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Letter

A Major Effect QTL Determined by Multiple Genes in Epileptic EL Mice

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The EL mouse strain provides a polygenic model for epilepsy. Previous mapping experiments between EL and nonepileptic ABP mice identified, and a congenic strain confirmed, a quantitative trait locus (QTL), *El2*, which lowered the threshold to seizures induced by gentle rhythmic tossing. To narrow the map interval further we used a nested strategy to analyze a series of recombinants derived from the congenic strain. The recombinant strains revealed a complex pattern of inheritance, with at least two independent regions of Chromosome 2 necessary for rhythmic tossing seizures and additional regions associated with unusual gender effects. Similar results obtained using a completely independent paradigm, pentylenetetrazole-induced tonic-clonic seizures, exclude the possibility that the genetic complexity was a unique property of the testing assay. Thus, although conventional QTL mapping efforts detected and appeared to confirm a trait locus with effects large enough for fine-structure mapping, subsequent dissection revealed multiple loci. Although at least one of these loci was mapped to a 1-cM interval, its individual effect is small, perhaps approaching the practical limits for further study. Our results in the EL mouse may be prophetic for similar assaults on other polygenic, composite neurological behaviors which vary among inbred strains, begging the consideration of alternative strategies toward gene identification in these models.

Epilepsy is a group of chronic disorders characterized by recurrent seizures (Wyllie 1993). Approximately 2.5 million people in the United States have epilepsy, and 200,000 people have seizures more than once a month making epilepsy second only to stroke as the leading neurological disorder. As epilepsy is known to be a genetically heterogenous disorder in humans (Anderman 1982; Anderson et al. 1999; Greenberg and Delgado-Escueta 1993), the availability of genetically diverse and complex mouse models for epilepsy provides an important experimental resource. Studying these models may provide essential information about the biochemical and physiological abnormalities involved in epilepsy.

The EL/Suz (EL) mouse has been described as a genetic model for human complex partial seizures with secondary generalizations (Brigande et al. 1989; Seyfried et al. 1992). Seizures originate in either the parietal cortex (Ishida et al. 1993) or the hippocampus (Mutoh et al. 1993) and then generalize to other brain regions. At ~90 days of age, EL mice become exquisitely susceptible to seizures following routine handling, and several variations of a physical rhythmic tossing procedure have been applied to achieve a more objective measure of seizure susceptibility (Fueta et al. 1983; Flavin et al. 1991; Frankel et al. 1994). In addition, EL mice are more susceptible than other strains to pharmacologically-induced convulsions including GABAa antagonists such as pentylenetetrazole (PTZ) (Sugaya et al. 1986; Naruse et al. 1960; Nakamoto et al. 1996). Although there appears to be a loss of inhibition in EL compared to control strains (DDY), pharmacologically GABAa receptor function is intact (Wang et al. 1997).

Quantitative trait locus (QTL) mapping in segregating crosses of EL with the nonepileptic strain ABP/Le (ABP), using the rhythmic tossing method to induce seizures, has identified seizure frequency QTL on multiple chromosomes (Rise et al. 1991; Frankel et al. 1995a). For the QTL *El2*, which had the largest effect on seizure frequency in any of these crosses, previously we found that a congenic strain constructed with the high (EL) susceptibility allele bred to a low (ABP) background inherits a major portion of the epilepsy susceptibility from the parental EL strain (Frankel et al. 1995b). Here, we attempted to narrow the *El2* map interval further by using a series of derivative recombinant strains, a robust strategy whereby multiple individuals are evaluated for each recombinant genotype. In this work we examined tonic-clonic seizures induced in two independent seizure threshold paradigms.

RESULTS

Nested Recombinant Strains to Dissect the *El2* Region

From initial QTL mapping experiments, the chromosomal localization of *El2* was very imprecise (2-lod confidence interval of >20 cM) and a large portion of Chromosome 2 was transferred in the construction of congenic strains. This imprecision resulted partly from the inherent difficulty of QTL mapping in modest-size single generation mapping crosses where trait charac-

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teristics of a given genotype can be determined only once, and partly from the segregation of unlinked loci. Therefore, as a prelude to high resolution crosses for fine-structure mapping of *El2*, we decided to break-up the *El2* congenic interval with a series of strains containing nested recombinations, anchored on either the centromeric or telomeric end by the recombination from the EL2E congenic strain. As each recombinant strain is homozygous, multiple individuals could be tested by independent measures of seizure susceptibility to determine which region harbors the main effect. In addition, recombinant strains which retained high seizure susceptibility would serve as renewable resources for further investigation. Individual mice containing recombinations were identified from crosses between EL2E and ABP, outcrossed to ABP, and then inbred lines were established to retain a particular recombination. Simple sequence length polymorphisms (SSLP) found between EL and ABP strains were used to define the recombination points of each strain. The result was eight derivative strains, four containing recombinations on the telomeric half of the *El2* congenic interval and four on the centromeric half (Table 1).

Multiple Determinants for Rhythmic Tossing Seizures

Seizure susceptibility of recombinant and parental strains was first assessed by analyzing the development of seizures over the duration of the rhythmic tossing assay (Fig. 1A). This plot is useful for qualitative assessment of seizure incidence differences between strains and genders, as well as a starting point for further analyses. In both males and females, the seizure onset of the parental strain EL2E was earlier than in all other strains, and individuals in recombinants 5 and 6 began to seize near the midway point of the testing paradigm, although recombinant 5, which contains more of the donor strain interval, had a steeper onset curve. Recombinant 4 had an earlier onset in females than in males and further delays in seizure onset were seen in other recombinant strains, as well as the ABP parent. In general, the females were more susceptible than males, although this difference was stronger in some regions than in others.

El2 was mapped initially in conventional backcrosses following a single, arbitrary, quantitative measurement, mean seizure frequency per 20 rhythmic tossing tests. For a quantitative assessment in the present study, where different congenic strains and derivative recombinants were compared to each other we first determined the effective test number required for 50% of each group to experience a seizure (ET_{50}). This is a more standardized, albeit conservative approach. Comparisons of ET_{50}s show that no single interval accounts for the high seizure susceptibility of the EL2E strain. Although recombinant 5 did have an ET_{50} similar to that of EL2E, neither recombinants 4 or 6, which overlap to together span the interval, was as susceptible (Fig. 1B). These data provided the first direct evidence that at least two genes on Chromosome 2 contribute to *El2*.

To assess the significance of quantitative differences between recombinant strains and at the same time to minimize nongenetic effects, for example, test procedure on outcome (Poderycki et al. 1998), we limited the window of analysis to the earliest test number (15 tests) that showed a maximal difference between parental strains. These data again show that only nested recombinant 5 was as seizure susceptible as EL2E, whereas other strains show only partial susceptibility at best, depending upon gender and the chromosomal interval retained. (Results were not substantially different from those determined after 20 tests; data not shown.) These results are consistent with and extend the ET_{50} results, showing that at least one gene from the proximal half of the interval and another from the distal half together comprise the seizure susceptibility of the parental congenic strain.

Table 1. Genetic Markers that Define the Recombination Boundaries of Congenic Strains

Strain	Proximal boundary	Distal boundary	Interval size (cM)
EL2E	*D2Mit5–D2Mit7*	*D2Mit71–D2Mit51*	52.5
1	*D2Mit422–D2Mit21*	from EL2E	14.7
2	*D2Mit133–D2Mit30*	from EL2E	23.0
3	*D2Mit132–D2Mit103*	from EL2E	26.2
4	*D2Mit479–D2Mit58*	from EL2E	28.4
5	from EL2E	*D2Mit258–D2Mit136*	34.9
6	from EL2E	*D2Mit224–D2Mit193*	33.9
7	from EL2E	*D2Mit41–D2Mit43*	21.9
8	from EL2E	*D2Mit435–D2Mit299*	17.5

See Fig. 1B for a graphical representation. Strains 1–8 are derived from the EL2E strain, as described in the text. Interval size was approximated from the MGD consensus map position midway between the two markers (see http://www.informatics.jax.org).

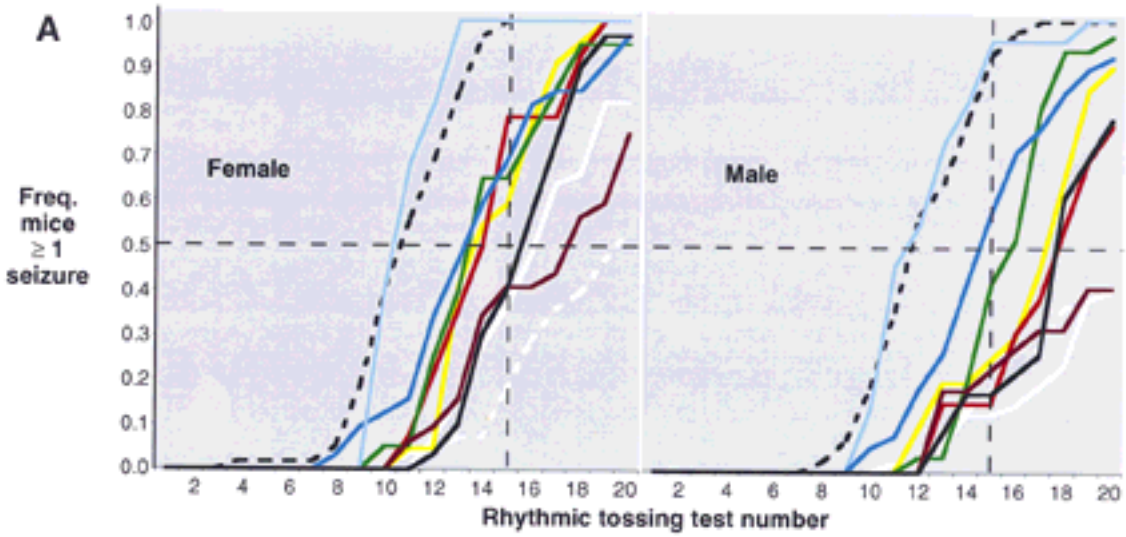

Figure 1 Cumulative onset for rhythmic tossing-induced seizures. (*A*) The frequency of the number of seizures in females or males per strain is plotted for each rhythmic tossing test and points connected by colored lines (key is shown in *B*). Thin broken horizontal lines within the plot highlight the 50% mark and the fifteenth seizure test, respectively, chosen for subsequent analysis as described in the text. (*B*) Graphic representation of the EL-like interval (in black; actual values are detailed in Table 1) retained in each of the congenic and recombinant strains, on its ABP background (white), with the Chromosome 2 centromere depicted as a ball and the colored lines used for each in *A*. The text table gives the effective number of tests for 50% (ET_{50}) of each strain to have experienced at least one seizure, as determined by Probit analysis described in the text.

Common Determinants for PTZ and Rhythmic Tossing Threshold Seizures

To determine whether this apparent genetic complexity was unique to the rhythmic tossing assay, susceptibility to PTZ induced seizures was determined in the same strain set (Fig. 2B). Interestingly for PTZ, the gender bias was reversed when compared to rhythmic tossing, with males more sensitive than females. Nevertheless, the relative patterns of seizure incidence were similar in recombinants which retain the telomeric half of the interval, the main difference being the much higher susceptibility (represented by recombinant strains 7 and 8 in Fig. 2B) of the centromeric half to PTZ threshold seizures. In particular, recombinant strains 4 and 6, which contain each respective half-interval, have an increased seizure frequency which is statistically different from ABP in both testing paradigms. Nevertheless, because the recombinant 7 is already sufficient for the PTZ threshold phenotype of the EL2E parent, it is unclear whether the same gene(s) in the centromeric half of the interval are responsible for both rhythmic tossing and PTZ responses.

Homozygous ABP Alleles Enhance Gender Effects

In both seizure testing paradigms, within-strain gender effects (Fig. 2) were enhanced by recessive inheritance of homozygous ABP alleles in the region defined between recombinants 4 and 8. Although the recombinant strain data do not distinguish whether one or more genes are responsible for this enhancement of gender effect, the large difference in susceptibility to PTZ seizures between (EL2E × ABP)F_1 and (4 × 7)F_1 hybrids (Fig. 2B); the latter, which showed similar effects to the homozygous recombinant 4 strain itself, suggests that at least one recessive gender effect locus acts as an interaction with heterozygous EL alleles in

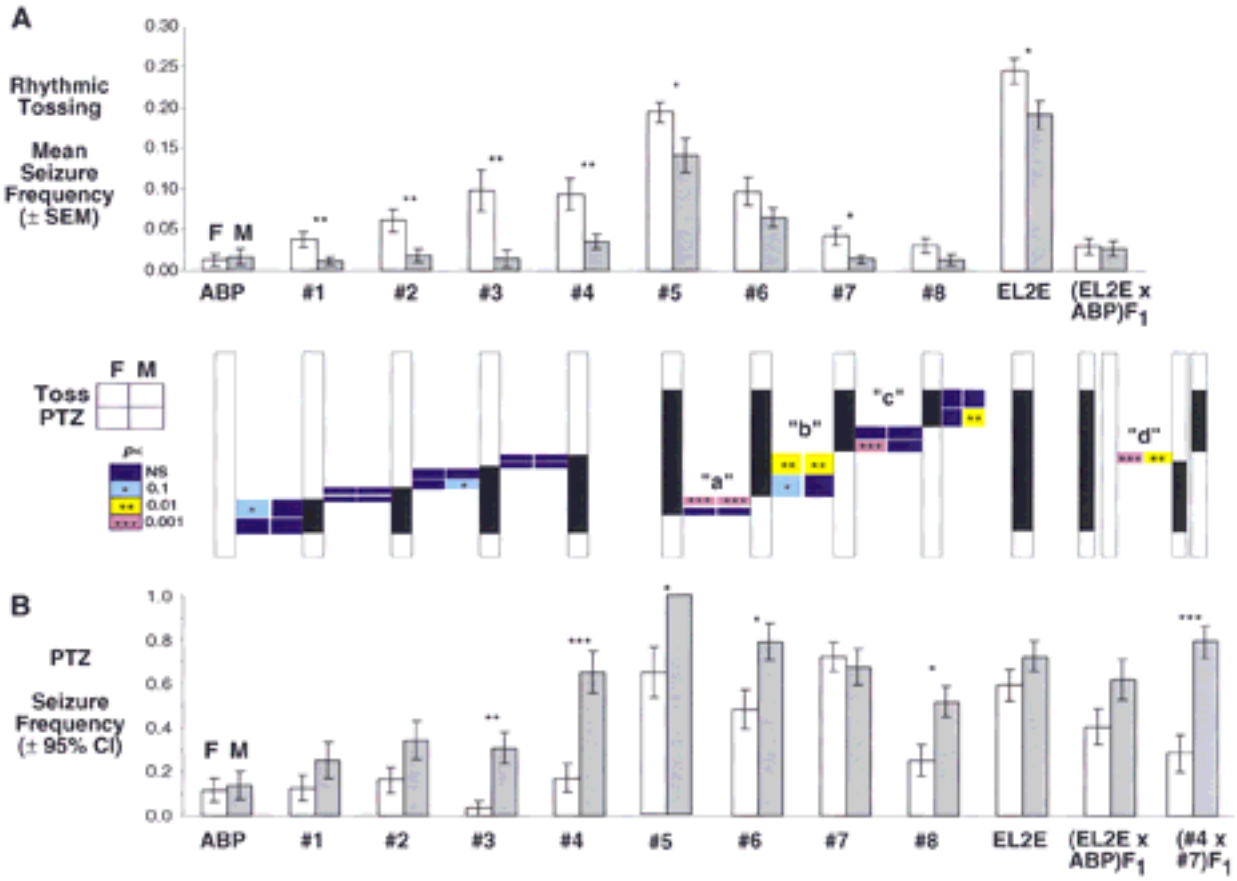

Figure 2 Comparison of seizure frequency utilizing rhythmic tossing. (*a*) vs. PTZ seizure testing (*b*), in recombinant strains. The Chromosome 2 area spanned by recombinant strains 1–8, EL2E, and ABP is depicted graphically (actual values given in Table 1), between *a* and *b* by a white bar for ABP-like and a black bar for EL-like. Females are shown on the *left* of each histogram pair (white bar) and males on the *right* (gray bar). Numbers of animals in each rhythmic tossing group were as follows (F,M): ABP (28,22); 1 (44,47); 2 (22,20); 3 (14,13); 4 (20,31); 5 (22,22); 6 (32,38); 7 (32,22); 8 (30,23); EL2E (59,42); (EL2E × ABP)F_1 (16,18). Numbers of animals in each PTZ-tested group were as follows: ABP (35,29); 1 (32,28); 2 (37,29); 3 (29,42); 4 (35,26); 5 (17,12); 6 (21,34); 7 (47,31); 8 (36,25); EL2E (47,40); (EL2E × ABP)F_1 (37,29); (4 × 7)F_1 (28,29). Pairwise differences in seizure frequency between strains with adjacent recombinations were determined as described in Methods and are represented by quadrants, comparing genders (female, *left* quadrants; male, *right*) and seizure tests (rhythmic tossing, *top* quadrants; PTZ, *bottom*); significance is depicted by a different color or number of asterisks (dark blue, no asterisk, not significant; light blue, *, $P < 0.1$; yellow, **, $P < 0.01$; pink, ***, $P < 0.0001$). Asterisks above histograms denote within-strain gender differences (same code). The quadrants to the *right* of strain 8 are from tests in comparison with the ABP/Le strain. Only the bottom quadrants are shown in the pairwise comparison between F1 hybrids because only PTZ was tested in both. Regions defined as *a*, *b*, *c*, and *d* are described in the text.

the telomeric half of the *El2* interval. Nevertheless, the strong difference in PTZ seizure frequency between females (but not males) of recombinant strain 7 and 8, in view of the lack of any within-strain gender difference for recombinant 7, suggests that additional gender responsive alleles exist.

DISCUSSION

Seizure susceptibility genes on Chromosome 9 (*El1*, *El4*), Chromosome 2 (*El2*), and Chromosome 10 (*El3*) were initially mapped as QTL in crosses between the EL and ABP mouse strains (Rise et al. 1991; Frankel et al. 1995a). Congenic strains were then produced containing the EL allele of each of these chromosomes transferred onto an ABP strain background by genotypic selection, and these strains were tested for sensitivity to seizures induced by rhythmic tossing (Frankel et al. 1995b). The relative seizure frequencies of these strains were *El2* > *El1*,*El4* > *El3*, parallel to respective effects in initial backcrosses. In particular, the EL2E congenic strain (Frankel et al. 1995b) showed that EL strain-derived alleles correlated well with seizure susceptibility in the context of a nonepileptic strain background. This is consistent with the notion that *El2* is a robust, major seizure locus suitable for higher resolution mapping.

To reduce the size of the *El2* critical interval, we constructed and tested nested recombinant strains derived from the EL2E congenic. Surprisingly, these stud-

ies revealed a more complex pattern of inheritance. The most obvious result was that none of the recombinant strains recapitulated the full rhythmic tossing seizure phenotype of EL2E, as determined first by a conservative analysis of phenotype (Fig. 1B) and then by seizure frequency itself (Fig. 2A). It was unclear whether this apparent gene interaction between linked determinants is epistatic or additive. For example, in females, incremental differences between recombinant strains suggested a continuum of loci with additive effects, that is, increasing seizure susceptibility with each interval inherited (Fig. 2A). However, these increments were not usually statistically significant and the effect was less apparent in males (Fig. 2A). Therefore, we conclude that at least one gene in the telomeric half (between *D2Mit58* and *D2Mit30*) and one in the centromeric half (between *D2Mit7* and *D2Mit41*) interact to give the full parental congenic strain phenotype. However, given the apparent trend for seizure frequencies to respond incrementally with added genetic material from the EL strain, it is possible that multiple genes contribute to each.

Parallel experiments were performed utilizing an independent seizure stimulus, PTZ. In the recombinants, PTZ mirrored the rhythmic tossing results in the telomeric half of the interval and had a similar (albeit opposite) effect on gender overall (Fig. 2). The fact that similar patterns were obtained for both seizure paradigms lessens the likelihood that at least some of the gene(s) underlying *El2* are merely artifacts of the rhythmic tossing procedure, as suggested by others (Poderycki et al. 1998). However, with respect to localization of essential seizure determinants, PTZ proved similarly confusing as rhythmic tossing. For example, PTZ genes in either half of the interval were sufficient to recapitulate the EL2E phenotype, at least in males (Fig. 2B, strain 4 vs. strain 6 or 7). In addition, mice containing only the centromeric half of the EL2E congenic interval appeared exquisitely sensitive to PTZ, that is, they did not respond to rhythmic tossing per se (Fig. 2, strains 7 and 8). Nevertheless, as we speculated earlier, by inference there must be a gene(s) in the centromeric half of the interval which contributes to the overall EL2E for rhythmic tossing phenotype by interacting with more telomeric loci. It is therefore possible that the underlying genes are the same, but that PTZ as stimulus is sufficient to push their effects above threshold, whereas rhythmic tossing is not. The alternative hypothesis is that unique PTZ-responsive determinants reside in the centromeric half.

An interesting area in the middle of the EL2E interval negates the gender bias in PTZ seizures, conferring equal sensitivity to both males and females (Fig. 2, EL-derived region of strain 7 vs. strain 8). Additionally, an adjacent area (between strains 4 and 7) seems to exacerbate the gender bias. One or both effects could be explained by the presence of a gene which modulates the effect of PTZ on the GABAa receptor. Gender differences in sensitivity to PTZ, but not in GABAa receptor binding, have been previously noted (Kokka et al. 1992). Female rats were shown to have a higher threshold than males to seizures induced by PTZ as a result of neurosteroid modulation by metabolites of progesterone. Further studies would be necessary to distinguish this possibility from the alternative explanation, namely gender limited expression of seizure susceptibility alleles. However, if there are gender bias-enhancement loci, they specifically interact with seizure susceptibility alleles located distally, and are not necessarily a property of the ABP strain background. For example, comparable recombinant strains made by introgressing Chromosome 7 alleles from the epileptic SWXL-4 strain onto an ABP background do not show the same pattern of gender bias (M.E. Legare and W.N. Frankel, unpubl.).

Despite these daunting complexities, several seizure determinants were narrowed to small areas of Chromosome 2. At least two strain pairs with adjacent recombinations show significant differences in seizure susceptibility to either PTZ or rhythmic tossing (Fig. 2). For example, an ~1 cM region (Fig. 2, region "a") between recombinants 5 and 6 differed for rhythmic tossing seizures, and an ~5 cM region between recombinants 6 and 7 had a more modest effect (Fig. 2, region "b"). In addition, a similar size interval between strains 7 and 8 females showed a significant difference in PTZ seizures (Fig. 2, region "c"). Finally, a small interval between strains 4 and 7 might contain an interesting gender-bias enhancement locus (Fig. 2, region "d"). However, although effects delimited to 1 cM are encouraging, in light of the overall nature of our results one cannot assume that the region is defined by a single genetic variant. Moreover, the effect of region "a," while statistically significant, is small and further systematic fine-mapping will require a great deal of progeny-testing of individual recombinants. Further electrophysiological and pharmacological characterization of strain pairs which differ in these regions may help determine whether a region is worth pursuing by a positional candidate approach, and several candidate genes within these intervals could be tested directly.

It is clear that in some polygenic models, such as the epileptic EL mouse, conventional QTL mapping approaches suffice to confirm trait loci with large effects. However, further dissection may reveal them to be multiple loci each with smaller effects. Although among the six seizure frequency QTLs mapped in EL we have evaluated *El2* most thoroughly, we are now completing studies on the Chromosome 7 locus *Szf1* from the epileptic SWXL-4 mouse and have observed similar results (M.E. Legare and W.N. Frankel, unpubl.). Many published QTL show multiple likelihood

curve peaks, suggestive of several underlying loci that are either more loosely linked to each other than in *El2*, that differ in additive or dominance components, or that exhibit different types of interaction with unlinked loci, any of which may be at least partly resolved by conventional QTL mapping programs, e.g., as for *El5*. (Frankel et al. 1995a). Nevertheless, in retrospect, our results are not particularly surprising. Based on the number of gene-targeted mouse mutations reported recently to have seizures, one can extrapolate that hundreds of genes would potentially give rise to seizure phenotypes when appropriately mutated. Consequently, between two common mouse strains, a functional polymorphism in a seizure gene might occur every 5–10 cM. Given the low resolution of conventional QTL mapping crosses and the tendency to select for further study regions with large effects, such approaches to detecting and confirming QTL might systematically capture several trait loci.

This phenomenon can be generalized for any trait where many segregating genes could potentially influence phenotype. There are two types of solution for such problems. First, although it is often desirable to study an intact phenotype, for example, tonic-clonic seizures, a study of component phenotypes should reduce the genetic complexity. This approach seems to work for certain systemic complex disorders, such as diabetes (Leiter et al. 1998), although it may be difficult or impractical for composite neurological behaviors. Second, alternatives to conventional QTL detection in mouse strains of choice have been suggested in recent years, including approaches such as advanced intercross (AI) lines (Darvasi and Soller 1995) and heterogeneous stocks (HS) (Talbot et al. 1999). In these cases, the retained nonrecombinant segments are much smaller than in conventional crosses. Thus, local linkages are more readily disrupted and mapped QTL are less likely the result of multiple linked genes. Nevertheless, some of these high-resolution approaches are not compatible with the type of control that is often necessary to understand the mechanism of a genetic effect (i.e., provide a strain pair where the only genetic variable is the locus in question), nor do they address the additional dilemma that many of these natural polygenic variants may be inherently difficult to study because effect sizes are usually quite small. The likelihood that common human genetic disorders are of a similar nature while perhaps justifying the approach, does not solve the problem. Moreover, polygenic models are many generations away from being suitable for genetic isolation and bear the extra burden of proof necessary for distinguishing between strain polymorphism and causative mutation. For seizure disorders, given the large number of target genes and the uncertainty of using multiple crosses to sort through the many possible natural variants to find those that are the most appropriate models for study, the systematic screening for de novo mutations induced by random chemical mutagenesis on a known strain background (King et al. 1997; Brown 1998; Schimenti and Bucan 1998) is an attractive alternative. Mutagenesis screens allow one to rapidly select from a set of single gene mutations of interest those that have the strongest effects and move directly from high-resolution mapping to gene identification.

METHODS

Mouse Strains

The inbred mouse strains EL/Suz and ABP/Le have been maintained in our colony by brother-sister mating. The ABP.EL–*El2*^{e}/Frk (abbreviated herein EL2E/Frk or EL2E) congenic strain was created by successive backcrossing using EL as donor and ABP as recipient by forced heterozygosity at the genetic markers *D2Mit11*, *D2Mit43*, *D2Mit30*, *D2Mit21*, *D2Mit71*, and *D2Mit55*, followed by intercross–backcross to yield mice homozygous for the EL allele at these markers. Congenic EL2E strains carrying recombinations within the *El2* interval were created from either (ABP × ABP.EL–*El2*^{e})F_2 or (ABP × ABP.EL–*El2*^{e})F_1 × ABP.EL–*El2*^{e} (N_2) progeny with genotypic selection. All mice were kept in plastic cages at controlled temperature and humidity with a 12-hr light cycle. They received standard diet and water ad libitum. Mice were weaned at 21–28 days of age, with males and females housed separately. The average litter size of ABP-derived strains ranged between six and seven.

PTZ Threshold Seizures

Convulsions evoked by a single-dose PTZ injection method have been shown to be a precise experimental model for some types of epilepsy, including the EL mouse (Sugaya et al. 1986). Advantages of PTZ over other chemo-convulsants include the rapidity and clarity of behavioral responses and its short biological half-life. Initial dose-response testing was performed on EL and ABP mice as the frequencies, latencies to, and qualitative aspects of seizure stages can be quite variable among common mouse strains and also at different doses of PTZ (M.E. Legare, K. Bartlett, and W.N. Frankel, unpubl.). At 60 mg/kg PTZ, 99% of all EL mice experienced a tonic–clonic seizure, whereas only 12% of ABP mice seized. PTZ (Sigma) solutions were prepared fresh on the day of each experiment in physiologic saline and administered at neutral pH in a volume of 0.01 ml/gm body weight. At 45 ± 5 days of age, each animal received a single, mid-dorsal, subcutaneous injection using a 0.5 ml syringe and 26-gauge needle. Mice were then placed in a clear plastic chamber and behavior was observed for 30 minutes. Parameters monitored included latency to first minimal seizure (twitch or clonus) and latency to first tonic-clonic seizure with loss of righting reflex. The initial episode of clonus was often very short or culminated directly in a tonic clonic seizure. In EL and ABP derived strains, loss of righting reflex always coincided with the generalized clonus, and was not dissociated from it, as is the case in other strains (i.e., C57BL/6J). PTZ seizure tests on parental and recombinant strains were done over a period of 3 years.

Rhythmic Tossing Seizures

A standard method for inducing tonic–clonic seizures by

gentle rhythmic tossing was used (Rise et al. 1991; Flavin et al. 1991; Frankel et al. 1994, 1995). At 30 days of age, mice were subjected to tossing with ~1.0-cm vertical displacement at a rate of 256 cycles per min in a quiet, mechanically driven plastic mouse box. Each test lasted no longer than 30 sec and was repeated once every 3 days for 60 days. For each test, mice were scored as either plus or minus for a full tonic-clonic seizure episode, including loss of righting and Straub's tail reflex. Rhythmic tossing tests for recombinant strains were done over a period of 3 years; those for parental strains were done over a period of 6 years.

Statistical Analysis

Data were stored and analyzed using Excel spreadsheets (Microsoft, Inc., Redmond, WA). ET_{50} values on rhythmic tossing data were determined by Probit analysis (Wardlaw 1985). To assess differences among groups in mean seizure frequency following rhythmic tossing or seizure frequency following PTZ injection, Student's *t*-tests or χ^2 contingency analyses were used, respectively.

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