Seizure Suppression by \textit{shakB}^{2}, a Gap Junction Mutation in \textit{Drosophila}

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\textbf{INTRODUCTION}

Gap junction proteins, called connexins, are organized into intercellular channels that span the plasma membranes of closely apposed cells and mediate the direct ionic current flow underlying electrical synaptic transmission (reviewed in Kumar and Gilula 1996; Swenson et al. 1989). Connexins are encoded by a gene family containing 20 members in mammals with 11 of the genes expressed in the CNS (Goodenough et al. 1996; Simon and Goodenough 1998). Gap junctions may be involved in several CNS disorders, including brain ischemia, brain hemorrhage, Alzheimer’s disease, Parkinson’s disease, brain tumor, and epilepsy (Anderson et al. 2003; Carlen et al. 2000; Chen and Swanson 2003; Lin et al. 2002; Nagy et al. 1996; Nakase and Naus 2004; Naus et al. 2001; Rufer et al. 1996). In particular, for epilepsy, there is pathological synchronous spiking activity during seizure that involves many thousands of nerve cells. Electrical synaptic transmission through gap junctions is an important mechanism for synchronizing signaling in the brain, combining with field effects, chemical synaptic transmission mechanisms, and ionic channel mechanisms in the generation and maintenance of seizures (Carlen et al. 2000). Experimental observations reinforce the notion of gap junction contributions to seizures. Pharmacology that reduces electrical transmission diminishes seizures, and enhanced electrical transmission increases the frequency and severity of seizures (Carlen et al. 2000; Jahromi et al. 2002).

For example, carbenoxolone, a gap junction blocker, has been shown to reduce seizures in several animal models of epilepsy (Gajda et al. 2003; Hosseinzadeh and Nassiri Asl 2003; Jahromi et al. 2002; Ross et al. 2000). Seizures are also reduced by other inhibitors of electrical transmission, such as intracellular acidosis, sodium propionate, 1-octanol, and NH\textsubscript{4}Cl (Jahromi et al. 2002). However, to date, there are no anticonvulsants that are known to target gap junction function. Gap junction blockers used experimentally, such as carbenoxolone, have other actions and are not specific for any particular connexin type (Carlen et al. 2000). This has been a limitation in determining precisely the contribution of electrical transmission to epilepsy and the development of promising anticonvulsant therapy based on targeting gap junction communication.

Gap junction proteins in \textit{Drosophila} are encoded by a family of eight genes called the “innexin” family (Phelan and Starich 2001). This study examines the effect of gap junction mutations on seizure susceptibility in \textit{Drosophila}, in particular, mutations of the innexin family member \textit{shakB}. The \textit{shakB} locus produces different transcripts by differential splicing and alternative promoter usage (Crompton et al. 1992, 1995; Krishnan et al. 1993, 1995). A subset of transcripts called “\textit{shakB (neural)}” are all essential for viability; loss-of-function mutants do not survive past the first larval stage. In contrast, transcripts called “\textit{shakB (neural)}” are nonessential; loss-of-function mutations are viable and have nervous system defects including perturbed electrical synaptic transmission, loss of dye-coupling, and alterations in neuroconnectivity (Baird et al. 1990, 1993; Phelan et al. 1996; Sun and Wyman 1996; Thomas and Wyman 1984). The \textit{shakB (neural)} allele used here, \textit{shakB}\textsuperscript{2}, is a loss-of-function mutation in which a T to A substitution inserts a stop codon within the signal sequence (Zhang et al. 1999). Preliminary results have shown previously that \textit{shakB}\textsuperscript{2} mutation can affect seizure susceptibility in \textit{Drosophila} model of epilepsy (Kuebler et al. 2000, 2001). Flies carrying the \textit{shakB}\textsuperscript{2} mutation are especially seizure-resistant and have an especially high-threshold to evoked seizures. In addition, in some double mutant combinations with “epilepsy” mutations, \textit{shakB}\textsuperscript{2} may be a seizure-suppressor mutation. This paper provides a detailed examination of \textit{shakB}\textsuperscript{2} as a seizure-suppressor mutation by testing its ability to suppress large and diverse collection of \textit{Drosophila} epilepsy mutations. The approaches used include quantification of seizure susceptibility levels in a variety of BS mutants, as well as the seizure suppression levels in double mutants of \textit{shakB}\textsuperscript{2} with various BS mutants. The data presented here provide insights into the nature of seizure susceptibility in \textit{Drosophila}.

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seizure suppression and examine criteria that help us evaluate gap junction targeting as an approach to anticonvulsant drug development.

METHODS

Fly stocks

WILD-TYPE STRAINS AND BS MUTANTS. Drosophila strains were maintained on standard cornmeal agar medium. They were reared and examined at room temperature (22–24°C). The wild-type strain was Canton Special (CS). Seizure-sensitive mutants were of the bang-sensitive (BS) paralytic class of behavioral mutants. The tko (techni-

kcal knockout) gene is located at 1–0.99 and encodes a mitochondri-

dal ribosomal protein (Royden et al. 1987). The sda (slamdance) gene is located at 3–95.9 and encodes the fly homolog of aminopeptidase N (Zhang et al. 2002). The bss (bang senseless) gene is located at 1–54.6 (Ganetzky and WU 1982); two alleles bss1 and bss2 were examined. The bas (bang sensitive) gene is located at 1–50.7 (Ganetzky and Wu 1982; Lee and Wu 2002); the bas1 and bas2 alleles examined were gifts from Dr. C.-F. Wu (University of Iowa). The jbug (jitterbug) gene is located at 2-58F-59A (X. Ren and M. A. Tanouye, unpublished observations). The kdn (knockdown) gene is located between cv (1–13.7) and v (1–33).

THE SHAKB2 MUTANT. The shakb (shaking-B) gene is located at 1–64 and encodes a gap junction protein (Phelan et al. 1998). The shakb2 allele is a null mutation that acts as a seizure-suppressor mutation (Kuebler et al. 2001). The shakb2 mutation has been shown to interfere with gap junction formation in the adult giant fiber (GF) system neurocircuit (Krishnan et al. 1993; Phelan et al. 1996; Sun and Wyman 1996; Thomas and Wyman 1984). Thus in the mutant there is a loss of electrical synaptic transmission between the GF and the tergotrochanter muscle motoneuron (TTMmn) and the GF and the peripherally synapsing interneuron (PSI). The shakb2 mutant has a behavioral defect: lack of escape jump to a light-off stimulus. In addition, the shakb2 mutant has an electrophysiological defect compared with wild-type flies (Fig. 1); electrical stimulation of the GF fails to drive the dorsal longitudinal muscle (DLM; Fig. 1B); and the GF drives the TTMmn with an abnormally long latency (Fig. 1B) (Krishnan et al. 1993; Phelan et al. 1996; Sun and Wyman 1996; Thomas and Wyman 1984).

DOUBLE MUTANTS. Standard genetic methods with multiple marked chromosomes and balancers were used for constructing double mut-

ants with the shakb2 suppressor mutation in combination with each of the BS mutations. The following methods were used to verify the presence of the shakb2 suppressor mutation and the BS mutation in the double mutant. A defective GF system electrophysiological phenotype was used to determine the presence of the shakb2 mutation for each double mutant. That is, the absence of the DLM response and an abnormally long-latency TTM response indicated the presence of a homozygous (or hemizygous) shakb2 mutation in a double mutant since abnormal GF system electrophysiology was never observed for any of the BS mutant strains. The presence of the BS mutation in double mutants was tested by backcrossing to the BS parental strain. Thus for flies of the putative double mutant genotype bss+/shakb2 bss2 shakb2, backcrossing yields flies of the genotype bss+/shakb2 bss2 shakb2. These latter flies show a restoration of normal GF system electrophysiology—a recessive shakb2 phenotype. The flies also show the BS behavioral and electrophysiological phenomenologies of bss2, suggesting that shakb2 acts as a recessive suppressor mutation. Similarly, phenotypes that resembled those of homozygous BS flies were observed for the genotypes. All the following genotypes from backcrosses showed BS phenotypes that resembled homozygous BS flies: bss+/shakb2 bss2 shakb2, bss1 shakb2 bss2 shakb2, tko shakb2/tko shakb2, kdn shakb2 kdn shakb2, and shakb2/shakb2. jbug/jbug.

TRANSGENIC FLY LINES. Rescue of shakb2 was conducted by expression of shakb2 using the GAL4-UAS system as described previously (Curtin et al. 2002; Osterwalder et al. 2001). For constitutive expression of shakb2+, we examined flies of the genotypes: P (ELAV-GAL4) shakb2; P (US-shakb2+) and P (ELAV-GAL4) shakb2; P (US-shakb2+); sda. These flies were compared with various nonexpressing controls as described in the text. For conditional expression of shakb2+, flies of the genotype shakb2 P (ELAV-GAL4); P (US-shakb2+)/−; P (tubP-GAL80ts)/+ were examined. P (US-shakb2+) is a cDNA construct on the second chromosome described by Curtin et al. (2002) and provided as a gift by Dr. K. Curtin (Yale University). We constructed a P (ELAV-GAL4) shakb2 first chromosome whereby P (ELAV-GAL4) drives UAS expression in a nervous system lacking electrical synapses because of the shakb2 background. A third chro-

mosome P (tubP-GAL80ts) construct was obtained from the Bloom-

ington Drosophila stock center (line 7018). In the TARGET system, GAL4-UAS is conditionally regulated by a temperature-sensitive allele of GAL80 (McGuire et al. 2004). To regulate expression, flies were maintained at 18°C to repress GAL4-mediated transcriptional activation of the UAS-shakb2+ transgene and shifted to 32°C for 1–2 days to induce transgene expression.

BS behavior

Behavior tests were performed on flies 3 days after eclosion that were allowed to recover for 2 h after CO2 anesthesia. For testing, 10 flies were placed in a clean vial and allowed to rest for 30 min. They were stimulated (10 s) with a VWR vortex mixer at maximum speed. Recovery from BS paralysis was monitored by counting the number of flies standing at different intervals following vortex. Recovery time was the time where 50% of flies had recovered. In brief, BS paralytic mutants undergo seizures characterized by brief hyperactivity (2 s)

![FIG. 1. Electrophysiology of the giant fiber (GF) system. Each panel shows a recording from the tergotrochanter jump muscle (TTM; top trace) and the dorsal longitudinal flight muscle (DLM; bottom trace). A: normal GF system response recorded from a wild-type Canton Special (CS) fly. Single-pulse electrical stimulus (0.2-ms duration) delivered to the brain activates the GF. TTM and DLM responses share a common threshold. A short-latency response is seen with the TTM occurring slightly before the DLM response. B: abnormal response recorded from a shakb2 fly. TTM response is driven at long and variable (data not shown) latency. There is no DLM response in this mutant fly. Calibration: 10 mV. 1 ms.](image-url)
and temporary paralysis (30–300 s); during a refractory period lasting \( \leq 1 \) h, flies cannot be re paralyzed by a second mechanical stimulation. Bang-sensitive paralytic behaviors for homozygous and hemizygous BS mutants were similar to that described previously (Ganetzky and Wu 1982; Pavlidis and Tanouye 1995). For \( bss^2 \), 100% of flies paralyzed with a recovery time of 170 s. For \( bas^4 \), 100% of flies paralyzed with a recovery time of 150 s. BS paralysis was less extreme for other BS mutants: \( tko \) (100% of flies paralyzed, recovery time 71 s), \( kdn \) (81% of flies paralyzed, recovery time 45 s), \( bas^4 \) (30% of flies paralyzed, recovery time 23 s), and \( jbug \) (43% of flies paralyzed, recovery time 12 s).

**Electrophysiology**

All flies used for electrophysiology were 3 days after eclosion. For BS paralytic mutant strains, flies were preselected for behavioral paralysis before electrophysiological testing. Preselecting seems to have a large effect on the \( jbug \) strain, with a lower average seizure threshold with less variability than for unselected flies (X. Ren and M. A. Tanouye, unpublished observations). After preselection, flies of the \( kdn \) strain continued to display considerable variation in seizure threshold. Flies were mounted for electrophysiology without anesthesia by capturing and immobilizing them with a vacuum line. Immobilized flies were attached to a pin glued the dorsal thorax with cyanoacrylate adhesive. Two uninsulated tungsten electrodes were inserted into the brain for stimulation. The ground electrode was inserted into the abdomen. Recording electrodes were uninsulated tungsten electrodes inserted into the DLM or TTM muscle identified by their thoracic insertion sites (Tanouye and Wyman 1980). Two types of electrical stimulation were used: single-pulse stimulation and high-frequency (HF) stimulation. Single-pulse stimuli (0.2-ms duration, 0.5 Hz) were delivered to the brain to drive the GF. Evoked DLM and TTM potentials in wild-type and BS flies have the same threshold to single pulse stimulation and characteristic latency relationships as described previously (Kuebler and Tanouye 2000; Pavlidis and Tanouye 1995). During the course of each experiment, the GF was stimulated continuously with single-pulse stimuli to assess GF circuit function. HF stimulation (0.5-ms pulses at 200 Hz for 300 ms) was used to elicit seizures. Seizure-like activity in *Drosophila* is observed as uncontrolled, high-frequency (>100 Hz) neuronal firing, most conveniently examined in motoneurons. Seizures are extensive: >30 motoneurons innervating at least seven different thoracic muscle groups participate in seizures (Kuebler and Tanouye 2000). To determine seizure threshold, HF stimuli were initially given to flies at predicted intensities, depending on their genotypes. If the stimulus fails to elicit a seizure, the intensity was subsequently increased at 1-V increments until a seizure was induced. The threshold was determined for individual fly as the lowest intensity at which seizures occurred. The fly was allowed to rest 15 min after each HF stimulation. The figures in this paper display recordings from DLM indirect flight muscles. All electrophysiology experiments on homoyzgous or hemizygous flies were performed on males. All experiments on heterozygous flies were performed on females. Females are larger than males and generally have slightly higher seizure thresholds (Kuebler et al. 2001). For example, male CS flies have a seizure threshold of 33.83 ± 3.19 V, whereas female CS flies have a threshold of 44.14 ± 4.06 V.

**Drug feeding**

Carbenoxolone (Sigma) was dissolved in 5% sucrose at the concentrations indicated (25, 10, and 5 mM) and fed to flies using the short-term feeding method described previously (Tan et al. 2004). A small filter (Whatman glass microfilter) was saturated with carbenoxolone solution and placed in an empty food vial. Adult flies 2–3 days after eclosion were fed in batches of 10 for 24 h and tested for the BS behavior.

**RESULTS**

**Seizure susceptibility in Drosophila BS mutants and their heterozygotes**

Seizure-like activity in the *Drosophila* nervous system can be elicited by HF electrical stimuli delivered to the brain, with different wild-type and mutant strains exhibiting characteristic seizure susceptibilities (Kuebler and Tanouye 2000; Pavlidis and Tanouye 1995). In this paper, HF stimuli (0.5-ms pulses at 200 Hz for 300 ms) elicited seizures in wild-type CS flies with a threshold of 33.83 ± 3.19 V, similar to the values reported previously (~30 V; Kuebler et al. 2001). Examination of several different BS mutant strains showed different seizure susceptibilities (Table 1; Fig. 2). Two BS mutants, \( bss^2 \) and \( bas^2 \), were highly seizure-sensitive with very low seizure thresholds of 3.66 ± 0.29 and 3.76 ± 0.33 V, respectively (Table 1; Fig. 2). Four BS mutants, \( sda \), \( bas^4 \), \( tko \), and \( jbug \), were moderately seizure-sensitive with low seizure thresholds of 6.72 ± 0.84, 7.59 ± 1.35, 9.86 ± 2.55, and 10.45 ± 2.62, respectively. One BS mutant, \( kdn \), was not especially seizure-sensitive; its seizure threshold was 20.17 ± 7.83 V, approaching the wild-type range. These results are consistent with those presented previously: the BS paralytic behavioral phenotype is highly correlated with seizure sensitivity. The \( bss^2 \) and \( bas^2 \) mutants are similar to other BS mutants that are highly seizure sensitive, \( bss^2 \) and \( eas \) (Kuebler and Tanouye 2000). Interestingly, the two alleles of \( bas \) differ in their effects on seizure threshold: the \( bas^2 \) mutant is highly sensitive to seizure, whereas the \( bas^1 \) mutant is only moderately seizure-sensitive.

Two BS mutants, \( tko \) and \( kdn \), behave as recessive mutants for the seizure phenotype. Seizure thresholds of heterozygous females are in the wild-type range for \( tko^+/+ \) (43.64 ± 2.73 V) and \( kdn^+/+ \) (44.02 ± 2.67 V). Four BS mutants, \( bss^2 \), \( bas^2 \), \( jbug \), and \( bas^4 \), behave as semidominant mutations for the seizure phenotype. Seizure thresholds of heterozygous females

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seizure threshold (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( bss^2 )</td>
<td>3.66 ± 0.29</td>
</tr>
<tr>
<td>( bas^2 )</td>
<td>3.76 ± 0.33</td>
</tr>
<tr>
<td>( sda )</td>
<td>6.72 ± 0.84</td>
</tr>
<tr>
<td>( bas^4 )</td>
<td>7.59 ± 1.35</td>
</tr>
<tr>
<td>( tko )</td>
<td>9.86 ± 2.55</td>
</tr>
<tr>
<td>( jbug )</td>
<td>10.45 ± 2.62</td>
</tr>
<tr>
<td>( kdn )</td>
<td>20.17 ± 7.83</td>
</tr>
<tr>
<td>( bas^2^+ )</td>
<td>14.52 ± 2.07</td>
</tr>
<tr>
<td>( jbug^+ )</td>
<td>20.62 ± 2.74</td>
</tr>
<tr>
<td>( bss^2^+ )</td>
<td>21.93 ± 2.66</td>
</tr>
<tr>
<td>( bas^4^+ )</td>
<td>32.38 ± 3.72</td>
</tr>
<tr>
<td>( tko^+ )</td>
<td>43.64 ± 2.73</td>
</tr>
<tr>
<td>( kdn^+ )</td>
<td>44.02 ± 2.67</td>
</tr>
<tr>
<td>CS</td>
<td>44.14 ± 4.06</td>
</tr>
</tbody>
</table>

The seizure thresholds listed here are means ± SD in volts for the HF stimulus (\( n \geq 10 \) for each genotype). Seizures were elicited in an all-or-nothing manner by short wavetrains of high-frequency (HF) electrical stimuli (0.5-ms pulses at 200 Hz for 300 ms) delivered to the brain. For comparisons with other studies, homoyzgous mutants are all males. However, heterozygotes are all females (required for X chromosomal mutations) along with CS. Note that in genotypes that can be compared, females have been shown to have slightly higher seizure thresholds than males (Kuebler and Tanouye 2000; Kuebler et al. 2001). Compare, for example, the difference in seizure thresholds between CS females (Table 1) and CS males (Table 2).
have values between the homozygous mutant range and the wild-type range for bss°/+ (21.93 ± 2.66 V), bas°/+ (14.52 ± 2.07 V), jbug/+ (20.62 ± 2.74 V), and bas°/+ (32.38 ± 3.72 V). In contrast to the seizure phenotype, all of the BS mutations examined acted as recessives for the behavioral phenotypes of BS paralysis.

**Different extent of seizure suppression by shakB²**

The gap junction blocker carbenoxolone has been shown to decrease seizure activity in active epileptic foci (Szente et al. 2002). Additionally, connexin-36–deficient mice show reduced epileptiform discharges in hippocampal slice preparations (Maier et al. 2002). We tested the *Drosophila* gap junction mutation shakB² for its ability to suppress behavioral and electrophysiological phenotypes in the six BS mutants studied here (sda, bss², bas², tko, kdn, and jbug) by examining appropriate double mutant combinations (Table 2). The shakB² single mutant is resistant to neurological seizures compared with wild-type CS flies. The seizure threshold for shakB² is 80.6 ± 8.71 V. Interestingly, the effectiveness of shakB² as a suppressor mutation is different depending on the BS mutant tested. The shakB² mutation completely suppresses phenotypes in sda, jbug, and kdn. Behavioral testing of shakB²:sda double mutants, shakB²:jbug double mutants, and kdn shakB² double mutants showed a complete lack of BS phenotypes. The addition of sda, jbug, and kdn to a shakB² background raised the physiological seizure threshold to wild-type levels; seizures were triggered at the HF intensity of 31.83 ± 3.24 V in shakB²:sda double mutants, 38.24 ± 3.96 V in shakB²:jbug double mutants, and 41.61 ± 3.63 V in kdn shakB² double mutants. The tko shakB² double mutant displayed a partial suppression because the physiological seizure threshold of 26.83 ± 4.17 V falls between tko and wild-type range (Fig. 2). Behaviorally, the presence of the shakB² mutation suppresses the BS phenotype of >90% tko mutants. In the case of bss² and bas², the presence of the shakB² mutation had a small, but significant, effect on seizure susceptibility. There was slight difference in the physiological seizure thresholds between bss² (3.66 ± 0.29 V) and bss² shakB² double mutants (4.96 ± 0.65 V). These low physiological seizure thresholds are consistent with behavioral phenotypes; both bss² mutants and bss² shakB² double mutants are completely paralyzed by BS mechanical stimulation. There was a small elevation in the seizure thresholds between bas² and bas² shakB² double mutants in bas² shakB² double mutants, the seizure threshold was 5.82 ± 1.37 V, whereas in bas² mutants, the seizure threshold was 3.76 ± 0.33 V (Fig. 2). Despite the small elevation in physiological seizure threshold, the shakB² mutation cannot suppress the behavioral phenotypes of bas² mutants; the double mutants are paralyzed by BS mechanical stimulation.

Taken together, the double-mutant combinations of the shakB² mutation with different BS mutants (include the 3 mutants examined previously: eas, bss¹, and sda) displayed seizure thresholds that span a large range of voltages (Fig. 3).

**TABLE 2. Seizure suppression of various BS mutants by shakB²**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seizure threshold (V)</th>
<th>GF response</th>
</tr>
</thead>
<tbody>
<tr>
<td>bss² shakB²</td>
<td>4.96 ± 0.65</td>
<td>Defective</td>
</tr>
<tr>
<td>bas² shakB²</td>
<td>5.82 ± 1.37</td>
<td>Defective</td>
</tr>
<tr>
<td>tko shakB²</td>
<td>26.83 ± 4.17</td>
<td>Defective</td>
</tr>
<tr>
<td>shakB²; sda</td>
<td>31.83 ± 3.24</td>
<td>Defective</td>
</tr>
<tr>
<td>shakB²; jbug</td>
<td>38.24 ± 3.96</td>
<td>Defective</td>
</tr>
<tr>
<td>kdn shakB²</td>
<td>41.61 ± 3.63</td>
<td>Defective</td>
</tr>
<tr>
<td>w shakB²</td>
<td>80.63 ± 8.71</td>
<td>Defective</td>
</tr>
<tr>
<td>CS</td>
<td>33.83 ± 3.19</td>
<td>Normal</td>
</tr>
</tbody>
</table>

The seizure thresholds listed here are means ± SD in volts for the HF stimulus (n = 10 for each genotype). Seizures were elicited in an all-or-nothing manner by HF electrical stimuli. The flies examined here, including the CS flies, are males that have slightly lower seizure thresholds than females of the same genotype (Kuebler and Tanouye 2000, Kuebler et al. 2001). Single pulse stimuli were used to activate the GF. Normal and defective GF system responses are determined as shown in Fig. 1.
Despite this range, double mutants can be classified into three categories. The first category consists of those with a nearly completely suppressed BS phenotype and includes shakB\(^2\):sda, shakB\(^2\):jbug, and kdn shakB\(^2\) double mutants. These flies have seizure thresholds that are comparable to wild-type. The second category consists of those with a partially suppressed BS seizure-susceptibility phenotype and includes eas shakB\(^2\) and tko shakB\(^2\) double mutants. These flies have seizure thresholds that are above the BS level but below wild-type level. The final category consists of those double mutants that have a seizure threshold close to the parental BS mutants and includes bss\(^2\) shakB\(^2\), bas\(^2\) shakB\(^2\), and bss\(^1\) shakB\(^2\) double mutants.

Reduction of spontaneous seizures in bss\(^2\) shakB\(^2\) and bas\(^2\) shakB\(^2\) double mutants

In bss\(^1\) mutants, mechanical stimulation causes a remarkably long period of paralysis (\(~198\) s) that is characterized by alternating cycles of quiescence and seizure resembling tonic-clonic activity observed in some human epilepsies (Pavlidis and Tanouye 1995; Tan et al. 2004). In this study, two additional mutants display long periods of BS behavioral paralysis: bss\(^2\) and bas\(^2\) mutants are paralyzed for 172 and 150 s, respectively (Fig. 4). Similar to bss\(^1\), the long periods of BS paralysis in bss\(^2\) and bas\(^2\) are characterized by tonic-clonic activity. HF stimulation shows electrophysiological correlates of this tonic-clonic activity in all three mutations bss\(^1\), bss\(^2\), and bas\(^2\). After HF stimulation, there is an initial seizure followed by repeating bouts of synaptic failure and spontaneous seizures. Double mutant combinations with shakB\(^2\) eliminate the tonic-clonic activity associated with bss\(^1\), bss\(^2\) and bas\(^2\). Thus for bss\(^2\) shakB\(^2\) double mutants, 100% of animals show BS paralysis like the bss\(^2\) parental. However, unlike the parental, the double mutant is paralyzed for a shorter time (\(~92\) s; Fig. 4). Also, HF stimulation shows that, although the seizure threshold is similar in the bss\(^2\) single mutant and the bss\(^2\) shakB\(^2\) double mutant, the electrophysiological correlates of tonic-clonic activity are absent in the double mutant. Similar results are seen for the bss\(^1\) shakB\(^2\) double mutant (Kuebler et al. 2001) and the bas\(^2\) shakB\(^2\) double mutant (\(~100\)-s behavioral paralysis) compared with the bss\(^1\) and bas\(^2\) single mutant strains (Fig. 4): shorter periods of behavioral paralysis without significant changes in the number of flies showing BS paralysis and seizure thresholds.

Modification of seizure susceptibility by constitutive expression of shakB\(^+\)

Seizure susceptibility of sda may be modulated by targeted expression of the gap junction protein using a shakB\(^+\) construct driven in the Drosophila nervous system by P (ELAV-GAL4). Observations in vertebrates indicate that treatments enhancing electrical synaptic transmission increase the frequency and severity of seizures (Carlen et al. 2000; Jahromi et al. 2002). We examined the overexpression of shakB\(^+\) in a wild-type background and found that it causes seizure sensitivity. Thus male flies of the genotypes P (ELAV-GAL4)/Y; P (UAS-shakB\(^+\))/+ and P (ELAV-GAL4)/Y; P (UAS-shakB\(^+\))/P (UAS-shakB\(^+\)) have seizure thresholds of 21.1 ± 3.9 and 18.2 ± 3.4 V, respectively. This is not as low as found in BS mutants but is significantly more seizure sensitive than for wild-type CS males. We also examined sda and found that in this mutant, seizure sensitivity is increased even further by shakB\(^+\) overexpression. Thus flies of the genotype P (ELAV-GAL4):P (UAS-shakB\(^+\)); sda have a seizure threshold of 3.6 ± 0.4 V, significantly lower than the sda mutant itself (6.7 ± 0.8 V). These results suggest that overexpression of shakB\(^+\) product in the CNS generally makes flies more seizure-sensitive, possibly by the synchronization of neuronal firing. These results suggest further the possibility that sda and shakB\(^+\) overexpression may be using different mechanisms to increase seizure sensitivity.

Our general findings from experimental and control animals in a sda background are that genotypes with defective gap junctions show seizure thresholds at or around the wild-type range indicating seizure suppression. Genotypes expected to have functional gap junctions show seizure thresholds at or around the mutant sda range. Thus flies of the genotype: P (ELAV-GAL4) shakB\(^2\); P (UAS-shakB\(^+\)); sda showed a seizure threshold of 14.6 ± 4.7 V near the threshold of sda. Our
interpretation is that seizure suppression of the sda phenotype by the shakB2 mutation is modified by the gap junction protein provided by P (UAS-shakB) that has been targeted to the nervous system through P (ELAV-GAL4). Flies of the control genotype P (ELAV-GAL4) shakB2;sda show a seizure threshold of 34.5 ± 3.3 V, near the wild-type threshold. Our interpretation is that shakB2 is suppressing sda seizures because, in this genotype, there is no P (UAS-shakB) product to compensate. Flies of the control genotype shakB2;P (UAS-shakB) show a seizure threshold of 32.1 ± 4.5 V, near the wild-type threshold. Our interpretation is that shakB2 is suppressing sda seizures because, in this genotype, there is no P (ELAV-GAL4) to drive P (UAS-shakB) expression. Other control genotypes have seizure thresholds consistent with these interpretations (Table 3). It is interesting to note that, although targeted expression of shakB+ rescues the seizure suppression phenotype of shakB2, it fails to rescue the neuroconnectivity defects of the GF circuit. That is, all of our genotypes expected to provide the shakB+ product, the GF remained incapable of driving a normal TTM response or a normal DLM response.

Modification of seizure susceptibility by conditional expression of shakB+

Expression of gap junction protein may be made conditional using the TARGET system that activates expression by the temperature shift. Our findings from experimental and control animals in a shakB2 background are interpreted similarly to the results of constitutive expression experiments. That is, we infer that flies with seizure thresholds at or around the shakB2 range indicate defective gap junctions. We infer that flies with seizure thresholds at or around the wild-type range are indicating functional gap junctions. Our general findings are that defective gap junctions are rescued by shakB+ expressed in the embryo or the larva; they are not rescued by expression in the pupa or the adult. Thus flies of the genotype P (ELAV-GAL4) shakB2;P (UAS-shakB)+/+;P (tubP-GAL80ts)/+ show seizure thresholds of 47.7 ± 2.9 and 47.3 ± 2.6 V when temperature shift to 32°C is made during the embryonic and larval stages, respectively (Table 4). This is close to, although slightly lower than, the wild-type range of thresholds. Our interpretation is that temperature-induced expression of shakB+ in the embryo and larva is sufficient to restore some gap junction functions in
The seizure thresholds listed here are means ± SD in volts for the HF stimulus (n = 10 for each genotype). Seizures were elicited in an all-or-nothing manner by HF electrical stimuli. Normal and defective GF system responses are determined as shown in Fig. 1. Compared are responses of various lines contributing to GAL4/UAS expression of shakB+. Control flies that lack P (UAS-shakB+) or P (ELAV-GAL4) are not expected to express shakB+, and thereby fail to rescue seizure threshold suppression. Note that the response of the GF circuit is not rescued by shakB+ in any construct.

The adult fly as inferred by rescue of seizure threshold. In contrast, flies of the same genotype show seizure thresholds of 73.6 ± 3.3 and 76.8 ± 4.1 V, near the shakB+ threshold, when temperature shift to 32°C is made during pupal and adult stages, respectively (Table 4). Our interpretation is that temperature-induced expression of shakB+ at late stages of development is unable to restore gap junction functions in the adult fly as inferred by failure to rescue the seizure threshold. Although conditional expression of shakB+ rescues the seizure suppression phenotype of shakB−, it fails to rescue the neuroconnectivity defects of the GF circuit as was seen in the constitutive expression analysis.

**Carbenoxolone reduces recovery period in bss1 mutants**

We examined the effects of the gap junction blocker in three BS mutants: eas, sda, and bss1. These mutants were fed various concentrations of the drug over a 24-h time period, and their bang-sensitivity was examined with the vortex assay. In our studies, we did not see the elimination of the BS phenotype in any of our mutants treated with carbenoxolone. However, we did observe a reduction in the paralytic recovery period of bss1, the most severe BS mutant. Treatment with 25 mM carbenoxolone shortened the recovery period to 147 ± 48 s (n = 100) compared with the untreated (206 ± 46 s; n = 100) recovery period. The recovery period here was calculated by measuring the time from the beginning of the vortex until the flies resumed an upright standing position. These observations are similar to those observed previously with KBr (Tan et al. 2004). KBr (0.25%) decreased the recovery time of bss1 flies from 198 to 101 s. Also, KBr did not eliminate the BS phenotype of bss1, eas, sda, and tko mutants. In these experiments, lower concentrations of carbenoxolone (5 and 10 mM) did not affect any of the mutants examined.

**DISCUSSION**

We have examined mutations responsible for seizure sensitivity in *Drosophila* such that a general picture is beginning to emerge around different characteristics. These characteristics are seen in the seizure threshold, but magnified and more apparent when factoring in the nature of mutation (e.g., dominant, recessive), presence of tonic-clonic activity, and sensitivity to seizure-suppressor mutations. The most severe mutations, bss1, bss2, and bas2 (but not bas1), have low seizure thresholds (<4 V) and 100% behavioral paralysis that are not much ameliorated in heterozygotes or by shakB+ seizure suppression. These mutants display conspicuous tonic-clonic activity that is eliminated by shakB+ seizure suppression and also apparently by some pharmacological agents (Reynolds et al. 2004; Tan et al. 2004).

Two mutants are less extreme: eas (Pavlidis et al. 1994) and tko. For these, 100% of animals showed a behavioral paralytic phenotype. They have seizure thresholds of ~4–10 V. These mutations act as simple recessives: heterozygotes have no BS paralytic phenotypes; seizure thresholds are in the wild-type range. Mutant phenotypes are slightly improved in double mutant combinations with the shakB+ seizure-suppressor, but not to wild-type levels. The weakest seizure-sensitive mutants

**Table 3. Seizure thresholds and GF responses during constitutive expression of shakB+**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seizure threshold (V)</th>
<th>GF response</th>
</tr>
</thead>
<tbody>
<tr>
<td>P (ELAV-GAL4); P (UAS-shakB+)−; sda</td>
<td>3.6 ± 0.4</td>
<td>Normal</td>
</tr>
<tr>
<td>w; sda</td>
<td>6.7 ± 0.8</td>
<td>Normal</td>
</tr>
<tr>
<td>w; P (UAS-shakB+)−; sda</td>
<td>7.3 ± 1.9</td>
<td>Normal</td>
</tr>
<tr>
<td>P (ELAV-GAL4); P (UAS-shakB+)−; sda/+</td>
<td>12.5 ± 3.2</td>
<td>Normal</td>
</tr>
<tr>
<td>P (ELAV-GAL4); P (UAS-shakB+)−; sda</td>
<td>14.6 ± 4.7</td>
<td>Defective</td>
</tr>
<tr>
<td>P (ELAV-GAL4); P (UAS-shakB+)−</td>
<td>18.2 ± 3.4</td>
<td>Normal</td>
</tr>
<tr>
<td>P (ELAV-GAL4); P (UAS-shakB+)−/+</td>
<td>21.1 ± 3.9</td>
<td>Normal</td>
</tr>
</tbody>
</table>

**Table 4. Seizure thresholds and GF responses during conditional expression of shakB+**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seizure Thresholds of Embryonic Expression, 32°C</th>
<th>Seizure Thresholds of Larval Expression, 32°C</th>
<th>Seizure Thresholds of Pupal Expression, 32°C</th>
<th>Seizure Thresholds of Adult Expression, 32°C</th>
<th>GF Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>w; P(tubP-GAL80)</td>
<td>33.6 ± 1.8</td>
<td>34.3 ± 2.1</td>
<td>32.5 ± 2.3</td>
<td>34.2 ± 1.6</td>
<td>Normal</td>
</tr>
<tr>
<td>P(ELAV-GAL4)</td>
<td>37.8 ± 3.2</td>
<td>36.3 ± 2.8</td>
<td>37.4 ± 3.6</td>
<td>38.5 ± 2.4</td>
<td>Normal</td>
</tr>
<tr>
<td>P(UAS-shakB+)−</td>
<td>39.3 ± 2.9</td>
<td>40.1 ± 3.3</td>
<td>41.3 ± 3.7</td>
<td>39.8 ± 3.1</td>
<td>Normal</td>
</tr>
<tr>
<td>P(ELAV-GAL4) shakB+; P(UAS-shakB+)−/+</td>
<td>47.7 ± 2.9</td>
<td>47.3 ± 2.6</td>
<td>73.6 ± 3.3</td>
<td>76.8 ± 4.1</td>
<td>Defective</td>
</tr>
</tbody>
</table>

The seizure thresholds listed here are means ± SD in volts for the HF stimulus (n = 10 for each genotype). In the TARGET system, the combination of GAL4-UAS and GAL80ts (temperature sensitive) provides temporal control over the transcripational activity of GAL4. Expression can be induced at any time during development or in the adult. The analysis depicted was performed under two temperatures: 18°C (nonexpressing) and 32°C (expressing). Fliers were shifted between the two temperatures to achieve expression during the desired stage. Electrophysiology was performed on adult flies 2-3d old. Seizures were elicited in an all-or-nothing manner by HF electrical stimuli. Compared are responses of various lines contributing to GAL4/UAS expression of shakB+ by the TARGET conditional expression system as controls. In the flies with the genotype P (ELAV-GAL4) shakB+; P(UAS-shakB+)−/+; P(tubP-GAL80)−, expression of shakB+ at the embryonic and larval stages rescues the seizure resistance phenotype of shakB+. Expression of shakB+ at the pupal or adult stage fails to rescue the seizure resistance phenotype of shakB−. Normal and defective GF system responses are determined as shown in Fig. 1. Note that the defective response of the GF circuit is not rescued by shakB+.
are bas1, jbug, and kdn. For these, a variable fraction of animals show a behavioral paralytic phenotype. They have variable seizure thresholds of ~10 V or higher. BS paralytic behavioral phenotypes and seizure thresholds are made substantially more severe in double mutant combinations with seizure enhancer mutations (D. Hekmat-Scafe and M. A. Tanouye, unpublished observations). Mutant phenotypes are completely repressive and are also completely suppressed to wild-type levels in double mutant combinations with the shakB+ seizure suppressor.

The sda mutation has some features that are unusual in our collection. For sda, 100% of animals show a behavioral paralytic phenotype. It has a seizure threshold about 7 V. This mutation acts as a semidominant: ~2% of heterozygotes have a BS paralytic phenotype; their seizure thresholds are ~30 V (female flies were used here), which is not quite to the wild-type level. For heterozygotes, BS paralytic behavioral phenotypes and seizure thresholds are substantially more severe in double mutant combinations with seizure enhancer mutations (Zhang et al. 2002). The sda phenotypes are completely suppressed to wild-type levels in double mutant combinations with the shakB+ seizure suppressor.

Synapses in shakB mutant have been shown to be impaired in electrical transmission (Baird et al. 1990; Phelan et al. 1996; Sun and Wyman 1996; Thomas and Wyman 1984). Thus shakB may act to suppress seizures by a mechanism similar to that proposed for drugs such as carbenoxolone that block gap junction activity (Szente et al. 2002). Impaired transmission at electrical synapses could impair synchronous activation of neuronal populations leading to decreased seizure susceptibility. Observations presented here are generally consistent with such a mechanism. To test this, we expressed UAS-shakB+ with a constitutive ELAV-GAL4 driver and with a conditional driver to determine if shakB suppression of sda could be interrupted. The resulting flies, expected to contain functional gap junctions, regained seizure sensitivity (i.e., lost seizure suppression). These results are supportive of an electrical synaptic failure mechanism for shakB+ seizure suppression. Furthermore, initial findings indicate that this is a developmental effect: shakB+ must be expressed in the embryo or in the larva to have effects on seizure susceptibility; adult expression of shakB+ is apparently not sufficient for this. Future experiments will provide additional information about the developmental mechanisms that are being affected by shakB+ expression. One concern is that we have not shown that electrical synaptic transmission is rescued by UAS-shakB+. Indeed, there are at least some electrical synapses, those of the GF system, that clearly are not rescued. These GF system synapses are apparently not responsible for seizure suppression; however, the relevant connections remain unidentified from this analysis.

Some features of shakB+ seizure suppression remain perplexing (e.g., shakB+ affects on class I mutants). In these mutants, tonic-clonic activity is suppressed by shakB+, indicating a contribution from electrical transmission for this portion of the seizure phenotype. Surprisingly, however, shakB does not seem to affect seizure thresholds. This could suggest that electrical transmission does not play a role in overall seizure-sensitivity for class I mutants as it apparently does in all other genotypes. Thus in a formal genetic sense, bas and bss gene products would seem to act downstream of gap junctions, whereas the products of other BS genes would appear to act upstream of gap junctions. This does not seem reasonable and we suggest instead that contributions to seizure-genesis in bas and bss mutants are going to prove considerable more complex than for other genotypes. At present, we do not have a good handle on what these complications are, especially because we have not determined the molecular identity of the bas and bss products.

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SEIZURE SUPPRESSION BY THE GAP JUNCTION MUTATION


