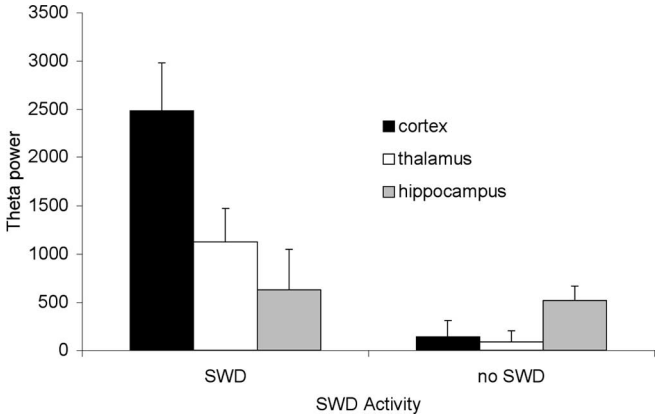

Figure 2. Localization of SWDs. SWD activity increases theta power in the cortex and thalamus but not in the hippocampus. Error bars represent SEM.

To determine which brain region(s) were most involved in generating these SWDs, we implanted depth electrodes into the hippocampus (CA3), the thalamus, or the cortex and assessed which regions were associated with SWDs in the burst frequency range around 7.5–9 Hz, their typical range. Amplitude estimates during SWDs ranged from 300 to 800 μV, with non-SWD activity ranging from 75 to 400 μV depending on region. The SWD duration ranged from 2.9 to 4.6 ± 1.6 s. Main effects of SWD ($F_{(1,10)} = 16$; $p = 0.002$) and placement ($F_{(2,10)} = 4.02$; $p = 0.052$) as well as a SWD by placement interaction ($F_{(2,10)} = 4.18$; $p = 0.047$) indicate that the seizure intensity is regionally specific. Planned comparisons reveal increased power during SWDs in the cortex ($F_{(1,10)} = 16.05$; $p = 0.003$) and thalamus ($F_{(1,10)} = 6.25$; $p = 0.032$) but not in the hippocampus ($F_{(1,10)} = 0.04$; $p = 0.841$) (Fig. 2).

Segregation analysis and trait locus mapping

To determine the mode of inheritance, we first crossed C3H/HeJ mice to B6 mice and examined F_1 hybrids: none exhibited SWDs, which is consistent with a recessive mode of inheritance (data not shown). We then crossed the F_1 hybrids to C3H/HeJ mice and assessed SWDs in the resultant backcross, or N_2 generation. A single-gene, Mendelian pattern of inheritance would have resulted in one-half of the N_2 mice exhibiting SWDs and one-half appearing normal. Instead, we observed that almost all of the N_2 mice exhibited SWDs, suggesting that that inheritance was non-Mendelian (Fig. 3*A*). However, in the N_2 population, the variation in SWD incidence was broad, with many mice exhibiting a much higher incidence and length of SWDs than any of the parental C3H/HeJ mice. For example, approximately one-half of the N_2 mice had >50 SWDs per hour; some SWDs lasted for as long as 10–15 s (Fig. 3*B*). Interestingly, most backcross mice exhibited SWDs throughout their recording period and not just during restful periods.

Despite the fact that the inheritance was not simple, the variance in SWD rate among N_2 mice encouraged us to attempt a genome scan to look for correlations between genotype and phenotype. More than 120 SNPs were genotyped across the genome in the majority of the 53 backcross mice, a robust linkage map was constructed using these markers, and marker regression was done to correlate genotype with the trait SWDs per hour. Strikingly, markers on centromeric Chr 9 showed a very high correla-

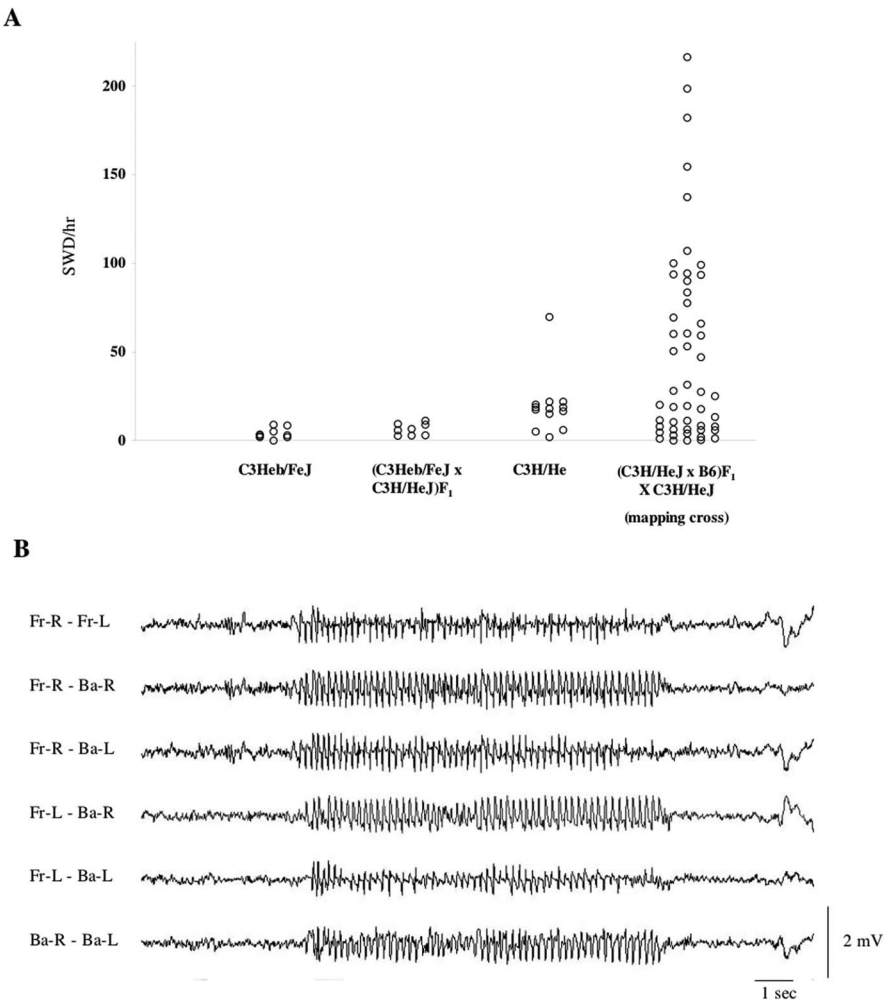

Figure 3. SWDs in an $F_1 \times$ C3H/HeJ backcross (N_2) population. ***A***, Distribution of SWDs per hour in N_2 mice compared with C3H/He and C3HeB/FeJ sublines and (C3H/He $\times$ C3HeB/FeJ)F_1 hybrids, showing higher incidence in the N_2 mice. ***B***, Representative SWDs of N_2 mice. Fr, Front; Ba, back; R, right; L, left.

tion with the phenotype, surpassing the highly significant genome-wide threshold as estimated using permutation tests (Fig. 4*A*). This locus, which we have termed *spkw1* (spike-wave 1), accounts for almost 40% of the total phenotypic variance in SWD rate in this backcross.

To begin to understand the non-Mendelian nature of the phenotype, we also performed a pairwise genome scan, testing whether loci act epistatically to affect SWDs. This test assesses whether pairs of loci, as opposed to one locus at a time, can interact with one another to account for the phenotype (Frankel and Schork, 1996). Using a permutation test to assess statistical significance (Churchill and Doerge, 1994), one pairwise interaction surpassed a significant ($p < 0.05$) genome-wide threshold, Chr 9 [National Center for Biotechnology Information (NCBI) refSNP accession number 3023202] by Chr 8 (NCBI refSNP accession number 3737220 or 3722665) (Fig. 4*B*). Furthermore, it was the B6 allele from Chr 8, and not the C3H allele, that was associated with high SWD incidence. Together, the Chr 9 single-locus effect and its interaction with Chr 8 accounted for 52% of the total phenotypic variance, as assessed by multiple regression analysis (data not shown). No other significant locus associations or epistatic effects were detected in the backcross population.

SWDs in backcross mice have hallmarks of absence seizures

Two criteria of absence seizures in model organisms are as follows: (1) They should be accompanied by an arrest of normal behavior. (2) They should respond to anti-absence-specific drugs, such as ethosuximide (ETX). Because the parental C3H/He mice were not very active when SWDs occurred, and because the act of injecting ETX would interfere with the rest activity pattern, we could not conclusively assess these criteria in these mice. But because many N_2 mice showed SWDs frequently and throughout the recording sessions, regardless of activity state, we were able to examine these important criteria. Indeed, the N_2 mice ceased normal behavior and become still for the duration of the SWD, except for the occasional whisker twitch, after which the mice resumed normal behavior. We treated N_2 mice showing various levels of SWDs with ETX (Fig. 5). Significantly fewer SWDs were observed in the 30 min period after ETX treatment than in the 30 min immediately preceding treatment ($p = 0.03$; repeated-measures ANOVA), after which the SWD incidence remained low or gradually resumed to previous levels (sample shown in Fig. 5*A*, all tests shown in Fig. 5*B*). Saline injection did not decrease the SWD incidence (Fig. 5*B*, stippled lines) Although some mice responded better to ETX than did others or recovered more quickly, the total number of SWDs observed in the mice 30 min after ETX treatment was very significantly lower than in saline-treated controls, compared with the incidence in the 30 min before treatment (2×2 contingency; $\chi^2 = 44$; $p < 0.0001$). The onset of SWDs after treatment was also significantly delayed in ETX- vs saline-treated mice (t test, $p < 0.02$; data not shown). Together, these results demonstrate that the SWDs of these mice are accompanied by the behavioral and pharmacological characteristics of absence seizures.

Discussion

In this study, we show that substrains and crosses involving C3H/He have a high incidence of SWDs, the hallmark electrophysiological characteristic of absence epilepsy. In humans, absence epilepsy has a strong genetic component, but progress in identification of the underlying genes has been slow, in part because it is often inherited as a genetically complex trait. Although a few genes for idiopathic generalized epilepsy in which some patients have absence seizures have been identified (Wallace et al., 2001), the best evidence for a genetic basis of absence epilepsy per se was finding mutations in the gene encoding *CACNA1H*, a T-type low-threshold VDCC, in Northern Chinese children (Chen et al., 2003). This important finding was not a surprise based on the known role of T-type channels in SWDs and was presaged by studies of *Cacna1g* knock-out mice, which are resis-

tant to chemically or genetically induced SWDs (Kim et al., 2001).

The manifestation of SWDs within C3H/HeJ adults was not typical for absence seizures, because they did not usually occur interspersed with periods of activity throughout an EEG recording session, but rather 30–40 min into the session, when mice were beginning to nestle in their shavings for a rest. During this transition, bursts of frequent, high-amplitude synchronous, bilateral SWDs were readily observed. However, when we recorded from C3H/HeJ × (C3H/HeJ × C57BL/6J)F_1 backcross (N_2) mice for segregation analysis and genetic mapping, we noticed that many exhibited SWDs very frequently, regardless of their activity state, suggesting that genetic modifier(s) from the C57BL/6J background relaxed the conditions under which SWDs are likely to occur. This broader manifestation of the phenotype made it possible for us to determine that these events have two of the hallmarks of absence seizures: behavioral arrest and pharmacological suppression by ethosuximide, which is selectively effective for absence epilepsy.

C3H/He mice represent an alternative to existing genetic models for studying absence epilepsy, because they do not have other major brain abnormalities, such as the movement disorders that impair the behavior of *stargazer*, *tottering*, and *lethargic* mutants, for example. The enhanced SWD phenotypes of many C3H/He × B6 backcross mice make it even more appealing. However, the B6-modified phenotype does not yet provide a useful working model, because backcross mice are not genetically uniform. Toward this end, we have begun to construct a congenic strain, containing mid-Chr 8 from C57BL/6J on a C3H/He strain background.

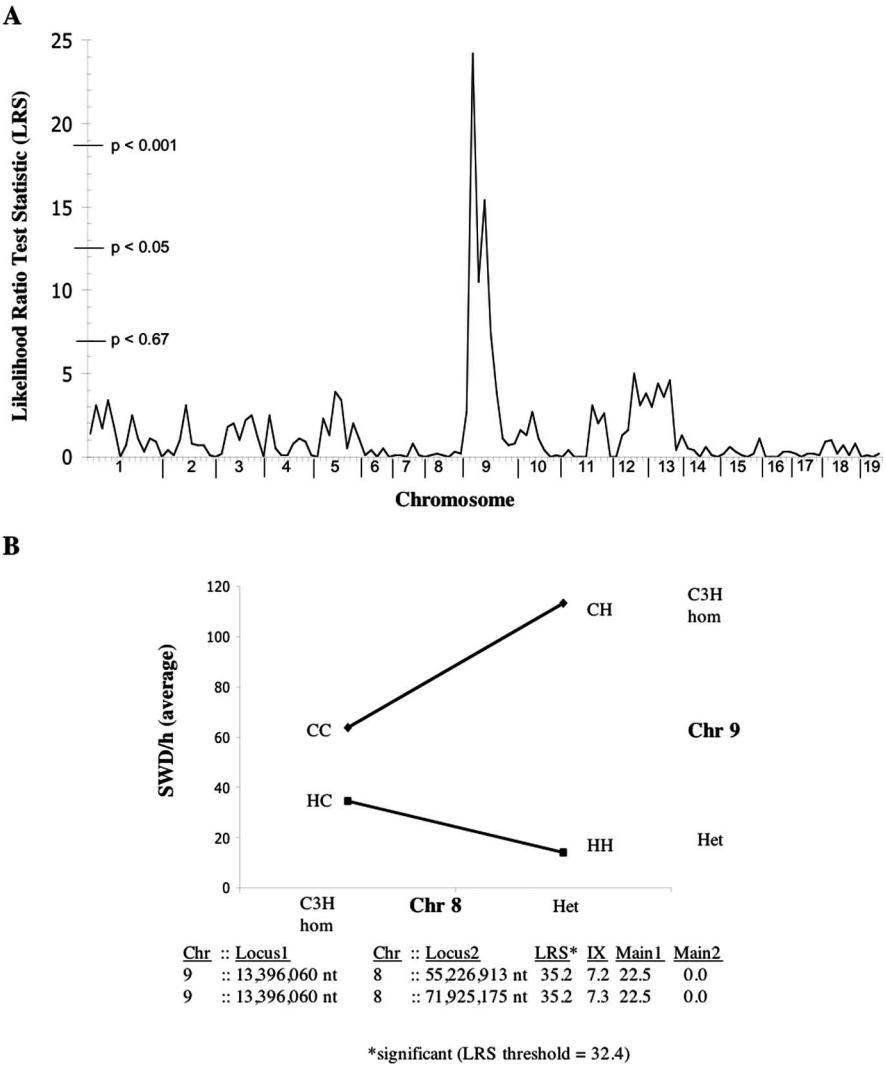

Figure 4. Results of a genome scan for SWD incidence in N_2 backcross mice. ***A***, Main effect. ***B***, Pairwise scan for epistatic interactions. The main effect assesses the correlation of individual markers, whereas the epistasis test assesses the correlation of pairs of markers with the SWD phenotype. Genome-wide significance thresholds were estimated by permutation testing (22); the threshold reached by the Chr 9 by Chr 8 interaction was significant at $p < 0.05$ genome-wide. CC, C3H hom Chr 8, C3H hom Chr 9; C3H hom Chr 8, het Chr 9; HC, het Chr 8, C3H hom Chr 9; H, het both chromosomes; hom, homozygous; Het, heterozygous.

Most of the genetic effect on the incidence of SWDs in the backcross mice comes from *spkw1*, a C3H/He-derived allele on centromeric Chr 9. By itself, it accounts for almost 40% of the total phenotypic variance, and together with a C57BL/6J-derived modifier on Chr 8, it accounts for >50%. Considering that there is some inherent procedural variance in measuring the EEG quantitatively in freely moving mice because of movement artifact, manual data review, or the occasional loose connection affecting the strength or integrity of the EEG signal, it is likely that the Chr 8 and Chr 9 effects account for most of the genetic variance (a parameter that cannot be measured directly in our study). That we were able to detect such strong effects at all in only 53 backcross mice also supports this model. However, a two-gene model cannot explain the entire effect, because no SWDs were seen in F_1 mice, and yet almost all of the N_2 mice (including Chr 9 heterozygotes) experience some level of SWDs; this suggests that there may be additional recessive alleles with small effects.

The C3H inbred strain was derived in the 1920s from a cross of a Bagg albino female with a DBA male. C3H is a very popular strain for research and is most well known for its high incidence of mammary tumors caused by an exogenous mouse mammary tumor virus. Like some other inbred strains, it also is homozygous for the retinal degeneration mutation, a very old mutation in the gene encoding PDE6B, causing severe visual impairment by young adulthood. The C3H/He substrain (He for Heston) arose in the 1940s. The substrains tested in this study were derived over the subsequent decade. We found that three of the four substrains showed a modest to high incidence of SWDs, but one, C3HeB/FeJ, showed a very low incidence. This suggests that the parental C3H strain already had a low-level predisposition to SWDs and that a new major mutation, likely *spkw1*, arose later but was not yet fixed in the germline until after 1948, when the C3HeB/FeJ substrain was derived. Considering that C3H/He is a popular strain, it is important to consider what effects these SWDs might have on behavioral studies. Although adult C3H/He mice are visually impaired and are therefore not usually given behavioral tests that rely on vision, one would expect to observe confounding effects on sleep and possibly eating or drinking behaviors attributable to this absence seizure phenotype.

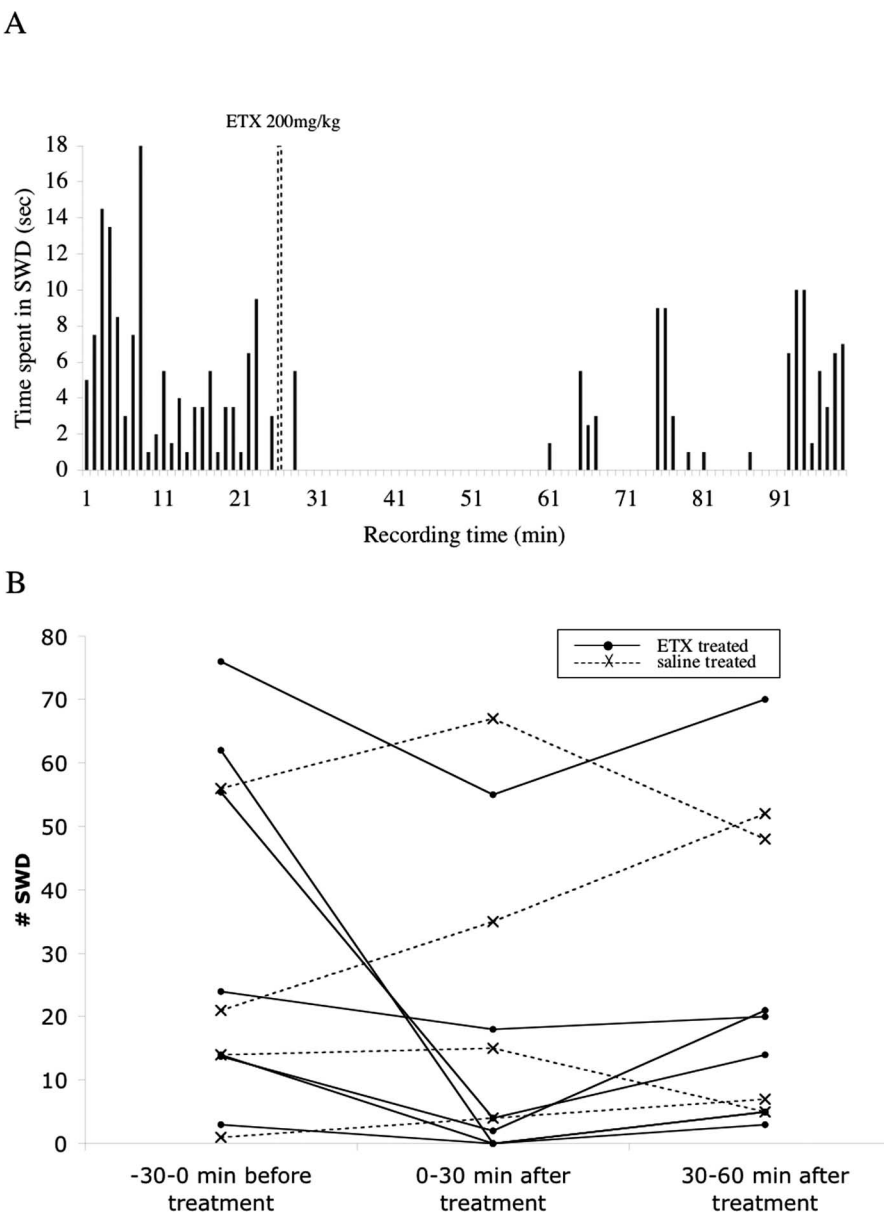

Figure 5. Ethosuximide suppresses SWDs in backcross mice. ***A***, Sample plot of a recording session from a backcross mouse in which ETX (200 mg/kg) reduced the number of SWDs. ETX was injected intraperitoneally ~25 min into the session. ***B***, Summary of SWD incidence of six ETX-treated (●, solid line) and four saline-treated (×, dashed line) backcross mice in the 30 min before treatment, in the 30 min immediately after treatment, and in a subsequent 30 min period.

At present, the 95% confidence interval for the location of *spkw1* on Chr 9 is quite broad (~30 Mb). We do note, however, that the centromeric third of this interval, where *spkw1* most likely resides, contains only 29 genes, although none encode ion channels known to be involved in epilepsy. The gene encoding the ionotropic AMPA receptor *Gria4* (also known as *Glur-4*), which is expressed in the cortex and in the thalamus, is located near the likelihood peak for *spkw1*. However, in this gene, no coding-sequence differences or gross expression differences were detected between C3H/He, C3HeB/FeJ, and B6 mouse strains (data not shown). Although we cannot exclude the possibility that a more subtle expression difference in *Gria4* is responsible for the phenotype, it is more likely that the underlying gene will be a novel target for absence epilepsy. Another ion channel that maps to the so far broad critical interval for *spkw1* is *Kcnj*5, also known as G-protein-activated inwardly rectifying K^+ channel (GIRK4). GIRK4 is expressed in discrete regions of the brain, including certain cortical and thalamic nuclei. GIRK4 knockout mice have been generated, and they exhibit deficiencies in several behavioral tests; however, their EEG has not been reported, so it is unknown whether they exhibit SWDs. Similarly, however, we have detected no coding-sequence variants in *Kcnj5* between the relevant mouse strains. To date, there are not many trait loci mapped for human absence epilepsy, and of those that are mapped, none reside on human chromosomes 11q or 19p, where the *spkw1* homolog maps. However, of the two polygenic rat models for absence epilepsy, one (GAERS) had a suggestive association with a marker on rat Chr 8, which is in the region orthologous to that in which *spkw1* resides. It will be interesting to determine whether and how *spkw1* is related to this minor locus from this popular rat model for epilepsy.

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