Genetic Suppression of Seizure Susceptibility in Drosophila

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Kuebler, Daniel, HaiGuang Zhang, XiaoYun Ren, and Mark A. Tanouye. Genetic suppression of seizure susceptibility in Drosophila. J Neurophysiol 86: 1211–1225, 2001. Despite the frequency of seizure disorders in the human population, the genetic and physiological basis for these defects has been difficult to resolve. Although many genetic defects that cause seizure susceptibility have been identified, the defects involve disparate biological processes, many of which are not neural specific. The large number and heterogeneous nature of the genes involved makes it difficult to understand the complex factors underlying the etiology of seizure disorders. Examining the effect known genetic mutations have on seizure susceptibility is one approach that may prove fruitful. This approach may be helpful both in understanding how different physiological processes affect seizure susceptibility and in identifying novel therapeutic treatments. In this study, we have taken advantage of Drosophila, a genetically tractable system, to identify factors that suppress seizure susceptibility. Of particular interest has been a group of Drosophila mutants, the bang-sensitive (BS) mutants, which are much more susceptible to seizures than wild type. The BS phenotypic class includes at least eight genes, including three examined in this study, bss, eas, and sda. Through the generation of double-mutant combinations with other well-characterized Drosophila mutants, the BS mutants are particularly useful for identifying genetic factors that suppress susceptibility to seizures. We have found that mutants affecting Na+ channels, mle\sup{\text{nos}} and para, K+ channels, Sh, and electrical synapses, shak-B\sup{\text{2}}, can suppress seizures in the BS mutants. This is the first demonstration that these types of mutations can suppress the development of seizures in any organism. Reduced neuronal excitability may contribute to seizure suppression. The best suppressor, mle\sup{\text{nos}}, causes an increased stimulation threshold for the giant fiber (GF) consistent with a reduction in single neuron excitability that could underlie suppression of seizures. For some other double mutants with para and Shk\sup{133} there are no GF threshold changes, but reduced excitability may also be indicated by a reduction in GF following frequency. These results demonstrate the utility of Drosophila as a model system for studying seizure susceptibility and identify physiological processes that modify seizure susceptibility.

INTRODUCTION

Seizure susceptibility is greatly influenced by genetic factors. At present, more than a dozen genes have been linked to various epilepsy syndromes in humans, while in mice more than 25 genetically mutated strains have epileptic phenotypes (McNamara 1999; Puranam and McNamara 1999). Genetic factors can also suppress seizures and epileptogenesis. Mice with mutations in the brain-derived neurotrophic factor gene (BDNF) or the immediate early gene c-fos display delayed onset of seizures following kindling (Kokaia et al. 1995; Watanabe et al. 1996). In addition, the variable penetrance of human epilepsy genes (Biervert et al. 1998; Durmer et al. 1991) indicates that other genetic factors can, in certain cases, suppress the development of spontaneous seizures.

The large number of disparate genes involved in seizures coupled with the heterogeneity of seizure disorders demonstrates the complexity of the problem and makes any study of the genetic factors that affect susceptibility to seizures difficult. The preponderance of a wide variety of seizure disorders (Commission on Classification and Terminology of the International League Against Epilepsy 1989; Hauser and Hesdorffer 1990) indicates that finding a simple molecular pathway responsible for the regulation of seizure susceptibility is unlikely. In fact, the high information-processing capability of the CNS may require it to be organized in such a fashion that it is extremely susceptible to chaotic discharges following small and unpredictable perturbations (Bak and Chen 1991; Bak and Paczuski 1995). If this is the case, then a large number of cellular and physiological processes should be able to affect seizure susceptibility. This is consistent with mouse knockout data, which has implicated genes as diverse as l-isoaspartyl methyltransferase, neuropeptide-Y, alkaline phosphatase, and a K,1.1 potassium channel in seizure disorders (Erickson et al. 1996; Smart et al. 1998; Waymire et al. 1995; Yamamoto et al. 1998). Despite the formidable complexity of the problem, an examination of the effect many well-characterized mutations have on seizure susceptibility has several potential benefits. Such a study may facilitate the identification of both novel physiological mechanisms by which seizure susceptibility is regulated and novel targets for the development of anti-convulsant drugs.

One system that avails itself to such a study is Drosophila because of the large collection of excitability and behavioral mutants available. In addition, recent studies have demonstrated the utility of using Drosophila as a model system for studying seizure disorders (Kuebler and Tanouye 2000). These studies have demonstrated that seizures in Drosophila share many characteristics with mammalian seizures and that genetic factors can alter seizure susceptibility levels.

We have begun an initial investigation of the genetic factors that suppress seizure susceptibility in Drosophila. This study has taken advantage of one group of mutants, the bang-sensi-
tive (BS) mutants (Benzer 1971; Ganetzky and Wu 1982; Pavlidis and Tanouye 1995) that are particularly sensitive to seizure: they are 5–10 times more susceptible to seizure following electrical shock than wild-type flies (Kuebler and Tanouye 2000). A comparison of this mutant class to the mammalian epilepsies has been made (Benzer 1971). The BS mutants used in this study were eas, which encodes an ethanolamine kinase involved in one pathway of phosphatidyl ethanolamine synthesis (Pavlidis et al. 1994), and bss and sda, which map to the first and third chromosomes, respectively, but whose products have not yet been described. The BS flies provide a particularly useful experimental tool for testing the ability of other genetic mutants to rescue a seizure-susceptible phenotype. Our approach consists of quantifying seizure susceptibility levels in a variety of mutant strains. Mutants that have seizure thresholds significantly different from wild type can then be tested in double-mutant combination with BS strains for the ability to suppress the BS seizure-susceptible phenotype. This approach allows us to not only identify mutations that suppress susceptibility but also to quantify the level of that suppression.

In this study, we have focused on mutants that affect processes thought to be involved in seizure generation. Na⁺ and K⁺ channel mutants were chosen because they should affect nervous system excitability (Loughney et al. 1989; Tanouye et al. 1981). Mutants that affect neural connections were also chosen as these may disrupt the putative positive feedback loops thought to be involved in seizure generation (McNamara 1994; Traynelis and Dingleline 1988). The data presented here quantify the ability of these mutants to suppress seizures and provide a baseline for a more exhaustive future study involving both forward and reverse genetic approaches to identifying suppressor mutations. We believe understanding how these various genetic factors suppress seizure susceptibility may be a gateway to dissecting the tremendously complex and heterogeneous problem of seizure disorders.

METHODS

Fly stocks

WILD-TYPE STRAINS AND BS MUTANTS. Wild-type Drosophila strains were Canton Special (CS), Oregon-R (OR), and Berlin. Three BS mutants were used eas, bss, and sda. The easily shocked gene (eas) is located at map position 1–53.5 and encodes an ethanolamine kinase (Pavlidis et al. 1994). The bang senseless gene (bss) is located at 1–54.6 and slumdance (sda) on the third chromosome at 97D8-9 (Ganetzky and Wu 1982; Zhang, unpublished observations); their gene products have not been described. The mutant behavioral phenotypes of seizure and paralysis, electrophysiological phenotypes of seizure and synaptic failure, and the threshold for seizure susceptibility have been described previously for the bss, eas, and sda mutant alleles (Ganetzky and Wu 1982; Kuebler and Tanouye 2000; Pavlidis and Tanouye 1995; Pavlidis et al. 1994).

TEMPERATURE-SENSITIVE PARALYTIC MUTANTS. Two temperature-sensitive paralytic mutations were used, mle<sup>apo</sup> and para. The maleless (mle) 2–56.2 gene encodes an RNA helicase-like protein (Kernan et al. 1991; Lee and HURWITZ 1993). The no-action potential temperature-sensitive allele (mle<sup>apo</sup>) is a gain-of-function mutation that causes a reduction in adult brain voltage-gated Na⁺ channels as assessed by tetrodotoxin-binding studies (Jackson et al. 1984; Kauvar 1982). As described previously, mle<sup>apo</sup> mutants show a loss of action potentials and behavioral paralysis at elevated (37°C) temperatures and increased action potential refractory period at room temperature (Wu and Ganetzky 1980; Wu et al. 1978). In double-mutant combinations, mle<sup>apo</sup> acts as a suppressor of behavioral phenotypes for BS and Sh mutants (Ganetzky and Wu 1982); it causes unconditional lethality in combination with para (Wu and Ganetzky 1980). The paralytic (para, 1–52.1) gene encodes a voltage-gated Na⁺ channel alpha subunit (Loughney et al. 1989; Ramaswami and Tanouye 1989). The para<sup>d</sup> allele causes a loss of action potentials and behavioral paralysis at elevated (29°C) temperatures and increased action potential refractory period at room temperature (Siddiqi and Benzer 1976; Suzuki et al. 1971; Wu and Ganetzky 1980). The para<sup>T76</sup> allele used here has been described previously (Siddiqi and Benzer 1976) and appears to be less severe than para<sup>el</sup> based on viability in a mle<sup>apo</sup> background (Ganetzky 1984). Finally, para<sup>el</sup> adults become paralyzed much quicker at 40°C than wild type (Hall 1973).

LEG-SHAKING MUTANTS. Two behavioral leg-shaking mutants were examined Sh and eag. The Shaker (Sh, 1–57.6) gene encodes several types of voltage-gated K⁺ channel alpha subunits (Baumann et al. 1987; Kamb et al. 1987; Tempel et al. 1987). For mutants under moderate ether anesthesia, legs shake abnormally, antennae twitch, and the abdomen pulsates. Mutations cause abnormal action potential repolarization of the adult giant fiber, repetitive firing of action potential in larval nerves, and prolonged transmitter release at the larval neuromuscular junction (Ganetzky and Wu 1982; Jan et al. 1977; Tonouye and Ferrus 1985; Tonouye et al. 1981). Mutants are abnormal in one class of K⁺ current (I<sub>KS</sub>) (Gautam and Tanouye 1990; Lichtinghagen et al. 1990; Salkoff and Wyman 1981). The Sh<sup>KS1.5</sup> allele is an extreme mutation due to a single amino acid substitution that causes a nonfunctional channel subunit with a complete loss of I<sub>K⁺</sub>. The Sh<sup>d</sup> allele is a less extreme mutation due to a amino acid substitution that changes I<sub>K⁺</sub> channel kinetics. The Sh<sup>KS1/2</sup> allele is the weakest mutation of the three and causes a slight decrease in I<sub>K⁺</sub>. In double-mutant combinations, mle<sup>apo</sup> has been shown to suppress Sh behavioral phenotypes (Ganetzky and Wu 1982). The ether-a-go-go (eag, 1–50) gene encodes a cyclic nucleotide-modulated K⁺ channel (Bruggemann et al. 1993; WARMKE et al. 1991). Similar to Sh, these mutants exhibit leg-shaking under ether and have abnormal K⁺ currents in larval muscles (Wu et al. 1983). The eag<sup>d</sup> mutation leads to the alteration but not the absence of four different K⁺ currents in larval muscles (Zhong and Wu 1991). In double-mutant combination with Sh, they show an enhancement of the Sh phenotype (Ganetzky and Wu 1983).

OTHER BEHAVIORAL MUTANTS. Two other behavioral mutants were examined slo and shak-B. The slowpoke (slo, 3–86) gene encodes a Ca<sup>2+</sup>-activated K⁺ channel (Atkinson et al. 1991). The mutants exhibit temperature-sensitive paralysis and are sluggish at room temperature (Elkins et al. 1986). The defect leads to a large reduction in the fast Ca<sup>2+</sup>-activated K⁺ current (I<sub>CT</sub>) in adults (Elkins et al. 1986) and larvae (Singh and Wu 1989). The shaking-B (shak-B, 1–64) gene encodes gap-junction proteins (Crompton et al. 1995; Krishnan et al. 1993; Phelan et al. 1998). The shak-B<sup>2</sup> allele prevents the formation of electrical synapses in the giant fiber (GF) system, in the flight circuit, and likely in many other parts of the nervous system (Phelan et al. 1996; Trimarchi and Murphey 1997). Mutations also display aberrant neuronal branching patterns and neuroconnectivity defects in the GF system (“Passover” defects) (Thomas and Wyman 1984). These lead to deficits in signaling (weak or no response) between the GF axon and the TTM motoneuron and in the GF to dorsal longitudinal muscle (DLM) pathway. Behaviorally, mutants show no escape response: flies are unable to jump into the air and fly away at a light off stimulus.

DOUBLE MUTANTS. The double mutants used in this study were tested to verify the presence of both the BS mutation as well as the putative suppressor mutation. The presence of either para or mle<sup>apo</sup> in the homozygous double-mutant stocks was verified by paralysis following 1-min exposure to 37°C, which is characteristic of these
mutations. The BS mutants do not paralyze under such conditions. The presence of ShK<sup>KS133</sup> in the homozygous double-mutant stock was verified by rapid leg shaking under ether, the behavioral characteristic of these mutants. The BS mutants do not exhibit rapid leg shaking under ether. The presence of shak-B<sup>-</sup> in the homozygous double-mutant stocks was verified by the absence of the normal giant fiber pathway response that is characteristic of shak-B<sup>-</sup> mutants.

The presence of the homozygous BS mutation in the double-mutant stocks was verified by back-crossing each double-mutant stock to males of the appropriate BS genotype. Female flies from these crosses, which should be homozygous for the BS mutation and heterozygous for the suppressor mutation, were then tested for the BS seizure phenotype. All of the following genotypes arising from back-crosses resembled BS homozygous flies in seizure threshold phenotype: bss/bss;male<sup>apo</sup>/+ (9 of 10 exhibited seizures at 4.5 V), bss/bss shak-B<sup>2</sup>/+ (10 of 10 exhibited seizures at 4.5 V), easles<sup>as</sup> shak-B<sup>2</sup>/+ (10 of 10 exhibited seizures at 6 V), shak-B<sup>2</sup>/+; sda/sda (8 of 10 exhibited seizures at 10 V), and male<sup>apo</sup>/sda/sda (10 of 10 exhibited seizures at 10 V). The lack of any obvious effects among the different background tests also indicated that alterations in the seizure thresholds reported here were due to the homozygous presence of suppressors in the double-mutant combinations and not likely due to nonspecific genetic background differences.

Flies of the genotype Sh<sup>KS133</sup>/+ sda/sda arising from the Sh<sup>KS133</sup> sda double-mutant backcross displayed a seizure threshold that was above the BS range. These flies have a seizure threshold of 13.8 ± 2.7 V, indicating a single copy of the semi-dominant Sh<sup>KS133</sup> allele can suppress seizures to a limited extent. The males from this backcross were examined to ensure the homozygous presence of sda in the original double mutant. All of the males from this cross were identical to sda homozygotes. Finally, flies of the genotype para<sup>+</sup>/ sda/sda arising from the para;sda double-mutant backcross displayed a seizure threshold, 14.7 ± 3.9 V, that was above the sda homozygote threshold. This value is much lower than the double-mutant threshold of 38.9 ± 8.0 V, but it does indicate that a single copy of para can suppress seizures to a limited extent. The males from this backcross were examined to ensure the homozygous presence of sda in the original double mutant.

Electrophysiology

The GF circuit was used to assess nervous system function. All experiments were performed at room temperature 22–24°C. Methods for handling and mounting flies, stimulating the GF with single pulses (0.2-ms duration, 0.5 Hz), and recording of evoked DLM potentials have been described previously (Kuebler and Tanouye 2000; Pavlidis and Tanouye 1995). During the course of these experiments, the GF was stimulated continuously to assess GF circuit function. Care was taken not to use flies from overcrowded vials as the reduced size of these flies could artificially lower the seizure threshold.

To determine following frequency of the GF circuit, 20 consecutive suprathreshold stimulus pulses (1.2–1.4 times the GF threshold) were delivered to the GF at a particular frequency. The following frequency was determined as the highest frequency at which the DLM responded to at least 19 of the 20 pulses. Between the different trials, flies were allowed to rest for at least 1 min. GF following frequencies were not determined for shak-B<sup>-</sup> (also called Passover) because the GF pathway is disrupted in this mutant.

Seizures were elicited by short wavetrains of high-frequency (HF) electrical stimuli (0.4-ms pulses at 200 Hz) delivered to the brain for 300 ms. Seizures consist of HF activity in at least seven different muscle groups and over 30 muscle fibers in the thorax (Kuebler and Tanouye 2000). Seizures are followed by a period of quiescence typified by synaptic failure. In all cases examined, the GF threshold in males (Table 1) was lower than that found previously in females (Kuebler and Tanouye 2000).

RESULTS

Seizure susceptibility in the BS mutants

Seizures in Drosophila following HF stimuli consist of aberrant HF firing in all muscle fibers and motoneurons that have been examined in the fly (Kuebler and Tanouye 2000) followed by a period of quiescence typified by synaptic failure.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seizure Threshold, V</th>
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<tbody>
<tr>
<td>bss</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>eas</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>sda</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>Berlin</td>
<td>25.5 ± 3.7</td>
</tr>
<tr>
<td>CS</td>
<td>30.1 ± 3.8</td>
</tr>
<tr>
<td>Oregon-R</td>
<td>39.3 ± 6.6</td>
</tr>
<tr>
<td>Sh&lt;sup&gt;+&lt;/sup&gt;</td>
<td>51.3 ± 6.1</td>
</tr>
<tr>
<td>slo</td>
<td>52.9 ± 7.4</td>
</tr>
<tr>
<td>eog</td>
<td>62.1 ± 10.3</td>
</tr>
<tr>
<td>para</td>
<td>65.0 ± 7.2</td>
</tr>
<tr>
<td>Sh&lt;sup&gt;KS133&lt;/sup&gt;</td>
<td>86.9 ± 8.5</td>
</tr>
<tr>
<td>shak-B&lt;sup&gt;2&lt;/sup&gt;</td>
<td>94.7 ± 10.2</td>
</tr>
<tr>
<td>mle&lt;sup&gt;apo&lt;/sup&gt;</td>
<td>72.2 ± 7.3</td>
</tr>
<tr>
<td>Sh&lt;sup&gt;KS133&lt;/sup&gt;</td>
<td>83.8 ± 12.8</td>
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Short wavetrains of high-frequency (HF) electrical stimuli (0.5-ms pulses at 200 Hz for 300 ms) are delivered to the brain at various voltages. Seizures are elicited in an all-or-nothing manner with the value of the threshold voltage indicated. Seizure activation is monitored by electrodes that record HF activity in the dorsal longitudinal muscle (DLM) reflecting aberrant firing of the DLM motoneuron. The genotypes are listed from most susceptible to least susceptible. The hang-sensitive (BS) mutants bss, eas, and sda have seizure thresholds that are substantially lower than the three wild-type strains Berlin, Canton Special (CS), and Oregon-R. All of the non-BS behavioral mutants examined here, Sh<sup>+</sup> (3 different alleles), slo, eog, para, shak-B, and mle<sup>apo</sup> have seizure thresholds that are higher than wild type. For Sh<sup>KS133</sup> and mle<sup>apo</sup> mutants (marked by an asterisk), seizures cannot be evoked with standard 300-ms HF stimuli at 100 V, the maximum voltage used here. Threshold voltages for these mutants are determined by using 400-ms HF stimuli. Values for seizure threshold are means ± SD in volts (n ≥ 13 for each genotype tested).
Previous studies have indicated that the ability of HF stimuli to elicit these seizures can be modified by genetic mutations (Kuebler and Tanouye 2000). BS mutants, including the three examined here bss, eas, and sda, have been shown to be more susceptible to seizures than wild type (Kuebler and Tanouye 2000). These mutants have seizure thresholds ranging from 3 to 7 V as opposed to the wild-type strains, Berlin, CS, and OR, which have seizure thresholds ranging from 26 to 39 V (Table 1, Figs. 1 and 2). Because of their phenotype, the BS mutants are a useful tool for investigating the types of genetic factors that can suppress seizure susceptibility.

Seizure thresholds in Na\textsuperscript{+} channel mutants

The first class of mutants we investigated consisted of two mutants that affect voltage-gated Na\textsuperscript{+} channels, \textit{mlce\textsubscript{naps}} and \textit{para}. These mutants seemed likely candidates for having alterations in seizure susceptibility as many anti-convulsants such as phenytoin, carbamazepine, and lamotrigine suppress seizures through interactions with voltage-gated Na\textsuperscript{+} channels (Kuo 1998; Kuo et al. 1997). Both of these mutants displayed decreased levels of seizure susceptibility compared with wild-type strains (Table 1). Examples of \textit{mlce\textsubscript{naps}} and \textit{para} seizures are shown in panels A and B.

![Graph showing seizure threshold comparison between sda and CS mutants](image-url)
Both mle$^{apts}$ and para suppress the BS seizure-susceptible phenotype

Because both mle$^{apts}$ and para were much less susceptible to seizures than wild type, both mutants were tested for the ability to suppress BS seizures (Table 2). The para mutation was able to suppress the BS seizure phenotype as the addition of $sda$ into a para background raised the seizure threshold to wild-type levels ($38.9 \pm 8.0$ V), well above the $6.2 \pm 0.8$ V $sda$ seizure threshold. The mle$^{apts}$ mutation was able to rescue the seizure susceptibility defect in two BS mutants tested, $bss$ and $sda$. The double-mutant combination $bss; mle^{apts}$ has a seizure threshold of $29 \pm 4.7$ V, which is in the range of wild type, as opposed to a $3.2 \pm 0.6$ V threshold for $bss$ (Fig. 2). The mle$^{apts}; sda$ double mutant had no appreciable seizures following 300- and 400-ms $HF$ stimuli had to be used. The value obtained, $89 \pm 10.2$ V, is much greater than wild-type values and is even slightly higher than that found for mle$^{apts}$ alone (Table 2). In both mle$^{apts}$ double mutants, $bss; mle^{apts}$ and mle$^{apts}; sda$, there was an absence of spontaneous seizure activity on recovery from the initial seizure and failure that may be related to the reduction in seizure susceptibility seen in these mutants. The BS flies always display intense spontaneous seizure activity during this period; however, the activity was absent in both mle$^{apts}$ double mutants $bss; mle^{apts}$ (Fig. 2) and mle$^{apts}; sda$ (not shown).

Seizure thresholds in K$^+$ channel mutants

The second group of mutants examined consisted of Drosophila K$^+$ channel mutants. We postulated mutants may have increased seizure susceptibility levels as K$^+$ channel defects can lead to seizure disorders in both mice and humans (Charlier et al. 1998; Singh et al. 1998; Smart et al. 1998). We tested a variety of K$^+$ channel mutants including eag, a mutant that affects a member of a family of voltage-gated K$^+$ channels (Wu and Ganetzky 1992) and slo, a mutant that affects the calcium-activated K$^+$ current, $I_{\text{CC}}$ (Atkinson et al. 1991). Surprisingly, we found that both eag and slo have seizure thresholds above the wild-type range, $62 \pm 10.3$ V for eag and $52.9 \pm 7.4$ V for slo (Table 1).

We also tested three different mutant alleles of the Sh K$^+$ channel gene and found that all three mutants were resistant to seizures in comparison to wild-type strains (Table 1). The most defective Sh mutant, $Sh^{-}\text{KS133}$, was resistant to seizures following 300-ms $HF$ stimuli, and its threshold following 400-ms $HF$ stimuli, $84 \pm 12.8$ V, was one of the highest seen in this study. The $Sh^{-}\text{KO120}$ mutant was also resistant to seizures compared with wild type as it was found to have a seizure threshold of $87 \pm 8.5$ V following 300-ms $HF$ stimuli. The $Sh^5$ mutant did not display much resistance to seizures; it has a seizure threshold of $51 \pm 6.1$ V, a value just above the wild-type range.

$Sh^{-}\text{KS133}$ can partially suppress the BS seizure-susceptible phenotype

We tested the ability of the $Sh^{-}\text{KS133}$ to suppress the BS seizure-susceptible phenotype even though K$^+$ channels had not previously been thought of as targets for anti-convulsants. We found that the introduction of $sda$ into a $Sh^{-}\text{KS133}$ background led to partial suppression of the $sda$ seizure-susceptible phenotype. The $Sh^{-}\text{KS133}; sda$ double mutant has a seizure threshold ($18.8 \pm 5.7$ V) that is above the values for the BS mutants yet below the wild-type range (Table 2, Fig. 3). $Sh^{-}\text{KS133}; sda$ flies also exhibited a decrease in the GF failure time following HF stimuli. The double mutant failed for $25 \pm 7.4$ s (Fig. 3), while $sda$ failed for $64 \pm 10.5$ s (Fig. 1). The presence of $Sh^{-}\text{KS133}$ appeared to suppress both failure and seizures although the $Sh^{-}\text{KS133}; sda$ flies were still more susceptible than wild type.
Mutant that affects neural connections is resistant to seizures

The mutant we investigated is *shak-B^2* that affects neural connections. Because neural connections and pathways are thought to be critical in both the generation and spread of seizures throughout the nervous system, mutations that disrupt these connections may affect seizure susceptibility. In addition, the *shak-B^2* mutant, which prevents the formation of electrical synapses throughout the nervous system (Phelan et al. 1996; Trimarchi and Murphey 1997), may inhibit the synchronization of neuronal firing, a trademark of seizure activity (Carlen et al. 1996; Perez-Velazquez et al. 1994). Here we found that the *shak-B^2* mutant has decreased susceptibility to seizures as the seizure threshold in this mutant, 95 ± 10.2 V, is well above wild-type levels (Fig. 4, Table 1).

Shak-B^2* mutation suppresses different BS mutants to varying degrees

The *shak-B^2* mutant was tested for its ability to suppress seizures in all three of the BS mutants studied here, *bss*, *eas*, and *sda* (Table 2, Fig. 4). In the case of *bss*, the presence of the *shak-B^2* mutation did not significantly alter the seizure susceptibility. There was little difference in the seizure thresholds between *bss* and *bss shak-B^2* double mutants: *bss* mutants have a threshold of 3.6 ± 0.7 V, while *bss* mutants have a threshold of 3.2 ± 0.6 V. The *eas* *shak-B^2* double mutant displayed partial suppression as it has a seizure threshold (15.3 ± 3.2 V) that falls between *eas* and the wild-type range (Tables 1 and 2). Finally, the addition of *sda* to a *shak-B^2* background raised the seizure threshold to wild-type levels (31.4 ± 5.2 V), well above the 6.2 ± 0.8 V seizure threshold.
HF stimuli are delivered to flies of different double-mutant genotypes, and their respective seizure thresholds are determined. The genotypes are listed from most seizure-susceptible to least susceptible. The genotypes can be roughly divided into categories: partially suppressed BS seizure phenotype with thresholds above the BS level but below wild type (genotypes: eas shak-B<sup>2</sup> and sh<sup>KS133</sup>;sda); completely suppressed BS phenotype with thresholds comparable to wild type (genotypes: bss;mle<sup>napt</sup>, shak-B<sup>2</sup>;sda, and para; sda); threshold comparable to the parental suppressor strain (genotype: mle<sup>napt</sup>;sda); and nonsuppressed with threshold comparable to the parental BS strain (genotype: bss shak-B<sup>2</sup>). For mle<sup>napt</sup>;sda mutants (marked by an asterisk), seizures cannot be evoked with standard 300-ms HF stimuli at 100 V, the maximum voltage used here. Threshold voltages for these mutants are determined by using 400-ms HF stimuli. Values for seizure threshold are means ± SD in volts (n = 13 for each genotype tested).

seen in the sda mutant (Table 2, Fig. 4). The reduction in excitability in shak-B<sup>2</sup>;sda mutants was also evident by the absence of spontaneous seizure activity during the recovery described previously for bss;mle<sup>napt</sup> and mle<sup>napt</sup>;sda.

Increased latency to seizures and reduction of spontaneous seizures in bss shak-B<sup>2</sup>

Despite the lack of ability to alter the seizure threshold in bss flies, the presence of the shak-B<sup>2</sup> mutation did lead to a significant increase in the latency to seizure onset in bss shak-B<sup>2</sup> flies following 4-V HF stimuli. In the case of the double mutant, the latency to seizure onset was 768 ± 371 ms, while the latency for bss was 295 ± 81 ms (n = 20). Previous studies (Kuebler and Tanouye 2000) have shown in bss, low-voltage HF stimuli give rise to seizures with longer latencies possibly because it takes some time for the activity in the few neurons activated by the 4-V HF stimulus to be amplified through positive feedback loops to generate a seizure. When higher voltage HF stimuli are used, we proposed that more neurons are recruited directly by the stimulus and therefore less time is taken for this activity to be amplified into a seizure. This also appears to occur in the double mutant as the latency to seizure decreases to 288 ± 25 ms (n = 5) following 12-V HF stimuli. It is possible that the absence of functional electrical connections in bss shak-B<sup>2</sup> flies increases the amount of time necessary to generate a seizure at 4 V by disrupting the amplification of the neural activity induced by the HF stimuli.

A reduction in excitability in the bss shak-B<sup>2</sup> mutant could also be seen on examining the activity seen on recovery from synaptic failure. Following electrically induced seizure and synaptic failure, bss mutants often undergo additional bouts of seizure and GF failure. These additional bouts of seizure and GF failure occurred in 87% of bss flies during recovery from a 4-V HF stimulus, while this activity only occurred in 12% of the bss shak-B<sup>2</sup> mutants. The absence of the electrical connections may disrupt the amplification or synchronization of the activity that occurs following synaptic failure such that it is much more difficult to generate further bouts of seizure and GF failure in these double mutants.

Double mutants: general features

Our general findings are that although all of the BS mutations examined could be suppressed in double-mutant combinations, they did not all appear to be suppressed equally well. The bss mutant was the most difficult to suppress, sda appeared to be the easiest to suppress, while eas was somewhere in between. Likewise, the mutations that we used to set the genetic background had varying abilities to act as suppressors. For the mutants tested, mle<sup>napt</sup> acted as the best suppressor, Sh<sup>KS133</sup> appeared to be the weakest of the suppressors, while the para and shak-B<sup>2</sup> mutations fell somewhere in between. In addition, the ability of a strain to suppress the BS phenotype

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**TABLE 2. Seizure thresholds for various double-mutant genotypes**

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<tr>
<th>Genotype</th>
<th>Seizure Threshold, V</th>
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<tr>
<td>bss shak-B&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3.6 ± 0.7</td>
</tr>
<tr>
<td>eas shak-B&lt;sup&gt;2&lt;/sup&gt;</td>
<td>15.3 ± 3.2</td>
</tr>
<tr>
<td>Sh&lt;sup&gt;KS133&lt;/sup&gt;;sda</td>
<td>18.8 ± 5.7</td>
</tr>
<tr>
<td>bss;mle&lt;sup&gt;napt&lt;/sup&gt;</td>
<td>29.2 ± 4.7</td>
</tr>
<tr>
<td>shak-B&lt;sup&gt;2&lt;/sup&gt;;sda</td>
<td>31.4 ± 5.2</td>
</tr>
<tr>
<td>para; sda</td>
<td>38.9 ± 8.0</td>
</tr>
<tr>
<td>mle&lt;sup&gt;napt&lt;/sup&gt;;sda</td>
<td>88.8 ± 10.2</td>
</tr>
</tbody>
</table>
The BS level but below wild type. The second category consisted of those with a completely suppressed BS phenotype and included \( bss;mlenapts;\ shak-B^2;\ sda \), and \( \text{para};\ sda \). These flies had seizure thresholds that were comparable to wild type. The final two categories each had only one representative and consisted of those double mutants that had seizure phenotypes that were consistent with one of the parental types. The \( \text{mle}^{\text{apts}};\ sda \) strain was the only double mutant that had a seizure threshold nearly identical to the parental suppressor strain, in this case \( \text{mle}^{\text{apts}} \). On the other hand, the \( bss;\ shak-B^2 \) strain was the only double mutant that had a seizure threshold comparable to the parental BS mutant, although the seizure phenotype of \( bss;\ shak-B^2 \) does differ slightly from \( bss \).

**GF threshold is not altered in most strains**

One possible explanation for the altered seizure thresholds seen in many of the double mutants is that the threshold of each individual neuron in these strains could be altered. If this was the case, different HF stimulus voltages could be required to recruit the same number of neurons in different genotypes. This would account for the different seizure thresholds seen here. To investigate this possibility, we examined the threshold of the GF neuron to determine if individual neuron thresholds were significantly altered in these strains. We found that the GF threshold differed in only three cases, \( \text{mle}^{\text{apts}};\ mlennapts;\ sda \), and \( bss;\ mlenapts \) (Table 3). In all three cases, the GF threshold is elevated compared with wild type, a factor that may contribute to the fact that \( \text{mle}^{\text{apts}} \) is the best suppressor we have studied. Despite this, the GF threshold did not necessarily correlate with the seizure threshold in these three cases: the double-mutant \( bss;\ mlenapts \) has a higher GF threshold than \( \text{mle}^{\text{apts}} \) alone despite the fact that the double mutant has a much lower seizure threshold (Tables 1 and 2). In addition, another neuron in \( \text{mle}^{\text{apts}} \) flies, the DLM motoneuron, showed no changes in stimulation threshold compared with wild type (Kuebler and Tanouye 2000).

Based on the preceding data, it is difficult to see a consistent pattern concerning individual neuron thresholds that could explain the wide range of seizure thresholds found here. It does remain a formal possibility though that other neurons not examined here may display altered thresholds that correspond more closely to the seizure threshold.

**Following frequency alterations**

During seizures, abnormal HF neuronal spiking spreads throughout the nervous system. If the nervous system is not capable of supporting HF spiking activity, then seizure suppression might occur. To examine this possibility, we chose the measurement of the GF following frequency, the maximum stimulation frequency the GF pathway can reliably follow, as an additional method for dissecting the excitability of the nervous system in the various strains. The first indication that GF following frequency may affect the seizure threshold is seen on examining the mutants with the highest thresholds, \( \text{Sh}^{\text{K}120} \) and \( \text{para} \) mutants, which have seizure thresholds that fall below wild-type and the high-threshold...
The GF was activated by single stimulus pulses (0.2-ms duration, 0.5 Hz) delivered to the brain. GF activation was monitored by electrodes that recorded evoked potentials in the DLM driven by the GF circuit. GF thresholds could not be obtained for shak-B2 mutants and their double mutants (genotypes: bss shak-B2, eas shak-B2, shak-B2;sda) by this procedure as they are defective in the GF pathway ("Passover" phenotypes) (Thomas and Wyman 1984). The GF thresholds for all genotypes are comparable except for mle napts mutants and their double mutants (genotypes: bss; mle napts and mle napts;sda) which are all higher. Values of GF threshold listed are means ± SD in volts (n ≥ 13 for each genotype tested).

The GF following frequencies were determined by delivering 20 consecutive suprathreshold stimulus pulses (1.2–1.4 times the GF threshold) to the GF at a particular frequency. GF following frequencies could not be obtained for shak-B2 mutants and their double mutants (genotypes: bss shak-B2, eas shak-B2, shak-B2;sda) by this procedure as they are defective in the GF pathway (Passover phenotypes) (Thomas and Wyman 1984). The following frequency was determined as the highest frequency at which the DLM responded to at least 19 stimuli. Values for the GF following frequency are listed for each genotype that was tested. Values listed are means ± SD in Hz (n ≥ 9 for each genotype).
In both cases the following frequency was reduced to levels well below the value found in wild-type flies. Calibration: 20 mV, 100 ms.

At this frequency of stimulation, 5 stimuli are ineffective in evoking responses of the GF pathway. The GF following frequency of the BS mutants is altered in some of these double mutants it does not always predict the seizure susceptibility level; however, it appears that modifications in following frequency could be one factor that contributes to the overall seizure susceptibility of the nervous system.

**DISCUSSION**

We have demonstrated that certain genetic mutations can both elevate the seizure threshold in *Drosophila* and suppress the seizure-susceptible phenotype seen in BS mutants. All of these mutants, in double-mutant combination with BS mutants, provided a genetic background that elevated the seizure threshold to values above those seen in the BS mutants alone. This is the first demonstration that mutations in *Na*⁺ channels, *K*⁺ channels, and a connexin protein can suppress seizures. Understanding how these mutations contribute to the seizure phenotype will be instrumental in furthering our knowledge of the complex array of factors that contribute to seizure susceptibility.

**Na⁺ channel mutants suppress seizures**

Seizures were found to be suppressed by altering voltage-gated-*Na*⁺ channels through mutations such as *mle*neo and *para* that lead to a reduction of functional *Na*⁺ channels (Kuroda et al. 1991; Loughney et al. 1989). The data presented here and in previous studies (Nelson and Wyman 1990) indicate that the reduction in *Na*⁺ channels leads to a decreased ability of the GF pathway to support HF firing. The loss of HF firing may contribute to the ability of these mutations to suppress BS seizures following HF stimuli. In every case, the suppression of seizures in these double mutants corresponds to a decrease in the GF following frequency compared with the following frequency seen in BS mutants alone. Although individual neuron (i.e., GF) excitability following single-pulse stimulation is altered in some of these double mutants it does not closely correspond to the changes in seizure susceptibility.

The reduction in the availability of active *Na*⁺ channels in *mle*neo and *para* suggests that they may inhibit seizures in much the same way as the anti-convulsants phenytoin and carbamazepine (Kuo 1998; McNamara 1999). During repetitive firing, these drugs are thought to stabilize the *Na*⁺ channel in the inactive state thereby reducing the number of channels that can be activated. This leads to an inhibition of HF firing (McLean and Macdonald 1983, 1986) similar to the GF following frequency defect in *mle*neo and *para*. The level of *Na*⁺ channel inactivation also accounts for seizures in patients who suffer from generalized epilepsy with febrile seizures plus...
(GEFS+), a disease resulting from a mutation in the Na\(^+\) channel beta1 subunit that causes the channel to inactivate much slower (Wallace et al. 1998).

**K\(^+\) channel mutants suppress seizures**

The finding that Sh and eag mutants displayed increased seizure thresholds compared with wild type was surprising given the hyperexcitability defects seen in these strains (Jan et al. 1977; Kaplan and Trout 1969; Tanouye et al. 1981). In fact, the Sh seizure phenotype in Drosophila is in stark contrast to the spontaneous seizure phenotype seen in a mouse knockout of the Shaker family channel K\(_{\text{v}1.1}\) (Smart et al. 1998). The difference in Sh mutant phenotypes could be due to the differences between the Sh loci in flies and mice. In mice, there are several Sh loci that are expressed differentially (Drewe et al. 1992; Lock et al. 1994; Wang et al. 1994) such that a mutation in one would presumably affect only a subset of neurons and could be partially compensated for by other loci. In flies, there is only one identified Sh locus that gives rise to a variety of Sh transcripts (Kamb et al. 1988) such that mutations affect most if not all the Sh channels in the fly. It is clear from mouse knockout studies that the specific Sh channel affected by mutation is critical to the seizure phenotype. For example, the Sh family K\(_{\text{v}4.1}\) knockout does not have a seizure susceptible phenotype (Ho et al. 1997), in contrast to the Sh K\(_{\text{v}1.1}\) knockout described in the preceding text. It is possible that mutations that affect one or a small subset of Sh transcripts could lead to a seizure susceptible defect in flies; however, mutations of this type have not been identified.

It is not obvious why the K\(^+\) channel mutants studied here have higher seizure thresholds than wild type nor why Sh\(_{\text{KS133}}\) can suppress sda seizures. One possible explanation centers on the ability of neural pathways in these mutants to support the type of HF activity seen during seizures. In Sh\(_{\text{KS133}}\) mutants, the ability of the GF to follow HF stimulation is severely impaired: it is reduced three to four times that of wild type (Tanouye et al. 1981). Among several possibilities,
delayed repolarization could prolong Na⁺ channel inactivation, thereby inhibiting HF activity in the GF pathway and possibly other pathways as well. Despite this correlation, it is apparent that other mechanisms must contribute to the seizure phenotype seen in these mutants for two reasons: Shk and ShkKO120 have similar GF following frequencies despite having different seizure thresholds and eag has no reduction in GF following frequency despite having an increased seizure threshold. Another possible contributing mechanism is that K⁺-channel mutants may disrupt the synchronization of synaptic components in positive feedback loops that are thought to be involved in the generation of the oscillations seen during seizures (Huguenard and Prince 1994; Traynelis and Dingledine 1988; Warren et al. 1994). The altered repolarization kinetics seen in these strains (for a review, see Wu and Ganetzky 1992) could affect firing patterns of certain neurons critical to the generation of seizures by causing components of the positive feedback loop to fire out of synch.

Mutations that affect neuronal connections

The involvement of positive feedback loops in seizure generation suggests that mutations altering neuronal connections within these loops might be expected to affect seizure susceptibility. One way to disrupt neuronal connections is to use shak-B² flies. These flies are defective in a neural-specific connexin gene that leads to the absence of electrical synapses or neuronal gap junctions in the fly (Crompton et al. 1995; Krishnan et al. 1993; Trimarchi and Murphey 1997). The absence of these leads to an increase in the seizure threshold in shak-B² mutants and an ability to suppress seizures in double-mutant combinations.

The effect shak-B² mutations have on seizure susceptibility may be related to the role neuronal gap junctions play in the synchronous firing of populations of neurons. It is known that gap junctions are involved in the generation of synchronous bursting in CA1 pyramidal cells in the in vitro calcium-free epilepsy model. The synchronous seizure-like discharges normally seen in this model are inhibited by methods that block gap junctions (Perez-Velaquez et al. 1994). In fact, electrical coupling of neurons mediated by gap junctions has long been proposed as a factor involved in generating the synchronous bursts seen during seizures (Carlen et al. 1996; Dudek et al. 1986).

The latency to seizure in bss shak-B² mutants as compared with bss is sometimes up to 2 s longer even though the voltage necessary to trigger seizure in the two strains is similar. This could be due to a lack of gap-junction-mediated synchronization in the positive feedback loops. In this case, the longer latency in bss shak-B² flies would occur because it takes longer for the activity triggered by the HF stimulus to be amplified within the feedback loop in the absence of the proper synchronizing connections. A similar mechanism could also explain the requirement of higher intensity HF stimuli to activate enough neurons synchronously to trigger a seizure in the shak-B², eas shak-B², and shak-B²; sda mutants as described previously (Kuebler and Tanouye 2000).

It is also possible that the removal of gap junctions in the nervous system of shak-B² flies controls seizures in much the same manner as the surgical removal of neural connections controls seizures in some patients that suffer from intractable epilepsy. This implies that seizures may occur in shak-B² mutants at the site of the stimulating electrodes, but due to lack of electrical connections in the nervous system, the seizure does not spread throughout the fly. This possibility is unlikely because seizures that spread to the thorax can be generated in shak-B² flies, albeit at high-voltages, and in bss shak-B² flies at very low voltages.

Model of Drosophila seizure susceptibility

A simple model that accounts for several major features of Drosophila seizure susceptibility and its modification by genetic mutations is depicted in Fig. 7. A neuron (labeled Sei) is capable of delivering seizure throughout the Drosophila CNS. Seizures are triggered in Sei by three presynaptic input neurons (labeled Wt, Bs, and Su). Although Sei, Wt, Bs, and Su are shown as single neurons, each may represent a population of neurons with similar properties or an extensive neural circuit. For convenience, the three input neurons are given separate names; however, they may actually be similar or identical to each other; we have not found features that distinguish them. Synaptic inputs from the presynaptic neurons show temporal and spatial summation in Sei.

In a normal wild-type fly, stimulation of two neurons (say: Bs and Wt) with a HF electrical stimulus wavetrain triggers a seizure. Synaptic potentials from Bs and Wt summate temporally and spatially in Sei to trigger the seizure. In a BS mutant, it is much easier to trigger a seizure and stimulation of only a single neuron (say: Bs) is sufficient. A low-voltage HF stimulus is sufficient to drive Bs, and its synaptic potentials summate temporally in Sei to trigger the seizure. In a suppressor mutant, it is much more difficult to trigger a seizure and necessary to stimulate all three neurons (Bs, Wt, and Su), thereby requiring a higher voltage HF stimulus. In a double mutant, susceptibility has been restored to the wild-type level and a seizure is triggered by stimulating only two neurons (say: Bs and Wt). These observations suggest that different synaptic inputs interact spatially and temporally to trigger seizures.

Suppressor mutations compromise nervous system signaling capabilities. Several mutations, mle⁹⁰⁵, para, and Sh, may suppress seizures because of an effect on firing frequencies.

FIG. 7. A model for seizure suppression in Drosophila. The model consists of 3 presynaptic neurons that act to trigger seizures (labeled Wt, Bs, and Su). These neurons drive a postsynaptic neuron (labeled Sei) that delivers the seizure throughout the nervous system. Although Sei, Wt, Bs, and Su are depicted as single neurons, each may represent a population of neurons with similar properties or an extensive neural circuit. For convenience, the input neurons are given separate names, however, we have found no qualitative features that distinguish them. Synaptic inputs show temporal and spatial summation in the Sei neuron.
Kuebler and Tanouye (2000) showed that the effectiveness of the triggering stimulus is dependent on the frequency of stimulus pulses in the HF wavetrain. A stimulus frequency of 200 Hz appears to be ideal, while a stimulus frequency of 150 Hz is substantially less effective. Mutations that compromise following frequency suppress seizures because 200-Hz HF frequencies cannot be maintained in input neurons and there is decreased temporal summation at the triggering synapses. To elicit seizures, additional inputs must be stimulated to increase spatial summation. For shak-B mutation, nervous system signaling capabilities are compromised because of neuroconnectivity defects. If the number of synapses or synaptic strength of Sei inputs are compromised, then decreased spatial summation is expected. To elicit seizures, additional inputs must be stimulated by higher voltage HF stimuli to compensate.

This simple model accounts for several major features of *Drosophila* seizure susceptibility and its modification by genetic mutations. However, it also appears to be an over-simplification because of some observations that are not well understood. The most fundamental of these is how BS mutations act to reduce the triggering requirements for Sei neuron activation from two neurons (Bs and Wt) to just one (Bs alone). For the most part, the nervous systems of BS mutants function completely normally generating predominantly normal behaviors. In all parts of the nervous system that we can record from, BS mutants do not show a higher capacity for following frequency; there are no obvious alterations in chemical synaptic transmission and there are no obvious supernumerary synaptic connections that would enhance synaptic strength. Despite our failure to discover an obvious cellular physiological defect, BS mutations have a profound effect on overall seizure sensitivity.

Other complexities that are not well explained by this simple model include high-voltage seizure suppression and changes in the latency between HF stimulus and the start of the seizure (Kuebler and Tanouye 2000). There is no indication of the potential importance of synchronous firing of neurons that could be important in seizure genesis and that could be disrupted in shak-B2 mutants leading to suppression. There is no indication whether or not synaptic failure (the quiescent period) might also play a role in seizure genesis (Pavlidis and Tanouye 1995).

Utility of using *Drosophila* to identify seizure suppressor genes

It is clear from this study that genetic factors can decrease seizure susceptibility in flies, while previous studies have demonstrated that genetic factors can increase seizure susceptibility (Kuebler and Tanouye 2000). The ability to identify these factors in mammals, especially in humans, is quite limited compared with the genetically tractable system of *Drosophila*. The generation of *Drosophila* double mutants described here allows one to test the ability of a variety of mutations to alter the seizure-susceptibility levels seen in BS mutants. In each case in which the BS seizure susceptible phenotype was altered, a single mutation, which becomes a possible target for the development of novel anti-seizure drugs, is responsible. Because the genetic defects as well as the phenotypic consequences of these mutations are known, these studies also determine how various physiological processes contribute to the overall seizure susceptibility of the fly. Further experiments of this type as well as the isolation of new suppressors may bring us closer to unraveling the complexity of seizure disorders.

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