Seizure Suppression by *shakB*², a Gap Junction Mutation in *Drosophila*

Juan Song¹ and Mark A. Tanouye¹,²

¹Department of Environmental Science, Policy and Management, Division of Insect Biology and ²Department of Molecular and Cell Biology, Division of Neurobiology, University of California, Berkeley, California

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First published September 28, 2005; doi:10.1152/jn.01059.2004. Gap junction proteins mediate electrical synaptic transmission. In *Drosophila*, flies carrying null mutations in the *shakB* locus, such as *shakB*², have behavioral and electrophysiological defects in the giant fiber (GF) system neurocircuit consistent with a loss of transmission at electrical synapses. The *shakB*² mutation also affects seizure susceptibility. Mutant flies are especially seizure-resistant and have a high threshold to evoked seizures. In addition, in some double mutant combinations with “epilepsy” mutations, *shakB*² appears to act as a seizure-suppressor mutation: *shakB*² restores seizure susceptibility to the wild-type range in the double mutant. In double mutant combinations, *shakB*² completely suppresses seizures caused by *slamdance (sda)*, *knockdown (kdn)*, and *jitterbug (jbug)* mutations. Seizures caused by easily shocked (eas) and technical knockout (tko) mutations are partially suppressed by *shakB*². Seizures caused by *bang-sensitive (bas)* and *bang-senseless (bsl)* alleles are not suppressed by *shakB*². These results show the use of *Drosophila* as a model system for studying the kinds of genetic interactions responsible for seizure susceptibility, bringing us closer to unraveling the complexity of seizure disorders in humans.

**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 NH₄Cl (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 *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 *Drosophila*, in particular, mutations of the innexin family member *shakB*. The *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 “*shakB (neural)*” are all essential for viability; loss-of-function mutants do not survive past the first larval stage. In contrast, transcripts called “*shakB (neural)*” are nonessential; loss-of-function mutants 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 *shakB (neural)* allele used here, *shakB*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 the *shakB*² mutation can affect seizure susceptibility in a *Drosophila* model of epilepsy (Kuebler et al. 2000, 2001). Flies carrying the *shakB*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, *shakB*² may be a seizure-suppressor mutation. This paper provides a detailed examination of *shakB*² as a seizure-suppressor mutation by testing its ability to suppress a large and diverse collection of *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 *shakB*² with various BS mutants. The data presented here provide insights into
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* (technical knockout) gene is located at 1–0.99 and encodes a mitochondrial 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 (Genetzkzy and Wu 1982); two alleles *bss*¹ and *bss*² were examined. The *bas* (bang sensitive) gene is located at 1–50.7 (Genetzkzy and Wu 1982; Lee and Wu 2002); the *bas*¹ and *bas*² 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 *shakB*² allele is a null mutation that acts as a seizure-suppressor mutation (Kuebler et al. 2001). The *shakB*² 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 tangrochanter muscle motoneuron (TTMmn) and the GF and the peripherally synapsing interneuron (PSI). The *shakB*² mutant has a behavioral defect: lack of escape jump to a light-off stimulus. In addition, the *shakB*² 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 mutants with the *shakB*² suppressor mutation in combination with each of the BS mutations. The following methods were used to verify the presence of the *shakB*² suppressor mutation and the BS mutation in the double mutant. A defective GF system electrophysiological phenotype was used to determine the presence of the *shakB*² 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) *shakB*² 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*¹ *shakB*²/*bss*² *shakB*², backcrossing yields flies of the genotype *bss*¹ *shakB*²/*bss*² *shakB*². These latter flies show a restoration of normal GF system electrophysiology—a recessive *shakB*² phenotype. The flies also show the BS behavioral and electrophysiological phenotypes of *bss*¹, suggesting that *shakB*² 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*¹ *shakB*²/*bss*² *shakB*², *bas*¹ *shakB*²/*bas*² *shakB*², *tko* *shakB*²/*tko* *shakB*², *kdn* *shakB*²/*kdn* *shakB*², and *shakB*²/*shakB*²:jbug/jbug.

**Transgenic fly lines.** Rescue of *shakB*² was conducted by expression of *shakB*² using the GAL4-UAS system as described previously (Curtin et al. 2002; Osterwalder et al. 2001). For constitutive expression of *shakB*², we examined flies of the genotypes: *P (ELAV-GAL4) shakB*²; *P (UAS-shakB*²) and *P (ELAV-GAL4) shakB*²; *P (UAS-shakB*²); *sda*. These flies were compared with various nonexpressing controls as described in the text. For conditional expression of *shakB*², flies of the genotype *shakB*²/*shakB*²; *P (ELAV-GAL4); *P (UAS-shakB*²) and *P (UAS-shakB*²) were examined. *P (UAS-shakB*²) 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) shakB*² first chromosome whereby *P (ELAV-GAL4) drives UAS expression in a nervous system lacking electrical synapses because of the *shakB*² background. A third chromosome *P (tubP-GAL80ts)* construct was obtained from the Bloomington *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-shakB*² 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 tangrochanter 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 *shakB*² 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.
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, bss² and bas², 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, bas¹, 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 bss² and bas² mutants are similar to other BS mutants that are highly seizure sensitive, bss¹ and eas (Kuebler and Tanouye 2000). Interestingly, the two alleles of bas differ in their effects on seizure threshold: the bas² mutant is highly sensitive to seizure, whereas the bas¹ 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, bss², bas², jbug, and bas¹, 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>bss²</td>
<td>3.66 ± 0.29</td>
</tr>
<tr>
<td>bas²</td>
<td>3.76 ± 0.33</td>
</tr>
<tr>
<td>sda</td>
<td>6.72 ± 0.84</td>
</tr>
<tr>
<td>bas¹</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>bas¹/+</td>
<td>14.52 ± 2.07</td>
</tr>
<tr>
<td>jbug/+</td>
<td>20.62 ± 2.74</td>
</tr>
<tr>
<td>bss²/+</td>
<td>21.93 ± 2.66</td>
</tr>
<tr>
<td>bas¹/+</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 ≥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, homoygous 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).
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).

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**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\textsuperscript{2};sda, shakB\textsuperscript{2};jbug, and kdn shakB\textsuperscript{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\textsuperscript{2} and tko shakB\textsuperscript{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\textsuperscript{1}shakB\textsuperscript{2}, bas\textsuperscript{2}shakB\textsuperscript{2}, and bss\textsuperscript{1}shakB\textsuperscript{2} double mutants.

Reduction of spontaneous seizures in bss\textsuperscript{2}shakB\textsuperscript{2} and bas\textsuperscript{2}shakB\textsuperscript{2} double mutants

In bss\textsuperscript{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\textsuperscript{2} and bas\textsuperscript{2} mutants are paralyzed for 172 and 150 s, respectively (Fig. 4). Similar to bss\textsuperscript{1}, the long periods of BS paralysis in bss\textsuperscript{2} and bas\textsuperscript{2} are characterized by tonic-clonic activity. HF stimulation shows electrophysiological correlates of this tonic-clonic activity in all three mutations bss\textsuperscript{1}, bss\textsuperscript{2}, and bas\textsuperscript{2}. After HF stimulation, there is an initial seizure followed by repeating bouts of synaptic failure and spontaneous seizures. Double mutant combinations with shakB\textsuperscript{2} eliminate the tonic-clonic activity associated with bss\textsuperscript{1}, bss\textsuperscript{2}, and bas\textsuperscript{2}. Thus for bss\textsuperscript{2}shakB\textsuperscript{2} double mutants, 100% of animals show BS paralysis like the bss\textsuperscript{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\textsuperscript{2} single mutant and the bss\textsuperscript{2}shakB\textsuperscript{2} double mutant, the electrophysiological correlates of tonic-clonic activity are absent in the double mutant. Similar results are seen for the bss\textsuperscript{1}shakB\textsuperscript{2} double mutant (Kuebler et al. 2001) and the bas\textsuperscript{2}shakB\textsuperscript{2} double mutant (~100-s behavioral paralysis) compared with the bss\textsuperscript{1} and bas\textsuperscript{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\textsuperscript{2}

Seizure susceptibility of sda may be modulated by targeted expression of the gap junction protein using a shakB\textsuperscript{2} 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\textsuperscript{2} 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\textsuperscript{2})/+ and P (ELAV-GAL4);Y;P (UAS-shakB\textsuperscript{2})/P (UAS-shakB\textsuperscript{2}) 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\textsuperscript{2} overexpression. Thus flies of the genotype P (ELAV-GAL4);P (UAS-shakB\textsuperscript{2})/sdax have a seizure threshold of 3.6 ± 0.4 V, significantly lower that the sda mutant itself (6.7 ± 0.8 V). These results suggest that overexpression of shakB\textsuperscript{2} 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\textsuperscript{2} 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\textsuperscript{2};P (UAS-shakB\textsuperscript{2})/sdax 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 \( P(\text{UAS-shakB}/\text{H11001}) \) that has been targeted to the nervous system through \( P(\text{ELAV-GAL4}) \). Flies of the control genotype \( P(\text{ELAV-GAL4}) \text{shakB2};\text{sda} \) show a seizure threshold of 34.5 ± 3.3 V, near the wild-type threshold. Our interpretation is that \text{shakB2} is suppressing \text{sda} seizures because, in this genotype, there is no \( P(\text{UAS-shakB}/\text{H11001}) \) product to compensate. Flies of the control genotype \( \text{shakB2};P(\text{UAS-shakB}/\text{H11001})/\text{H11001};P(\text{tubP-GAL80ts})/\text{H11001} \) 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 higher than, the wild-type range of thresholds. Our interpretation is that temperature-induced expression of \text{shakB⁺} in the embryo and larva is sufficient to restore some gap junction functions.

Modification of seizure susceptibility by conditional expression of \text{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 \text{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 \text{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 \text{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 \( P(\text{ELAV-GAL4}) \text{shakB2};P(\text{UAS-shakB⁺})+/++;P(\text{tubP-GAL80ts})+/++ \) 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 higher than, the wild-type range of thresholds. Our interpretation is that temperature-induced expression of \text{shakB⁺} in the embryo and larva is sufficient to restore some gap junction functions in
in an all-or-nothing manner by HF electrical stimuli. Compared are responses of various lines contributing to GAL4-UAS and GAL80ts (temperature sensitive) provides temporal control over the transcriptional activity of GAL4. Expression can be induced at any time.

**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>
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<td>P (ELAV-GAL4); P (UAS-shakB); sda/+</td>
<td>12.5 ± 3.2</td>
<td>Normal</td>
</tr>
<tr>
<td>P (ELAV-GAL4) shakB; P (UAS-shakB)</td>
<td>14.6 ± 4.7</td>
<td>Defective</td>
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<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) j/+</td>
<td>21.1 ± 3.9</td>
<td>Normal</td>
</tr>
<tr>
<td>shakB2; sda</td>
<td>31.8 ± 3.2</td>
<td>Defective</td>
</tr>
<tr>
<td>shakB2; P (UAS-shakB); sda</td>
<td>32.1 ± 4.5</td>
<td>Defective</td>
</tr>
<tr>
<td>P (ELAV-GAL4) shakB2; sda</td>
<td>34.5 ± 3.3</td>
<td>Defective</td>
</tr>
<tr>
<td>w; P (UAS-shakB)</td>
<td>40.4 ± 3.8</td>
<td>Normal</td>
</tr>
<tr>
<td>P (ELAV-GAL4) shakB2; P (UAS-shakB)</td>
<td>42.7 ± 3.2</td>
<td>Defective</td>
</tr>
<tr>
<td>P (ELAV-GAL4) shakB2</td>
<td>78.5 ± 6.7</td>
<td>Defective</td>
</tr>
<tr>
<td>shakB2; P (UAS-shakB)</td>
<td>79.8 ± 8.2</td>
<td>Defective</td>
</tr>
<tr>
<td>w shakB</td>
<td>80.6 ± 8.7</td>
<td>Defective</td>
</tr>
</tbody>
</table>

The seizure thresholds listed here are means ± SD in volts for the HF stimulus (a ≥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 shakB2 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.

**D I S C U S S I O N**

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 shakB2 seizure suppression. These mutants display conspicuous tonic-clonic activity that is eliminated by shakB2 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 shakB2 seizure-suppressor, but not to wild-type levels. The weakest seizure-sensitive mutants

**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-shakB2)</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) shakB2; P(UAS-shakB2) j/+;</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>
<tr>
<td>P(tubP-GAL80)j/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
are bas', 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 recessive and are also completely suppressed to wild-type levels in double mutant combinations with the shakB2 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 shakB2 seizure suppressor.

Synapses in shakB2 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 shakB2 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-shakB2 with a constitutive ELAV-GAL4 driver and with a conditional driver to determine if shakB2 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 shakB2 seizure suppression. Furthermore, initial findings indicate that this is a developmental effect: shakB2 must be expressed in the embryo or in the larva to have effects on seizure susceptibility; adult expression of shakB2 is apparently not sufficient for this. Future experiments will provide additional information about the developmental mechanisms that are being affected by shakB2 expression. One concern is that we have not shown that electrical synaptic transmission is rescued by UAS-shakB2. 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 shakB2 seizure suppression remain perplexing (e.g., shakB2 affects on class I mutants). In these mutants, tonic-clonic activity is suppressed by shakB2', indicating a contribution from electrical transmission for this portion of the seizure phenotype. Surprisingly, however, shakB2 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 bss and bas products.

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