

## Use of chromosome substitution strains to identify seizure susceptibility loci in mice

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### Abstract

Seizure susceptibility varies among inbred mouse strains. Chromosome substitution strains (CSS), in which a single chromosome from one inbred strain (donor) has been transferred onto a second strain (host) by repeated backcrossing, may be used to identify quantitative trait loci (QTLs) that contribute to seizure susceptibility. QTLs for susceptibility to pilocarpine-induced seizures, a model of temporal lobe epilepsy, have not been reported, and CSS have not previously been used to localize seizure susceptibility genes. We report QTLs identified using a B6 (host) × A/J (donor) CSS panel to localize genes involved in susceptibility to pilocarpine-induced seizures. Three hundred fifty-five adult male CSS mice, 58 B6, and 39 A/J were tested for susceptibility to pilocarpine-induced seizures. Highest stage reached and latency to each stage were recorded for all mice. B6 mice were resistant to seizures and slower to reach stages compared to A/J mice. The CSS for Chromosomes 10 and 18 progressed to the most severe stages, diverging dramatically from the B6 phenotype. Latencies to stages were also significantly shorter for CSS10 and CSS18 mice. CSS mapping suggests seizure susceptibility loci on mouse Chromosomes 10 and 18. This approach provides a framework for identifying potentially novel homologous candidate genes for human temporal lobe epilepsy.

### Introduction

The importance of genetic factors in the etiology of human epilepsy is well accepted, but the specific genes responsible have been identified for only a few rare Mendelian epilepsy syndromes (Baulac et al. 2001; Charlier et al. 1998; Cossette et al. 2002; De Fusco et al. 2000; Escayg et al. 2000; Harkin et al. 2002; Haug et al. 2003; Heron et al. 2002; Kalachikov et al. 2002; Kananura et al. 2002; Morante-Redolat et al. 2002; Singh et al. 1998; Steinlein et al. 1995; Sugawara et al. 2001a, b; Suzuki et al. 2004; Wallace et al. 1998, 2001a, b, 2003). Most common forms of epilepsy are not explained by the genes discovered so far, and their genetics appears to be “complex,” resulting from multiple genes of small to moderate effect. Temporal lobe epilepsy (TLE), a common type of epilepsy that is frequently refractory to medical therapy, presents similar challenges because of its genetic complexity (Engel 2001; Fuerst et al. 2001; Semah et al. 1998; Stephen et al. 2001).

Mouse models are ideally suited for the investigation of complex diseases like TLE. Prolific breeding and short gestation period are critical for identification of susceptibility loci because linkage methods rely on naturally occurring recombination (Palmer and Phillips 2002; Phillips et al. 2002). Large numbers of offspring with controlled breeding histories allow parsing of the genome into segments that can be genotyped using markers and analyzed for association with a susceptibility phenotype. In addition, the availability of genetic maps with thousands of molecular markers and increasingly available sequence data for inbred strains facilitate genetic research in mice. Finally, chromosome segment conservation in mouse and human genomes

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allows genetic discoveries in the mouse to be applied to human populations.

Chromosome substitution strain (CSS) analysis is a recently developed method of QTL identification that has been applied successfully to the study of anxiety (Nadeau et al. 2000; Singer et al. 2004), germ-cell tumors (Matin et al. 1999; Youngren et al. 2003), hypertension (Cowley et al. 2001; Liang et al. 2002), response to barbiturates (Stekiel et al. 2006), obesity, and sterol levels (Singer et al. 2004). Chromosome substitution strains are strains in which a single chromosome from one inbred strain (donor) has been transferred onto a second strain (host) by repeated backcrossing. A panel of CSS in which each chromosome is represented may be used to identify QTLs that contribute to a phenotype. Compared with traditional QTL mapping in an  $F_2$  or  $N_2$  population, CSS analysis has the advantage of quick and efficient localization at the chromosomal level without the need for cross-breeding or genotyping (Nadeau et al. 2000).

Inbred mouse strains differ in susceptibility to developing seizures in response to toxic, electrical, and auditory insults to the nervous system, and these differences are heritable (Fehr et al. 2002; Ferraro et al. 1995, 1997, 1998; Gershenfeld et al. 1999; Hain et al. 2000; McKhann et al. 2003; Neumann and Collins 1991, 1992). Heritability has been demonstrated by breeding studies in which offspring of parental crosses develop intermediate phenotypes, and loci underlying strain differences in susceptibility have been identified using QTL mapping in these models (Table 1). C57BL/6J (B6) mice have been shown to be resistant to seizures; the A/J strain has been less studied but appears more susceptible to seizures. There is also evidence for strain-specific susceptibility to seizure-induced hippocampal damage (Chen et al. 2005; McKhann et al. 2003; Schauwecker 2002; Schauwecker and Steward 1997; Schauwecker et al. 2004). QTL mapping has also identified modifier loci that determine the severity of the epilepsy phenotype related to mutations in voltage-gated sodium channels (Bergren et al. 2005).

Pilocarpine, a muscarinic cholinergic agonist, induces an initial period of severe limbic seizures (status epilepticus) in rodents, followed by cell loss similar to that observed in TLE as well as later chronic spontaneous limbic (temporal lobe) seizures (Borges et al. 2003; Leite et al. 2002; Turski 2000; Turski et al. 1989). Limbic seizures have not been studied in A/J, and susceptibility to pilocarpine-induced seizures has not been studied with QTL mapping in any strains. The model's similarities to human TLE make it an excellent target for discovery

of susceptibility genes that can subsequently be tested in human populations.

It is important to note that examining susceptibility to limbic seizures (acute seizures caused by pilocarpine) is different from examining susceptibility to chronic spontaneous seizures that occur after recovery from pilocarpine. However, seizure susceptibility is an important phenotype and is likely to be related directly to epilepsy susceptibility. All investigators who have reported murine QTL studies have examined seizure susceptibility rather than epilepsy susceptibility in mice. In part this is because spontaneous seizures are extremely difficult to measure. The measurement requires long observation times (many continuous hours over latency periods of weeks to months) for each mouse, and collection of a statistically powerful sample population is challenging.

Despite the difference between seizure susceptibility and epilepsy susceptibility, identification of genes influencing seizure susceptibility is crucial to understanding human epilepsy (Noebels 2003). Rodent models provide evidence that seizure and epilepsy outcomes are linked; spontaneous seizures have been shown to develop in mice and rats after toxin-induced limbic seizures. In addition, there is evidence to suggest that seizures damage the brain and lead to further seizures, in both human and animal models (Pitkanen and Sutula 2002; Shorvon 2002; Sutula and Pitkanen 2001). In the human, TLE may result from genetic susceptibility to develop seizures or epilepsy in response to a provocative stimulus or early seizures. Further support for the applicability of mouse seizure susceptibility genes to human epilepsy comes in the form of successful translation of the mouse candidate gene (*KCNJ10*) identified by QTL mapping to genetic association in two independent human epilepsy populations (Buono et al. 2004; Lenzen et al. 2005). Future studies in a smaller subset of animals kept for long-term observation may address this problem more directly, but examination of seizure susceptibility remains an important avenue for discovery.

To our knowledge, CSS have not been previously used to identify seizure susceptibility loci. In this article we report QTLs identified using a B6 (host)  $\times$  A/J (donor) CSS panel to localize genes involved in susceptibility to pilocarpine-induced limbic seizures.

### **Materials and methods**

All methods were approved by the Institutional Animal Care and Use Committee of Columbia University and met the guidelines of the National Institutes of Health.

**Table 1. Initial mapping studies of murine susceptibility (not including fine mapping)**

<i>1st Author/year</i>	<i>Strains</i>	<i>Stimulus</i>	<i>Seizure type</i>	<i>Variable used</i>	<i>Mouse QTLs identified</i>
Ferraro 1999	C57BL/6j × DBA/2J	PTZ	Focal and generalized clonic	Latency to seizure stages	Chr 1, 17, 5 significant
Ferraro 1997	C57BL/6j × DBA/2J	Kainate	Limbic (TLE)	Latency	Chr 3, 4, 6 suggestive Chr 1 significant Chr 11, 15, 18, 4, 5, 7 suggestive
Ferraro 2001	C57BL/6j × DBA/2J	Maximum electroshock threshold (MEST)	Generalized	Threshold current for maximal seizure	Chr 1, 2, 5, 15 significant
Hain 2000	C57BL/6j × DBA/2J	Cocaine	Tonic/clonic	Doses to induce tonic and clonic seizures	Chr 9, 14, 15 significant
Gershenfeld 1999	C57BL/6j × A/J	8-CCM	Generalized	Latency and dichotomous occurrence of seizure	Chr 9, 15 suggestive Chr 10 (confirmed in backcross)
Clement 1996	C3XtEso and JE/1e;	8-CCM	Clonic-myoclonic-myotonic	Latency to occurrence of myoclonic seizure	Chr 4, 7 suggestive Ch 4, 13 significant
Martin 1995	ABP/Le, C57BL/6J, and C57BL/6jBy]				Chr 9 suggestive
Neumann 1991, 1992	A/J, C57BL/6j DBA/2J	Audiogenic	Wild running/clonic-tonic	Occurrence of convulsions	Chr 4, 7, 12, significant Chr 7 confirmed
Buck 1999	B6D2 F <sub>2</sub> cross BXD RI strains	Pentobarbital withdrawal	Handling-induced tonic/clonic convulsions	Severity of convulsions	Chr 1 significant Chr 4, 11 suggestive
Buck 1997, Fehr 2002	B6D2 selected lines D2.B6 Interval-Specific Congenics	Alcohol & Pentobarbital Withdrawal	Tonic/tonic-clonic convulsions	Severity of handling-induced convulsions	Chr 1, 4, 11 significant Chr 2 suggestive
Bergren 2005	C57BL/6j X SJL	Modifier of seizure susceptibility with sodium channel mutations	Focal	Susceptibility to spontaneous seizures	Chr 11, 19 (confirmed in backcross)

Neumann and Collins 1991, 1992; Gershenfeld et al. 1999; Ferraro et al. 1997, 1999, 2001; Hain et al. 2000; Fehr et al. 2002; Buck and Finn 2001; Buck et al. 1997, 1999; Martin et al. 1995; Clement et al. 1996; Bergren et al. 2005

**Table 2. Limbic seizure stages in pilocarpine-treated mice**

Stage 1	Immobililty/lying low
Stage 2	Twitching/tremor/shaking of tail/head/body/ or limbs, <b>not continuous</b> , forelimb and/or tail extension, rigid posture, repetitive movements, head bobbing
Stage 3	Continuous tremor/clonic seizures of body and tail while retaining posture
Stage 4	Rearing/hyperexcitability/running/falling, tonic extension/clonic seizures <sup>a</sup> with loss of posture
Stage 5	Status epilepticus (continuous stage 4 seizures)

<sup>a</sup>Tonic seizures are characterized by whole-body stiffening and extension and clonic seizures by repetitive rhythmic jerking. These two phenomena are typically combined in whole body convulsive seizures.

**Animals.** A/J, B6, and CSS breeder pairs for the X chromosome and the 19 autosomal strains were purchased from The Jackson Laboratory, and colonies of each strain were bred in our laboratory. Mice were housed in a temperature- and humidity-controlled environment, with a 12-h light/dark schedule and food and water available *ad libitum*. Three hundred fifty-five adult male mice—at least ten of each CSS, 58 B6, and 39 A/J—were tested for susceptibility to pilocarpine-induced seizures between 10 and 12 weeks of age. Animals from the two parental inbred strains, B6 and A/J, were tested throughout the time period of CSS animal testing to minimize the effects of environmental variation on results. Only two CSS13 were tested because of difficulty breeding and limited availability from The Jackson Laboratory. The Y chromosome consomic strain was omitted.

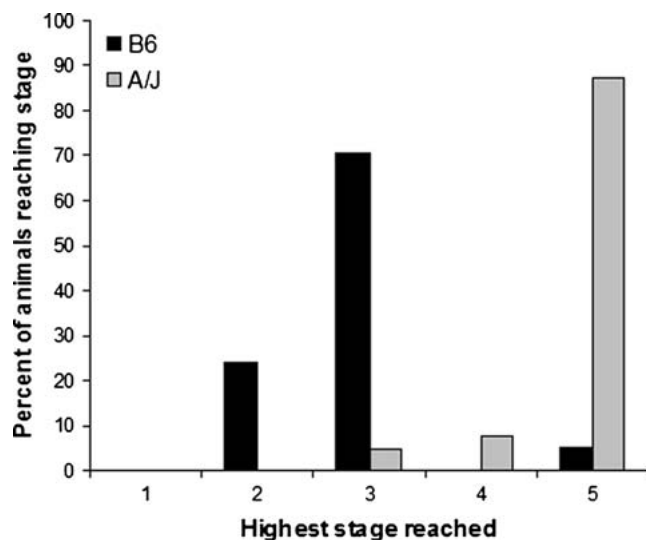
**Convulsant administration and measurement of seizure behaviors.** Adult male mice (10-12 weeks old) were transferred to individual test cages, brought to a quiet procedure room, and administered atropine methylbromide (1 mg/kg IP) or atropine methylnitrate (1 mg/kg IP) 30 min before pilocarpine hydrochloride (250 mg/kg IP). Atropine is given routinely to limit the peripheral side effects of pilocarpine. During the testing of the CSS panel, atropine methylbromide became unavailable from the supplier and was replaced by the methylnitrate formation. The type of atropine used did not affect results (described in the *Results* section). All animals were observed continuously for 3 h after pilocarpine administration, and seizure behaviors were recorded with their time of onset in minutes from injection. Using a seizure staging system adapted from established (Racine 1972) rodent seizure scales, highest stage reached and latency to each stage were recorded for all mice (see Table 2 for seizure stage definitions). Animals that had status epilepticus (continuous seizures without recovery) were administered diazepam (5 mg/kg IP, Henry Schein, USA) 1 h after the onset of status epilepticus to truncate seizures. Time of day, tester, type of atropine, and age at testing were recorded for covariate analysis.

We selected the 250-mg/kg dose after dose-response testing in 132 animals from three mouse strains—A/J, C57Bl/6J, and DBA/2J—in a prior study (unpublished data). In that study we measured incidence of and latency to status and neuroanatomic and neurochemical changes in the brain after status across strains. We particularly wished to examine the response of A/J animals to pilocarpine as a potentially valuable strain for mapping of pilocarpine-induced seizure susceptibility QTLs. We chose the 250-mg/kg dose because it maximized the occurrence of seizures/status across strains while limiting early mortality.

**Statistical analysis.** Significance of results in the CSS panel screen was determined by nonparametric comparison of highest stage reached (Mann-Whitney). The Mann-Whitney test was used because of the nonparametric nature of the seizure stage variable. Cox proportional hazards regression was used to generate hazard ratios and *p* values for latency variables, because not all animals reached all stages. The Cox proportional hazards model is a type of multivariate analysis used to identify a factor or combination of factors that best predicts outcome. This analysis is used when the outcome is the time to an event. The hazard ratio is defined as the relative risk of an endpoint at any given time. We tested more B6 (background) mice to improve power to detect a QTL; the most efficient ratio of background to CSS mice has been shown to be 4.5:1 (Belknap 2003).

## Results

A total of 58 B6 and 39 A/J animals were tested for pilocarpine-induced seizure susceptibility. Animals of both strains demonstrated characteristic behavioral manifestations of motor limbic seizures. The parental inbred strains differed dramatically in both seizure severity and latency to and between seizure stages (Fig. 1, Table 3). For B6 animals, the mean highest stage reached (on a scale of 1-5) was 2.95 (SD = 0.97) and median highest stage was 3.0. For A/J animals, the mean highest stage reached was 4.82 (SD = 0.51) with a median of 5.0. The difference in

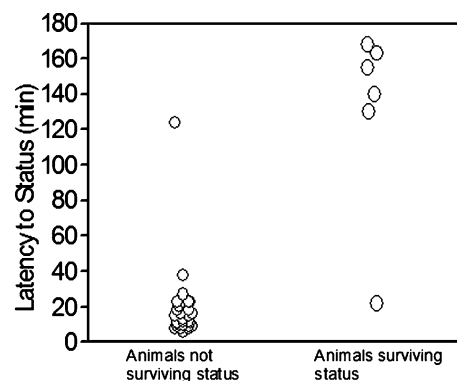


**Fig. 1.** Distribution of highest stage reached for A/J and B6 parental strains.

highest stage reached by Mann-Whitney was highly significant ( $Z = -8.27$ ,  $p < 0.01$ ). Latencies to stages, particularly higher stages (3, 4, and 5), were significantly longer for B6 than for A/J animals. Hazard ratios and  $p$  values for latencies are summarized in Table 3. Stage 1, initial immobility, is not included in this table. It occurs in all animals injected with pilocarpine within 1-2 min of injection and there is no evidence from either prior investigators or our own EEG studies (unpublished data) that this stage corresponds to electrographic seizures.

Latencies varied even within inbred strains. In A/J, a bimodal distribution of latencies was observed, with most animals reaching late stages quickly and then dying with severe seizures; a few animals survived with much longer latencies to status (Fig. 2). We have observed this phenomenon previously (unpublished data). Latency to status cannot be examined satisfactorily in B6 animals because they rarely attain status, but latency to stage 3 seizures varied from 3 to 49 min in B6 animals attaining stage 3.

Figure 3 shows the number of animals tested in each CSS strain. At least ten animals were tested from each strain except CSS13 (because of limited availability and poor breeding of the CSS13 strain).



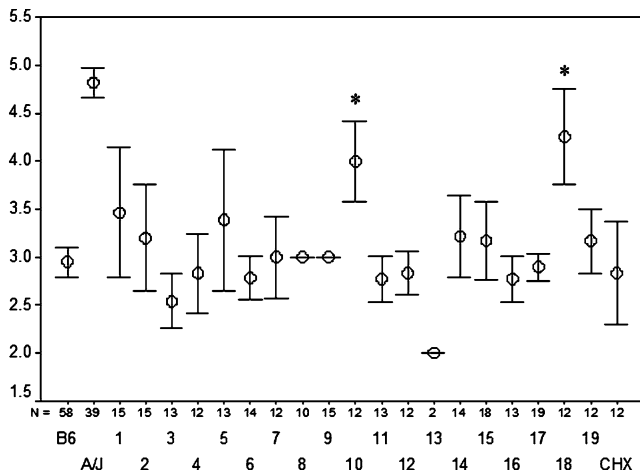
**Fig. 2.** Occurrence of status in this figure includes animals with continuous severe seizures leading to early mortality before 3 min of continuous seizure, as well as those with sustained status epilepticus requiring truncation by diazepam at 1 h. A dose-response effect, with shorter latencies at higher doses, is evident.

CSSY strains were not tested. Results of screening in the remaining CSS panel (18 autosomes plus CSSX) revealed significant contributions to seizure susceptibility on Chromosomes 10 and 18 (Fig. 3). The CSS for Chromosomes 10 and 18 progressed to the most severe seizure stages, diverging dramatically from the B6 seizure-resistant phenotype. The mean highest seizure stage reached for CSS10 was 4.0 (SD = 0.739), with a median highest stage of 4.0; for CSS18 the mean highest stage was 4.25 (SD = 0.866), with a median of 4.5. The CSS10 and 18 highest stage were both significantly different from the B6 highest stage reached using Mann-Whitney for comparison ( $Z$  score CSS10 vs. B6 =  $-4.7$ ;  $Z$  score for CSS18 vs. B6 =  $-4.86$ ). These results persisted after Bonferroni correction for multiple testing (multiplying by 20, the number of CSS strains tested), with corrected  $p$  values of less than 0.02. Latencies to seizure stages, particularly stages 3-5, were also significantly shorter for CSS10 and CSS18 mice (Table 3).

To determine what effects nongenetic factors (e.g., age) might have on this phenotype, we examined and controlled for the effects of several covariates: age (in days) at testing, time of day, tester (RK, MN, SS, MW), and type of atropine (methylbromide vs. methylnitrate) used. We found that age had a notable effect on latency when incorporated into the

**Table 3.** Unadjusted hazard ratios and  $p$  values for latencies to and between seizure stages in A/J, CSS10, and CSS18 animals (using B6 as the comparison group; hazard = 1)

Strain	Time to Stage 2		Time to Stage 3		Time to Stage 4		Time to Stage 5	
	Hazard ratio	$p$ value	Hazard ratio	$p$ value	Hazard ratio	$p$ value	Hazard ratio	$p$ value
A/J	1.75	0.02	24.6	<0.0001	48.1	<0.0001	31.5	<0.0001
CSS10	1.83	0.07	14.24	0.0001	31.5	<0.0001	5.3	0.04
CSS 18	1.43	0.27	30.43	<0.0001	24.1	<0.0001	10.2	0.0001



**Fig. 3.** Mean highest stage reached and number of animals tested per strain. Error bars represent the standard error of the mean (SEM). Asterisks are used to designate CSS for which the highest stage reached was significantly different from B6 by Mann-Whitney. SEMs for CSS lines 8 and 9 were zero because all animals had the same value (3) for last stage reached within those two substrains.

proportional hazards model (older animals took longer to reach seizure stages). Less prominent effects were also seen for type of atropine and tester. However, the main effect of strain persisted after inclusion of all these variables in the Cox proportional hazards model. Type of atropine also had no effect on likelihood of early progression to seizures and death in A/J animals.

An estimate of the heritability of the seizure susceptibility phenotype can be derived from the CSS data itself, using the pooled within-strain variance for the CSS to estimate the environmental variance. This is best estimated as the within mean square from a one-way analysis of variance (ANOVA) (0.544 in this case). Methods of estimating heritability generally assume that the between-strain variance is almost entirely the result of increasing alleles all coming from one progenitor strain at all important QTLs, which may not be the case. However, this assumption is more likely to be met in each CSS vs. B6 comparison because there may be only one QTL on each chromosome. Heritability estimates are also generally based on parametric statistics and equal interval scale variables; however, the stage variable is nonparametric and nonequal interval. Despite these caveats, calculation of heritability may be useful for determining the degree of effect of environmental variables on the phenotype observed. The between-progenitor-strain variance can be used to estimate the genetic variance, as  $\frac{1}{4} * (4.82 - 2.86)^2 = 0.96$ . (This equation is derived from the simple definition of sample variance,

$s^2 = \Sigma(X - M)^2/N$ , where the between-strain variance is based on an  $N$  of only two strains,  $X$  is the strain mean,  $\Sigma$  is the summation sign, and  $M$  is the mean of the two strain means.) Therefore, the estimated heritability is  $0.96/(0.96 + 0.544) = 0.64$ . Another method for calculating heritability uses the expression  $h^2 = t^2/(t^2 + df)$ , where  $t$  is Student's  $t$  for the two-group (strain) comparison and  $df$  is the degrees of freedom, or  $N = 2$  (Belknap 2003). Here,  $N$  refers to individual mice in both groups pooled together, not strains. This method produces nearly the same result.

## Discussion

To our knowledge this is the first use of CSS for mapping seizure susceptibility genes and the first mapping of QTLs for pilocarpine-induced seizures. Using the CSS A/J  $\times$  B6 panel, we have found evidence on mouse Chromosomes 10 and 18 of QTLs contributing to seizure susceptibility. Two prior seizure-related overlapping QTLs have been reported on Chromosome 18 for susceptibility to kainic acid-induced hippocampal cell death and limbic seizures (Ferraro et al. 1997; Schauwecker et al. 2004) in C57Bl/6  $\times$  FVB/N and C57Bl/6  $\times$  DBA/2J crosses (Schauwecker et al. 2004). Identification of a QTL for multiple different seizure and/or cell death susceptibility phenotypes (and for kainic acid pilocarpine) could suggest shared genetic contributions to these different limbic outcomes and give insight into pathophysiology. One QTL on Chromosome 10 confirmed by backcrossing has been reported in A/J  $\times$  B6, but for a generalized (rather than limbic) seizure model using  $\beta$ -CCM to induce seizures, and no fine mapping or gene identification has been published subsequent to that initial report (Gershfeld et al. 1999). Most QTL mapping studies of seizure susceptibility have used B6 and DBA/2J, classically described as being at opposite ends of the seizure susceptibility spectrum (Table 1). Use of less studied strains may reveal novel loci. The kainic acid model of limbic seizures has been used to map QTLs in B6  $\times$  DBA but not the A/J strain (Ferraro et al. 1997).

In this study both the severity variable (determined by highest stage reached) and latency variable identified the same two CSS and distinguished the parental inbred strains, i.e., greatest severity traveled together with shorter latencies. However, this may not always be the case. In particular, latency, though a commonly used variable in mapping studies (Table 1), must be used cautiously because of its variability within some strains. In A/J animals that we describe here, the bimodal distribution of latency

(Fig. 2) and the occurrence of early mortality in animals progressing rapidly may point to pharmacologic variables that do not directly reflect seizure susceptibility. This might include injection site variability, absorption rates, and variations in metabolism, or penetration of the blood-brain barrier. Prior pilot studies of A/J using the same protocol but in a different laboratory (unpublished data) have also identified this variability in latency with early mortality in some animals after progression to severe seizures. Future studies should incorporate these concerns in variable selection for fine mapping susceptibility loci.

It is certainly possible that QTL mapping of susceptibility to pilocarpine may reflect pharmacologic variables relating to drug metabolism in addition to seizure susceptibility. However, brain metabolites of kainic acid have been shown not to differ between susceptible and resistant strains in one QTL study, proving that strain differences are not related to strain-specific variation in metabolism or blood-brain barrier transport of kainate (Ferraro et al. 1995). Furthermore, identification of a QTL on Chromosome 10 using an entirely different drug (8-CCM) with a different mechanism of action also suggests a QTL that directly affects seizure susceptibility, independent of specific pharmacologic variables (Gershenfeld et al. 1999). It is still possible that some QTL within the chromosomes identified might be pharmacokinetic, while others are likely to be directly related to seizure susceptibility. This could be addressed in future studies by screening potential candidates for brain-specific expression *in vitro* or in expression databases.

Nongenetic variables can affect the results of mapping studies. In our analysis of covariates in the Cox model, we found that older age at testing, even within a two-week period, can affect latency to seizure stages, although our results did not change after adjustment for age. Limiting age to a narrower range in future studies therefore is warranted to avoid potential confounding and spurious results.

QTL mapping of seizure susceptibility genes can provide a framework for identifying potentially novel homologous candidate genes for human epilepsy. Recent success in this realm has been demonstrated in the identification of a maximum electroshock seizure susceptibility locus on mouse Chromosome 1, subsequent fine mapping, selection of a potassium channel candidate gene, and testing using an association study model in a human epilepsy population (Buono et al. 2004; Ferraro et al. 2001, 2004). Replication has been successful in an independent population (Lenzen et al. 2005). This success provides support for the use of QTL mapping to maximize the

prior probability of identifying relevant candidate genes to minimize false-positive results. In the future, fine-mapping studies can be performed easily using backcrosses of CSS animals of interest with the host strain in the hopes of identifying loci or genes that can translate to understanding human seizure susceptibility.

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