Neurobiology of Disease

Mutations in the K⁺/Cl⁻ Cotransporter Gene kazachoc (kcc) Increase Seizure Susceptibility in Drosophila

Daria S. Hekmat-Scafe,¹,² Miriam Y. Lundy,¹ Rakhee Ranga,¹ and Mark A. Tanouye¹,²
¹Department of Environmental Science, Policy and Management, Division of Insect Biology, ²Department of Molecular and Cell Biology, Division of Neurobiology, and ³Division of Genetics and Development, University of California, Berkeley, California 94720

During a critical period in the developing mammalian brain, there is a major switch in the nature of GABAergic transmission from depolarizing and excitatory, the pattern of the neonatal brain, to hyperpolarizing and inhibitory, the pattern of the mature brain. This switch is believed to play a major role in determining neuronal connectivity via activity-dependent mechanisms. The GABAergic developmental switch may also be particularly vulnerable to dysfunction leading to seizure disorders. The developmental GABA switch is mediated primarily by KCC2, a neuronal K⁺/Cl⁻ cotransporter that determines the intracellular concentration of Cl⁻ and, hence, the reversal potential for GABA. Here, we report that kazachoc (kcc) mutations that reduce the level of the sole K⁺/Cl⁻ cotransporter in the fruitfly Drosophila melanogaster render flies susceptible to epileptic-like seizures. Drosophila kcc protein is widely expressed in brain neuropil, and its level rises with developmental age. Young kcc mutant flies with low kcc levels display behavioral seizures and demonstrate a reduced threshold for seizures induced by electroconvulsive shock. The kcc mutation enhances a series of other Drosophila epilepsy mutations indicating functional interactions leading to seizure disorder. Both genetic and pharmacological experiments suggest that the increased seizure susceptibility of kcc flies occurs via excitatory GABAergic signaling. The kcc mutants provide an excellent model system in which to investigate how modulation of GABAergic signaling influences neuronal excitability and epileptogenesis.

Key words: seizure; epilepsy; K⁺/Cl⁻ cotransporter; GABA_A receptor; genetics; Drosophila

Introduction

Synaptic inhibition in Drosophila, as in mammals, is mediated primarily by the neurotransmitter GABA (Mody et al., 1994; Hosie et al., 1997). GABAergic neurons are found throughout the CNS of Drosophila adults and at all stages of development (Buchner et al., 1988; Jackson et al., 1990; Harrison et al., 1996). Inhibitory GABAergic signaling occurs primarily via ionotropic GABA_A receptors encoded by the Resistance to dieldrin (Rdl) gene (Hosie et al., 1997; Lee et al., 2003). The homomeric Rdl GABA_A channel is the target of several commercially important insecticides; a single Rdl point mutation is responsible for nearly all known cases of resistance to the series of insecticides that target GABA_A receptors (Buckingham et al., 2005). As with other GABA_A receptors, binding of GABA to Rdl leads to the opening of an internal channel that conducts primarily Cl⁻ ions (Buckingham et al., 2005). Consequently, synaptic inhibition is contingent on the electrochemical gradient for Cl⁻ of the GABAergic neuron, which determines the GABAergic reversal potential of the neuron (E_GABA, the voltage at which GABAergic currents change their direction).

Recent investigations of GABAergic inhibition in the mammalian CNS have focused considerable interest on the interplay between the GABA_A receptor and a K⁺/Cl⁻ cotransporter termed KCC2. KCC2 is the neuronal member of a family of four vertebrate KCCs, all of which concomitantly extrude K⁺ and Cl⁻ from the cell (Mount et al., 1998; Hebert et al., 2004). Because the level of KCC2 affects E_GABA, expression of KCC2 greatly influences signaling mediated by GABA_A receptors (Miles, 1999; Lee et al., 2005; Zhu et al., 2005). In normal adult cortical neurons, KCC2 activity produces low intracellular Cl⁻ levels (Rivera et al., 1999; Stein et al., 2004). GABA-mediated opening of GABA_A Cl⁻ channels thus produces Cl⁻ efflux, resulting in hyperpolarization that, in turn, reduces the ability of the neuron to fire action potentials (Qian and Sejnowski, 1990; Staley and Mody, 1992; Mitchell and Silver, 2003). In immature neurons or under certain pathophysiological conditions, reduced expression of the KCC2 GABA switch results in an elevated intracellular Cl⁻ concentration (Katchman et al., 1999; van den Pol et al., 1996; Ben-Ari, 2002; Nabekura et al., 2002; Payne et al., 2003). GABA-mediated activation of GABA_A receptors can then lead to depolarizing outward Cl⁻ currents and, in some instances, produce synaptic ex-
Materials and Methods

Fly stocks. A list of *Drosophila* stocks used in this study is given in Table 1. Stocks were maintained on standard cornmeal–molasses medium in vials on an upper shelf at room temperature (25°C). Crosses were performed at 22°C if progeny were to be tested for bang sensitivity (unless otherwise specified); all other crosses were performed at 25°C. Three bang-sensitive (BS) mutations are included: *eas*, *bus*, and *sda*. The *eas* gene is located at cytological region 14B and encodes an ethanolamine kinase (Pavlidis et al., 1994). The recessive *eas* 

<table>
<thead>
<tr>
<th>Stock no.</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-5</td>
<td>Wild type</td>
</tr>
<tr>
<td>D505</td>
<td><em>w</em>1118, <em>kc</em>Δ2071/SMS Cy; <em>P(w+) bsd</em> / <em>LacZCG9925</em></td>
</tr>
<tr>
<td>D506</td>
<td><em>w</em>1118, <em>kc</em>Δ2071/YO</td>
</tr>
<tr>
<td>U036</td>
<td><em>w</em>; <em>SMS Cy; TM3; apXa</em></td>
</tr>
<tr>
<td>D225</td>
<td><em>w</em>; <em>TM3/TM6B</em></td>
</tr>
<tr>
<td>D245</td>
<td><em>w</em>1118; <em>ub</em> b scor16; <em>lt</em> stw1/YO</td>
</tr>
<tr>
<td>Z1A</td>
<td><em>y ac w; P(YES;YBB1688 = <em>y</em>1 CG9925</em> / *)</td>
</tr>
<tr>
<td>D572</td>
<td><em>w</em>; *kc<strong>Δ2071</strong>/KO; <em>sdai</em>Δ9/YTM68</td>
</tr>
<tr>
<td>MR047</td>
<td>w*; cso*</td>
</tr>
<tr>
<td>MR068</td>
<td>b 3/f</td>
</tr>
<tr>
<td>D547</td>
<td><em>w</em>; <em>SMS Cy; sdai</em>Δ9.2/Yap</td>
</tr>
<tr>
<td>D548</td>
<td><em>w</em>; <em>sdai</em>Δ9.2/Yap; +/KO</td>
</tr>
<tr>
<td>D508</td>
<td><em>w</em> 3/b; <em>CyO; +/Yap</em></td>
</tr>
<tr>
<td>D250</td>
<td>alp b pr csp sp</td>
</tr>
<tr>
<td>D253</td>
<td><em>w</em>; <em>CyO; Df Δ2-3/TM3</em></td>
</tr>
<tr>
<td>D254</td>
<td><em>y; cv bw sp</em></td>
</tr>
<tr>
<td>D206</td>
<td><em>dmp</em>Δ15/b; a[1260A1-10]SM6a Cy</td>
</tr>
<tr>
<td>5207</td>
<td><em>dmp</em>Δ15/b; a[1260A1-10]/G56a Cy</td>
</tr>
<tr>
<td>D562</td>
<td><em>dmp</em>Δ15/b; a[1260A1-10]CG9925</td>
</tr>
<tr>
<td>S242</td>
<td><em>Df(2R)106</em> / <em>SM5 Cy</em></td>
</tr>
<tr>
<td>16887</td>
<td><em>y</em>1 w*Δ23/Y; <em>P(w+) w</em>1118</td>
</tr>
<tr>
<td>EP2164</td>
<td><em>w</em>; *P(w+) = <em>EP(Y2)CG9925</em></td>
</tr>
<tr>
<td>13216</td>
<td><em>y</em>1 w*Δ23/Y; *P(w+) = <em>SUPor-P</em></td>
</tr>
<tr>
<td>D577</td>
<td><em>w</em>; *kc<strong>Δ2071</strong>/KO</td>
</tr>
<tr>
<td>D569</td>
<td><em>w</em>; <em>sdai</em>Δ9/YTM68</td>
</tr>
<tr>
<td>D582</td>
<td><em>w</em>; <em>sdai</em>Δ9/YTM68</td>
</tr>
</tbody>
</table>

Table 1. *Drosophila* stocks
stock were then crossed to D245 virgin females. Finally, male and virgin female progeny of the genotype w1118, kccDHS1/CyO were crossed to create our D506 balanced kccDHS1 stock.

Recombination mapping of kcc. In an initial recombination mapping experiment, virgin females from a multiply marked second chromosome mapping strain (D250) were crossed to D506 males. Groups of two (+/w1118, at dp 3 b pr c px sP/kccDHS1) virgin female progeny were then crossed to D250 males. Recombinant male progeny were then individually crossed to D506 and the progeny scored for bang sensitivity. These experiments revealed that kcc is very near sp [at 107 map units (m.u.)].

We then performed three-point mapping experiments to further refine the position of the kcc gene. In these experiments, D506 virgin females were crossed to D254 males. The resultant kccDHS1/CyO bw sp female progeny were then crossed in groups of three to D506 males, and the male progeny were tested for bang sensitivity. The 115 bang-sensitive (kccDHS1/kccDHS1) male progeny were then individually crossed to D254 virgin females, and the progeny were scored for the bw and sm markers. Five recombinants (three bw kcc and two kcc sp) were identified, indicating that the gene order is as follows: bw kcc sp.

Creation of a new kcc allele by imprecise P-element excision. The kcc(ML1) allele was produced by imprecise excision of a whiteP-element (SUPOR-P) located 5 bp downstream of the kcc 3′ untranslated region (UTR) as follows: first, no. 13216 virgin females were crossed to D253 males. Next, the resulting P(w1118;+/CyO); D2–23/+ male progeny were crossed to U036 virgin females, and the male progeny were screened for those with white eyes, which presumably had undergone excision of the P(w1118;+) element. From ~2400 male progeny examined, we obtained 41 independently derived white-eyed males. Each of these white-eyed males was then separately crossed to D562 virgin females at 23°C, and both the Cy and non-Cy progeny were screened for bang sensitivity. Six of these crosses yielded some bang-sensitive non-Cy (but not Cy) progeny, and another produced few non-Cy progeny. Balanced stocks for each of the seven lines were created as follows: First, the seven original white-eyed males of interest were individually crossed to D245 virgin females. CyO male and virgin female progeny were then crossed to create the balanced stock. A fraction of the homozygous flies from four of the resultant lines were bang sensitive (8B, 8%; 19A, 1%; 21A, 2%; 23A, 2%); another no homozygous flies, indicating the presence of a lethal mutation on the second chromosome.

Other genetic analysis. In our deficiency mapping experiments, D506 virgin females were crossed to balanced Df(2R) males and at least 30 of the nonbalanced kccDHS1/Df(2R) progeny were tested for bang-sensitive paralysis. We concluded that a deficiency uncovered kcc if a significant fraction of the kccDHS1/Df(2R) progeny were bang sensitive. In our duplication mapping experiment, D506 virgin females were first crossed to y w1118, 13, where the 13 bp insertion is located. The thermocycling program used was as follows: 50°C for 30 min; followed by 94°C for 2 min; and then one cycle of 72°C for 7 min. AmpliTaq DNA polymerase (Roche, Basel, Switzerland) was used according to the manufacturer's instructions. Twelve primer pairs, which lead to the production of 12 overlapping ~600 bp products, were used to amplify almost the entire kcc gene from homozygous kccDHS1/kccDHS1 D506 flies. (The ~1 kbp intronic regions flanking each of the alternative exons were not examined.) Two primers, CG14-5 (5′-CAATTTGTAACCCATATTGAGC) and Pwht1 (5′-GAACGCCTCACTCCGACAGTCACA), were used to amplify the kcc gene/P-element junction in homozygous kccDHS1/kccDHS1 D577 flies. CG14-5 is in an alternative exon ~1 kbp from the 3′ end of kcc, whereas Pwht1 occurs in SUPOR-P ~10 kb downstream of the kcc gene. As anticipated, no CG14-5/Pwht1 PCR product was obtained using DNA from the original no. 13216 stock. However, a 1.5 kb CG14-5/Pwht1 PCR product was obtained using D577 DNA, indicating an imprecise excision that removed the 3′/5′ portion of the P-element (the end proximal to the kcc gene). All PCR products were purified using the QiAquick PCR Purification kit (Qiagen, Valencia, CA) and sequenced at the University of California, Berkeley, DNA Sequencing Facility using the corresponding 5′ and 3′ PCR primers.

RT-PCR analysis. Homozygous kccDHS1 D506 and CS-5 control flies were allowed to ingest either the control or picrotoxin-containing sucrose for ~24 h after eclosion. Fly heads were then collected in vials containing three 2.4 cm Whatman GF/A filters saturated with 600 μl of 5% sucrose containing green food coloring with or without 1 μm picrotoxin (PTX) (Sigma, St. Louis, MO). The flies were placed in vials containing three 2.4 cm Whatman GF/A filters saturated with 20 μg/ml RNase A. PCR amplifications were performed on approximately one fly-equivalent of DNA. The following PCR conditions were used: 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 2 min, and then one cycle of 72°C for 7 min. AmpliTaq DNA polymerase (Roche, Basel, Switzerland) was used according to the manufacturer's instructions. Twelve primer pairs, which lead to the production of 12 overlapping ~600 bp products, were used to amplify almost the entire kcc gene from homozygous kccDHS1/kccDHS1 D506 flies. (The ~1 kbp intronic regions flanking each of the alternative exons were not examined.) Two primers, CG14-5 (5′-CAATTTGTAACCCATATTGAGC) and Pwht1 (5′-GAACGCCTCACTCCGACAGTCACA), were used to amplify the kcc gene/P-element junction in homozygous kccDHS1/kccDHS1 D577 flies. CG14-5 is in an alternative exon ~1 kbp from the 3′ end of kcc, whereas Pwht1 occurs in SUPOR-P ~10 kb downstream of the kcc gene. As anticipated, no CG14-5/Pwht1 PCR product was obtained using D577 DNA, indicating an imprecise excision that removed the 3′/5′ portion of the P-element (the end proximal to the kcc gene). All PCR products were purified using the QiAquick PCR Purification kit (Qiagen, Valencia, CA) and sequenced at the University of California, Berkeley, DNA Sequencing Facility using the corresponding 5′ and 3′ PCR primers.
Figure 7 was quantified using ImageJ 1.34 (http://rsb.info.nih.gov/ij). The relative kcc transcript levels in the test and control lanes were determined by normalizing the signal intensity of the kcc band to that of the corresponding Act79 band. A separate set of RT-PCRs was performed using 1 μg of each total RNA sample, the kcc (but not Act79b) primers and 35 PCR cycles. The resulting RT-PCR products were excised from a 0.9% agarose gel stained with ethidium bromide, purified using the QIAquick PCR Purification kit (Qiagen), and sequenced at the University of California, Berkeley, DNA Sequencing Facility using the kcc-15-5 and kcc-15-3 primers.

**Western analysis.** For the initial Western blot (see Fig. 8), flies were reared at room temperature (−24°C), collected using CO2, and frozen at −80°C. For the developmental Westerns (see Fig. 9), flies were reared at 22°C, collected using CO2, and returned to 22°C for 0–4 additional days before freezing at −80°C. Fly heads were detached by vigorous shaking of flies frozen at −80°C. For each strain, equal numbers of male and female heads were collected in the presence of liquid nitrogen and again stored at −80°C. Subsequently, extraction buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, and 100 μM PMSE) was added to each set of heads, and the heads were homogenized at 4°C using a Teflon pestle. The homogenates were microfuged at 4°C for 30 min, and the supernatants were collected. For each set of heads, the clarified homogenate from three fly-head equivalents was separated on a 7% SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) by standard methods (Sambrook et al., 1989). The kcc protein bands were visualized using the Protoblot Western Blot AP System (Promega, Madison, WI), according to the manufacturer’s instructions with two modifications: the blocking step was done for 2 days at 4°C, and the incubation with the primary antibody was done overnight at 4°C. The primary antibody was a polyclonal rabbit antiserum, α-SA4 (Su et al., 1999), directed against a peptide (RGGGREVTIYS) found at the C terminus of mammalian KCC1, as well as *Drosophila* kcc (Payne et al., 1996; Filipov et al., 2003), and was diluted 1:5000. The secondary antibody, alkaline phosphatase-conjugated goat α-rabbit IgG (Promega), was diluted 1:7500. This same Western blot was subsequently rebloked and incubated with a mouse anti-α-tubulin monoclonal antibody (Sigma), diluted 1:5000, and then HRP-conjugated goat α-mouse IgG. The signal was visualized with 0.5 mg/ml DAB and 0.03% hydrogen peroxide. For the developmental Western shown in Figure 9A, the initial Western blot was subsequently incubated with peroxidase-conjugated horse α-goat IgG and peroxidase-conjugated horse α-mouse IgG (both 1:1000 and obtained from Vector Laboratories, Burlingame, CA), and proteins were detected using the ECL system (Amersham Biosciences) according to the manufacturer’s directions. Band signal intensities were quantified using ImageJ 1.34 (http://rsb.info.nih.gov/ij). We then obtained an approximation of the relative kcc protein levels in each lane by normalizing the signal intensity of the kcc band to that of the corresponding α-tubulin band.

**Immunohistochemistry.** Heads from 4- to 5-d-old CS-5 flies reared at 22°C were manually dissected and frozen in OCT embedding medium (TissueTek). Microtome sections (8 μm) were collected on lysine-coated slides and fixed for 15 min in Histochoice (Electron Microscopy Sciences, Fort Washington, PA). Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer’s directions except that both the blocking and the primary antibody incubation steps were done overnight at 4°C. The α-SA24 primary antibody (Su et al., 1999) was diluted 1:150.

**Electrophysiology.** Homozygous kcc<sup>DNHS</sup>/kcc<sup>DNHS</sup> and heterozygous kcc<sup>DNHS</sup>/CyO flies from D506 bottles grown at 22°C were collected <24 h after eclosion for electrophysiological testing. Brain stimulation and recording of both giant fiber (GF)-driven muscle potentials and seizures was performed essentially as described previously (Kuebler and Tanouye, 2000) except that the flies were mounted using soft wax (Godenschwege et al., 2002). Both single-pulse stimuli and high frequency (HF) wave trains were delivered to the fly’s brain using bipolar tungsten stimulating electrodes. Single-pulse stimuli (0.5 ms duration; 0.8 Hz) were used to drive the GF, and the GF-driven muscle potentials were recorded from the dorsal longitudinal muscles using tungsten recording electrodes. The GF threshold was considered to be the lowest voltage at which the GF pathway responded to a single pulse stimulus. The GF was stimulated continuously to assess circuit function during the course of each experiment, and flies were discarded if GF function appeared compromised. Seizures consist of HF activity in at least seven different muscle groups and over 30 muscle fibers in the thorax, and reflect the HF firing of the innervating motoneurons (Kuebler and Tanouye, 2000). We attempted to elicit seizures by delivering short wave trains of HF electrical stimuli (0.5 ms pulses delivered at 200 Hz for 300 ms) to the fly’s brain. The lowest intensity HF stimulus required to elicit a seizure was designated the “seizure threshold.” The two-tailed t test was used to determine the p values for differences in seizure threshold between the kcc<sup>DNHS</sup>/kcc<sup>DNHS</sup> flies and their kcc<sup>DNHS</sup>/CyO sibling controls.

**Results**

A novel neurological mutant with seizure phenotypes called *kazachoc* (kcc)

A novel *Drosophila* neurological mutant was identified serendipitously in a screen for seizure-enhancer mutations (Zhang et al., 2002). Flies from a putative seizure-enhancer line called D505 carry a P-element insertion upstream of the CG9925 gene in cytological region 88A on the third chromosome (Zhang et al., 2002). Genetic analysis revealed that the seizure-enhancer phenotype of D505 flies resulted primarily from an unmarked mutation on the second chromosome rather than the P-element insertion. We named the novel second chromosomal mutation *kazachoc* (kcc<sub>DNHS</sub>). The kcc<sub>DNHS</sub> mutation confers an incompletely penetrant BS paralytic phenotype, a behavioral indication of seizure sensitivity. At room temperature (23°C), 27% of homozygous kcc<sub>DNHS</sub>/kcc<sub>DNHS</sub> flies display BS paralysis (Fig. 1). The incomplete penetrance of the kcc<sub>DNHS</sub>/kcc<sub>DNHS</sub> BS phenotype appears to be a stochastic phenomenon: only 27% of flies that were BS after 1–2 d were still BS when retested the following day, and 9% of flies that were not BS when initially tested were subse-
The bang-sensitive behavioral phenotype is a useful measure of kccDHS1 function. The seizure susceptibility showed that the seizure threshold of homozygous kccDHS1 flies were collected <1 d after eclosion from a DS06 population reared at 22°C. The kccDHS1 flies (n = 65) were then placed in food vials at 22°C and tested for bang-sensitive paralysis at ~24h intervals.

**Figure 2.** The kazachoc (kcc) mutation displays an age-dependent decline in bang sensitivity. The fraction of kcc^DHS1^ flies that display the BS phenotype decreases after 2–3 d (from 38 to 22% BS) and then falls precipitously (to 3% BS) 1 d later. Homozygous kcc^DHS1^ flies were collected <1 d after eclosion from a D506 population reared at 22°C. The kccDHS1 flies (n = 65) were then placed in food vials at 22°C and tested for bang-sensitive paralysis at ~24h intervals.

**Figure 3.** kcc reduces seizure threshold. A, A seizure is elicited in a homozygous kcc^DHS1^/kcc^DHS1^ fly by a high-frequency stimulus at its seizure threshold (17.0 ± 1.4 V). This seizure activity is similar in appearance and time course to those previously observed in other BS mutants (Pavlidis and Tanouye, 1995; Kuebler and Tanouye, 2000; Zhang et al., 2002). The HF stimulus is a short wave train (0.5 ms pulses at 200 Hz for 300 ms) of electrical stimuli delivered to the brain. The vertical calibration bar is 20 mV, and the horizontal bar is 200 ms. In contrast, a higher-intensity HF stimulus (30 V) fails to elicit a seizure in a kcc^DHS1^/+ sibling control fly, because it is below the fly’s seizure threshold (32.7 ± 0.23 V). The seizure threshold of the kcc^DHS1^/+ control flies is comparable with the 34.3 V threshold of wild-type CS-5 flies (J. S. Tan et al., 2004), whereas the homoyzogous kcc^DHS1^/kcc^DHS1^ mutation produces almost a threefold reduction in seizure threshold.

**The variable penetrance of the kcc^DHS1^ seizure phenotype is especially sensitive to both rearing temperature and age.** The kcc^DHS1^ mutation is more severe at lowered temperatures (Fig. 1). Homozygous kcc^DHS1^ kcc^DHS1^ show a significant increase in the BS paralytic phenotype at 22°C compared with 23°C (44 vs 27% BS paralysis, respectively). At 18°C, homozygous kcc^DHS1^/kcc^DHS1^ flies are inviable. The kcc^DHS1^ A/Δ flies display a more extreme temperature sensitivity; they are inviable at temperatures of 22°C and lower. This cold-sensitive lethality implies that kcc is an essential gene. At any given temperature, the BS paralysis produced by kcc^DHS1^ is more pronounced in younger flies (Fig. 2). The fraction of kcc^DHS1^ flies that display the BS phenotype decreases significantly after 2–3 d after eclosion and thereafter, and few flies exhibit BS paralysis.

**The kcc mutant displays a reduced seizure threshold**

The bang-sensitive behavioral phenotype is a useful measure of seizure susceptibility in Drosophila flies that show a strong BS phenotype also display significantly reduced seizure thresholds at the electrophysiological level, whereas mutations that decrease behavioral bang sensitivity raise the fly’s seizure threshold (Pavlidis and Tanouye, 1995; Kuebler and Tanouye, 2000; Kuebler et al., 2001; Glasscock and Tanouye, 2005; Glasscock et al., 2005; Hekmat-Scafe et al., 2005; Song and Tanouye, 2006). Examination of seizure susceptibility showed that the seizure threshold of kcc^DHS1^ flies is almost one-third that of wild-type and control genotypes. Thus, kcc^DHS1^ shows a seizure threshold of 13.1 ± 4.67 V high-frequency stimulus (HFS), whereas their kcc^DHS1^/+ sibling controls show a seizure threshold of 32.7 ± 0.23 V HFS, comparable with wild-type CS flies [34.3 ± 1.9 V HFS after J. S. Tan et al. (2004)]. In contrast, flies carrying the fully penetrant BS mutations bss, eas, or sda have seizure thresholds of (3.2 ± 0.6, 3.4 ± 0.5, and 6.2 ± 0.8 V HFS, respectively), 5- to 10-fold lower than wild-type (Kuebler et al., 2001). Figure 3A shows a seizure recorded from the dorsal longitudinal muscle of a homoyzogous kcc^DHS1^ fly in response to a 17 V HF stimulus. Abnormal HF muscle potentials (>100 Hz) are observed and reflect seizure activity of the single motoneuron innervating this muscle fiber (Kuebler and Tanouye, 2000). This seizure activity is similar in appearance and time course to those previously observed in other BS mutants (Pavlidis and Tanouye, 1995; Kuebler and Tanouye, 2000; Zhang et al., 2002). Figure 3B shows a dorsal longitudinal muscle recording from a heterozygous kcc^DHS1^/+ fly in response to a 30 V HF stimulus. In this instance, no seizure activity can be seen, indicating that this higher intensity HF stimulus was not of sufficient strength to elicit a seizure in the heterozygote.

**The seizure susceptibility of a variety of seizure-prone flies is increased by kcc^DHS1^**

The kcc^DHS1^ mutation was uncovered in a screen for enhancers of sda+/+. Here, we examined more explicitly seizure-enhancer functions of kcc^DHS1^ (Fig. 4). We showed that approximately one-half (51%) of doubly heterozygous kcc^DHS1^/+; sda+/+ flies are BS. Because the singly heterozygous sda/+ flies fail to show a BS phenotype and heterozygous kcc^DHS1^/+ are also not BS, the indication is that there is a genetic interaction in the double heterozygous mutations that enhances the BS phenotype. We suggest that kcc^DHS1^ acts as an enhancer of sda. A more extreme phenotype attributable to interaction is observed in kcc^DHS1^/kcc^DHS1^; sda/+ flies with 82% of flies showing BS phenotype. The mutations also display synthetic lethality: the double mutant combinations sda/sda; kcc^DHS1^/+/ and sda/sda; kcc^DHS1^/ are inviable at temperatures ranging from 18 to 25°C (data not shown).

The kcc^DHS1^ mutation also enhances other BS mutations including bang-sensitive (bss) and easily shocked (eas) (Fig. 4). Both heterozygous and homozygous kcc^DHS1^ mutations produce significant enhancement of bss. Whereas 0% of either heterozygotes
The kcc mutation enhances a variety of other bang-sensitive mutations. Flies of the indicated different genotypes were examined for the bang-sensitive paralytic behavioral phenotype in response to a 10 s mechanical bang. The data reveal that the kccEH56 mutation produces significant enhancement of the BS mutations sda, bss, and eas. The kccEH56 mutation is recessive, and kcc/+ flies are normally not BS (Fig. 1). At 22°C, our sda, bss, and eas mutations are also recessive, and consequently sda/+; bss/+; and eas/+ flies are also not BS. However, the double heterozygotes of kcc with either sda or bss (kcc+/sda+; kcc+/bss+; kcc+/+) display significant bang sensitivity (51% and 48%, respectively). If the sda/+ or bss/+ heterozygotes are instead homozygous for the kccEH56 mutation (kcc/sda+; bss+; kcc/kcc), their bang sensitivity is even more pronounced (82 or 77%, respectively). Flies of the genotype eas+; kcc/kcc show a level of bang sensitivity (64%) that is greater than that of flies carrying either the eas+ or kcc mutations alone (0 or 44%, respectively), indicating moderate enhancement.

Figure 4. The kcc mutation enhances a variety of other bang-sensitive mutations. Flies of the indicated different genotypes were examined for the bang-sensitive paralytic behavioral phenotype in response to a 10 s mechanical bang. The data reveal that the kccEH56 mutation produces significant enhancement of the BS mutations sda, bss, and eas. The kccEH56 mutation is recessive, and kcc/+ flies are normally not BS (Fig. 1). At 22°C, our sda, bss, and eas mutations are also recessive, and consequently sda/+; bss/+; and eas/+ flies are also not BS. However, the double heterozygotes of kcc with either sda or bss (kcc+/sda+; kcc+/bss+; kcc+/+) display significant bang sensitivity (51% and 48%, respectively). If the sda/+ or bss/+ heterozygotes are instead homozygous for the kccEH56 mutation (kcc/sda+; bss+; kcc/kcc), their bang sensitivity is even more pronounced (82 or 77%, respectively). Flies of the genotype eas+; kcc/kcc show a level of bang sensitivity (64%) that is greater than that of flies carrying either the eas+ or kcc mutations alone (0 or 44%, respectively), indicating moderate enhancement.

kccDH51/ or bss/+ flies are BS, almost one-half (48%) of the double heterozygotes (bss+; kccDH51/+ ) are BS. An even greater fraction (77%) of bss/+; kccDH51/kccDH51 flies are BS. The kccDH51 enhancement of the BS phenotype for eas is modest. None of the double heterozygotes (eas+; kcc/kcc) are BS. However, there is genetic interaction because 64% of eas+; kccDH51/kccDH51 flies are BS (Fig. 4). This proportion is higher than that of kccDH51/ kccDH51 flies at this temperature (40%) (Fig. 1). The percent bang sensitivity of homozygous kccDH51/kccDH51 flies is further increased by either the P-element insertion in CG9925 (identified in line J) (Zhang et al., 2002) and the jitterbug (jbug) mutation identified in the same screen (data not shown). The jbug gene encodes Drosophila filamin; mutations in human filamin are associated with periventricular heterotopia, which presents with epilepsy (X. Ren and M. A. Tanouye, personal communication) (Fox et al., 1998).

Mapping and identifying the kcc gene
The kcc gene resides in a 57 kb segment at region 60A near the distal tip of chromosome 2R as revealed by genetic mapping. Recombination mapping of the BS phenotype shows that kcc is located between brown (bw) at 104.5 m.u. and speck (sp) at 107 m.u.; the apparent position of kcc is 106.5 m.u. Duplication and deficiency mapping with a number of aneuploid chromosomes showed that kcc lies between the distal break point of Df(2R)b23, which fails to uncover kcc, and Df(2R)bw41, which uncovers kcc. Superposition of our deficiency mapping with a molecular map of the region revealed that the kcc gene must map to a 57 kb region of chromosomal region 60A (Fig. 5B).

The DNA segment where kcc maps contains 15 genes (Fig. 5B). Our focus for kcc candidates was on the five essential genes because of kcc lethal phenotypes. Three candidates (ken, CG4882, and Nap1) were eliminated by complementation analysis (data not shown). Of the remaining two candidates (CG5594 and CG11183), kcc was shown to correspond to CG5594. This identification was based on a series of mutations that all failed to complement in different heterozygote combinations, indicating that they are allelic (Table 2). These include the mutants kccEH51, kccML1, CG5594EY08340, I(2)60A-Cp1, and I(2)60A-Cp100. Molecular lesions for several of the mutations have been localized to the same transcription unit (Fig. 6). Subsequently, we will use a consolidated terminology that refers to kcc lethal alleles as kccEY08340, kccML1, and kccEH51.

The kcc gene encodes a K+-Cl- cotransporter
The Drosophila kcc gene has been previously identified as a cation cotransporter closely related to the mammalian KCC2 cotransporter (Filippov et al., 2003). The kcc gene has four splice variants (A–D) (Drysdale et al., 2005) shown in Figure 6. The product of each of these splice variants has 12 predicted transmembrane domains, which is a canonical feature of vertebrate KCCs (Delpire and Mount, 2002), with which they share 53–59% amino acid identity. The major kcc splice variants appear to be B and D, which constitute the preponderance of cDNAs identified in a BLAST search (http://flybase.net/blast) of Drosophila expressed sequence tags (dbESTs). Our dbEST BLAST search indicated that the B variant is enriched in libraries prepared from adult heads, whereas the D variant is enriched in embryonic libraries. Proteins predicted for the A and C transcripts have a different N terminus than those predicted for the B and D transcripts. The N termini of KCCs are located intracellularly and apparently regulate cotransport (Delpire and Mount, 2002). The C and D variants have an additional exon not present in either the A or B forms. Consequently, the C and D products would carry an additional 31 aa in their C-terminal tails. The mammalian KCC2 differs from the other KCCs by carrying an additional ~100 aa domain at approximately the same C-terminal region (Payne et al., 1996; Hebert et al., 2004).

Sequence analysis identified molecular lesions within the kcc gene associated with kccEY08340, kccML1, and kccEH51 mutants (Fig. 6). The lethal kccEY08340 allele results from a P-element insertion within the last exon of the kcc gene causing a truncation of 47 C-terminal amino acid residues. In the mammalian ortholog KCC1, C-terminal truncation is known to completely abolish function (Casula et al., 2001). The kccEY08340 product also lacks a conserved tyrosine known to be essential in KCC2, KCC1, and KCC4 (Strange et al., 2000). The kccML1 allele is a deletion that removes 83 bp of 3'-UTR from the last kcc exon. The kccML1 mutation is a 13 bp insertion (ACTATGCTACTGT) after the seventh base pair in intron 11 of the kcc gene. Analysis of kcc RT-PCR products revealed that splicing of intron 11 is unaltered, but there is a 2.3-fold reduction in kcc transcript levels in the heads of kccML1 mutants relative to those of wild-type controls (Fig. 7).

kcc protein is reduced in kcc mutants
Western blot analysis showed that kcc protein is reduced in the heads of both kccEH51 and kccML1 flies (Fig. 8). Wild-type flies show a prominent band of apparently 125 kDa, slightly larger than the expected size of 114–119 kDa. This size difference may reflect posttranslational modification of the kcc protein: mammalian KCCs are extensively glycosylated and phosphorylated (Payne et al., 1996; Mount et al., 1998, 1999; Su et al., 1999). An estimate of the relative kcc levels shows that compared with wild-type flies, kcc protein is reduced ~4-fold and 1.9-fold in the kccEH51 and kccML1 mutants, respectively. Heterozygotes carrying one copy of the lethal kccEY08340 allele and one wild-type allele also
show a threefold reduction in kcc protein relative to wild-type flies.

The level of kcc protein in the heads of kcc<sup>CG594EY08304</sup> flies progressively increases with developmental age (Fig. 9A). By 4 d after eclosion, the level of kcc in the heads of kcc<sup>CG594EY08304</sup> flies has risen ~13-fold. This rise in kcc level likely explains the progressive decrease in bang sensitivity of the kcc<sup>CG594EY08304</sup> mutant (Fig. 2). Wild-type flies also display a developmental increase in kcc level, which nearly doubles in the fly’s first 4 d (Fig. 9B).

Immunohistochemical staining of Drosophila heads revealed that kcc is widely expressed in brain neuropil (Fig. 10). This pattern is consistent with kcc expression in neuronal dendrites and/or axons, although we cannot exclude the possibility of additional glial expression. Intense and specific immunostaining was observed in the protocerebrum, deutocerebrum, central brain (protocerebral bridge, ellipsoid body, and fan-shaped body), antennal lobe, and optic lobe (lamina, medulla, lobula, and lobula plate). In contrast to the strong immunostaining observed in most other neuropil regions, there was little or no kcc immunostaining in the mushroom body (Fig. 10A, B), a structure involved in learning and memory (Heisenberg, 2003). The kcc expression pattern in brain neuropil is strikingly similar to that observed previously for the Rdl GABA<sub>B</sub> receptor except that Rdl also displays strong expression in mushroom body (Aronstein and ffrench-Constant, 1995; Harrison et al., 1996).
**kcc-mediated seizure susceptibility requires GABAergic signaling**

The kcc seizure phenotype depends on signaling via the GABAA receptor suggesting that dysfunction is attributable to a disruption of the normal Cl− gradients that underlie GABAergic inhibition in the CNS. GABAA receptor involvement was initially tested using PTX. PTX is a GABAA receptor blocker and also a convulsant drug in mammals (Usunoff et al., 1969). PTX is also toxic for kcc flies than for their wild-type counterparts: kcc flies die with a longer t1/2 of 3.5 d (data not shown). Thus, a PTX block of GABA_A receptors has differential effects on wild-type and kcc flies. In wild-type animals, partial loss of GABA_A receptor function acts to enhance the seizure phenotype, whereas in kcc, partial loss of GABA_A receptor function acts to suppress seizures.

GABA_A receptor function was also reduced genetically using a Resistance to dieldrin (Rdl) mutation. Rdl encodes a Drosophila GABA_A receptor that is sensitive to block by organophosphate toxins such as dieldrin and to picrotoxin (Zhang et al., 1995; Hosie et al., 1997). Seizure sensitivity of kcc flies is significantly suppressed when the dosage of the Rdl GABA_A receptor gene is reduced twofold by the null Rdl mutation (Fig. 11 B). Here, 26% of homozygous kcc<sup>DHS1</sup>/kcc<sup>DHS1</sup> flies are BS. However, only ∼1% of their kcc<sup>DHS1</sup>/kcc<sup>DHS1</sup> Rdl<sup>+/+</sup> siblings show the BS phenotype (p < 0.001). Reducing the dosage of the GABA biosynthetic enzyme glutamic acid decarboxylase via the Gad1<sup>L352F</sup> mutation similarly reduces the seizure sensitivity of kcc flies (Table 3). Whereas 28% of homozygous kcc<sup>DHS1</sup>/kcc<sup>DHS1</sup> flies are BS, only 3% of their kcc<sup>DHS1</sup>/kcc<sup>DHS1</sup>; Gad1<sup>L352F</sup>/+ siblings display the BS phenotype (p < 0.001). We observed that a decreased dosage of the Rdl GABA_A receptor also produces modest reductions in the seizure susceptibility of heterozygous bss/+ and sda/+ flies and that bss/+ is partially suppressed by the Gad1 mutation (Table 3).

**Discussion**

We find that Drosophila kcc null mutations cause recessive lethal phenotypes, indicating that kcc is an essential gene. Partial loss-of-function kcc mutations cause seizure-sensitive phenotypes and act as potent seizure-enhancer mutations. Seizure sensitivity of kcc flies appears to depend on transmission by the ionotropic GABA_A receptor, the PTX-sensitive product of the Rdl gene. These observations confirm and extend recent investigations of mammalian KCC2 in mouse and humans. Reduced KCC2 function has also been found to cause lethality (null mutation) and epilepsy (partial loss-of-function mutations) (Hübner et al., 2001; Woo et al., 2002; Tornberg et al., 2005). Thus, the results in the present paper provide a link between human epilepsy and Drosophila and mouse models of seizure disorder. Furthermore, the present investigation suggests that seizure disorders in kcc and KCC2 mutants result from a dysfunction in GABAergic inhibition. We suggest that epilepsy phenotypes, in many instances,
might be traced back to a perturbation of the biology underlying the ontogenetic switch from excitatory to inhibitory GABAergic signaling.

Drosophila kcc mutations resemble those described for mouse KCC2. KCC2 is an essential gene that causes lethal phenotypes in knock-out mutants (Hübner et al., 2001). Seizure phenotypes are observed in mouse KCC2 knock-down mutants that reduce the normal level of neuronal K^+\text{}/\text{Cl}^- cotransporter (Woo et al., 2002; Tornberg et al., 2005). In the more severe knock-down mutant (5% normal KCC2 level), generalized seizures are frequently induced by the mild mechanical stimulation that occurs during handling (Woo et al., 2002). Hippocampal slices from heterozygous KCC2 disruption mice display a twofold increase in seizure susceptibility (Woo et al., 2002). The level of KCC2 is known to decrease after brain trauma in vertebrate models of epilepsy, which may explain why head trauma frequently produces seizures in humans (Payne et al., 2003). For example, reduced KCC2 protein level is seen after focal cerebral ischemia–exotoxicity (Thomas-Crusells et al., 2000). The resulting increase in intracellular Cl^- is associated with depolarizing GABA_A receptor-mediated responses after brain trauma (Katchman et al., 1994; van den Pol et al., 1996; Fukuda et al., 1998; Nabekura et al., 2002). Excitatory GABAergic transmission is also found in the mature hippocampus, a plastic structure which is also a frequent location for epileptic foci (Leinekugel et al., 1995; Obrietan and van den Pol, 1995; Khazipov et al., 1997; Fujiwara-Tsukamoto et al., 2003).

The seizure susceptibility of the Drosophila kcc mutants shows a marked age and temperature dependence (Figs. 1, 2). Mutant kcc_{DHS1} flies become progressively less bang sensitive with increasing age (Fig. 2). A plausible explanation for this observation is the concomitant increase in the level of kcc protein that occurs as the flies age (Fig. 9A). This may recapitulate the ontogenetic switch from excitatory to inhibitory GABAergic signaling that occurs during mammalian development (Miles, 1999; Ben-Ari, 2002). It is currently unclear why the kcc phenotype becomes more pronounced as the developmental temperature is reduced (Fig. 1). This may reflect the fact that Drosophila resting potentials become progressively more depolarized as the temperature decreases (Hosler et al., 2000). However, virtually all neural processes are temperature sensitive (Montgomery and MacDonald, 1990). It would be of interest to examine whether the mouse KCC2 mutants (Woo et al., 2002; Tornberg et al., 2005) display similar age- and temperature-dependent alterations in seizure susceptibility.

Pharmacological agents that act as convulsants increase overall CNS excitability by blocking synaptic inhibition or by promoting excess excitability (LaRoche and Helmers, 2004). Conversely, anticonvulsants work by decreasing excitation or by promoting inhibition (Woodbury, 1980). An unexpected finding in this study is that PTX, a potent GABA_A receptor blocker, can act as either a convulsant or an anticonvulsant in Drosophila depending on the genetic background (Fig. 11). As expected, in wild-type Drosophila, PTX acts as a convulsant. We expect that PTX interferes with GABAergic synaptic inhibition by blocking GABA_A

Figure 11. Reduced levels of GABA_A receptor suppress the kcc mutation. A, Feeding wild-type (WT) flies (n = 120) the convulsant PTX, which is a competitive inhibitor of the GABA_A receptor, produces a low-level bang sensitivity (1% BS). In contrast, PTX-fed kcc_{DHS1} flies (n = 74) displayed a significant reduction in bang sensitivity relative to their sibling controls (n = 65). Whereas 38% of control kcc flies were bang sensitive, only 16% of their PTX-fed siblings were bang sensitive (p < 0.05). B, The bang sensitivity of kcc_{DHS1} flies is suppressed in Rdl^{+/+} flies, which carry one null allele of the Rdl GABA_A receptor gene. Whereas 26% of control kcc_{DHS1} flies (n = 231) were bang sensitive, only 1% of their kcc_{DHS1}; Rdl^{+/+} siblings (n = 301) were BS (p < 0.05).
receptors, thereby promoting overall CNS excitability (Usunoff et al., 1969; Zhang et al., 1995). The surprising finding is that PTX acts as an anticonvulsant to suppress seizures in a kcc genetic background. At present, the anticonvulsant properties of PTX are difficult to explain. We assume that PTX continues to act by blocking GABA<sub>A</sub> receptors. We assume further that anticonvulsant properties reflect an overall decrease in CNS excitability. An attractive hypothesis is that, in kcc mutants, GABAergic transmission is mostly excitatory. This excitatory GABAergic transmission could act to promote seizure sensitivities and enhance the seizure susceptibility of other seizure-sensitive mutations. If so, then PTX could act as an anticonvulsant by blocking GABA<sub>A</sub> receptors and thereby reducing overall CNS excitability. Taken alone, our PTX results alone must be interpreted with some caution, because electrophysiological recordings of wild-type flies after short-term PTX feeding suggest that PTX may induce an atypical seizure-like state characterized by periodic bursts rather than stereotypical electrophysiological seizures (Lee and Wu, 2002). However, consistent with our hypothesis that PTX acts by reducing GABAergic excitation in our kcc mutants, we observed that a genetic reduction of GABA<sub>A</sub> receptor also suppressed kcc seizures (Fig. 11). Additional experimental evidence will depend on the development of a good electrophysiological preparation in Drosophila for examining synaptic inhibition; such a preparation is currently lacking. However, such paradoxical seizure suppression after application of a GABA<sub>A</sub> receptor antagonist has been observed previously in brain slices from patients with temporal lobe epilepsy as well as in rodent models of epilepsy in which excitatory GABAergic signaling is believed to underlie seizure activity (Cohen et al., 2002; Shinnar and Glauzer, 2002; Baulac et al., 2004).

An especially challenging problem in neurobiology is to determine how genetic and environmental factors interact to cause the expression of seizure phenotypes in epilepsy. The major difficulties are a combination of polygenic inheritance and incomplete penetrance: inheritance of particular combinations of mutations may predispose, but not cause epilepsy in a given individual (Noebels, 2003). For example, recent interest has focused on the genetics of juvenile myoclonic epilepsy (JME), an idiopathic generalized epilepsy characterized by myoclonic jerks and generalized tonic-clonic seizures (Zifkin et al., 2005). JME, like most idiopathic generalized epilepsies, displays multifactorial inheritance that reflects the additive effects of multiple susceptibility genes interacting with environmental factors to produce the final phenotype (Berkovic et al., 1998). Polymorphisms in a number of genes are suspected to predispose individuals to JME, including GABRA1, the a1 subunit of the GABA<sub>A</sub> receptor (Wallace et al., 2001; Cossette et al., 2002), CICN2, a voltage-gated Cl<sup>-</sup> channel primarily expressed in cerebral neurons inhibited by GABA (D’Agostino et al., 2004), and the KCNQ3 K<sup>+</sup> channel (Vijai et al., 2003). However, even in these cases of apparent monogenic inheritance, the phenotypic variation between families and even family members suggests that modifying genes and environmental factors interact with the predisposing mutations to produce JME, as well as other forms of idiopathic generalized epilepsy (Zifkin et al., 2005). Although genetic interactions appear to play a central role in several idiopathic generalized epilepsies, there are surprisingly few experimental observations providing confirmation (N. C. K. Tan et al., 2004). The Drosophila kcc mutation appears to provide an excellent model in which to investigate the ways interacting genes contribute to seizure disorder. The kcc seizure-enhancer mutation may be used to facilitate the identification of genes that contribute to seizure phenotypes via double mutant analysis. Contributing genes may then be separated from kcc and basal phenotypes examined to determine the extent to which phenotypes are dependent on interactions. In addition, the kcc mutant provides a model for how nongenetic factors such as age and temperature contribute to overall phenotypic expression of the seizure phenotype.

Our results suggest that seizure susceptibility in Drosophila is determined, in large part, by disruption of GABAergic signaling and, in particular, the kcc GABA switch. Genetic alterations that perturb the GABAergic pathway ameliorate the seizure susceptibility conferred by a variety of BS mutations (Table 3). The partial loss-of-function kcc<sup>DHS1</sup> and kcc<sup>M11</sup> mutations, which reduce the level of the kcc GABA switch, increase seizure sensitivity. Indeed, the degree of seizure sensitivity increases as the kcc level falls (Figs. 2, 8, 9A; Table 3). Both Rdl and kcc are expressed in similar regions of brain neuropil with the exception of mushroom body, where only low levels of kcc are found (Fig. 10). Consequently, the mushroom body could be particularly sensitive to a reduction in kcc level, resulting in GABAergic excitation. Mutations that reduce kcc function might increase seizure susceptibility, particularly if the level of kcc is already low. This may explain why kcc<sup>−/−</sup> acts as a seizure enhancer for a large variety of other BS mutations (Fig. 4). The BS mutations that interact genetically with both kcc and Rdl could reduce kcc function by any of number of molecular or cell biological processes including the following: inefficient transport activity because of abnormalities in phospholipid environment (i.e., loss of eas function), mislocalization because of deficiencies in cytoskeletal scaffolding (i.e., loss of jhbg filamin function), destabilization of the extracellular environment of the synapse (i.e., loss of the sda aminopeptidase), transcriptional, translational, or posttranslational effects on molecules affecting kcc expression levels or subcellular localization. Likewise, seizure resistance and seizure suppression could presumably be attributable to factors that increase kcc function, thereby decreasing intracellular Cl<sup>-</sup>. This could have the net effect of acting to sharpen or refine inhibitory GABA<sub>A</sub> function in the nervous system. For these reasons, we suggest that modulation of GABAergic signaling by kcc could be central to both seizure sensitivity and seizure resistance.

### References


<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Bang sensitivity</th>
<th>Control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>kcc&lt;sup&gt;−/−&lt;/sup&gt;; Rdl&lt;sup&gt;/+&lt;/sup&gt;</td>
<td>1 (301)</td>
<td>26 (231)</td>
<td>3.7 × 10&lt;sup&gt;−19&lt;/sup&gt;</td>
</tr>
<tr>
<td>ba&lt;sup&gt;d&lt;/sup&gt;; Rdl&lt;sup&gt;/+&lt;/sup&gt;</td>
<td>53 (146)</td>
<td>78 (137)</td>
<td>1.0 × 10&lt;sup&gt;−5&lt;/sup&gt;</td>
</tr>
<tr>
<td>sda&lt;sup&gt;d&lt;/sup&gt;; Rdl&lt;sup&gt;/+&lt;/sup&gt;</td>
<td>1 (144)</td>
<td>2 (80)</td>
<td>1.9 × 10&lt;sup&gt;−9&lt;/sup&gt;</td>
</tr>
<tr>
<td>eas&lt;sup&gt;−/−&lt;/sup&gt;; Rdl&lt;sup&gt;/+&lt;/sup&gt;</td>
<td>0 (104)</td>
<td>0 (94)</td>
<td>1.0 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
</tr>
<tr>
<td>kcc&lt;sup&gt;−/−&lt;/sup&gt;; Gad1&lt;sup&gt;L352F&lt;/sup&gt;</td>
<td>3 (72)</td>
<td>28 (47)</td>
<td>1.9 × 10&lt;sup&gt;−7&lt;/sup&gt;</td>
</tr>
<tr>
<td>kcc&lt;sup&gt;−/−&lt;/sup&gt;; Gad1&lt;sup&gt;L352F&lt;/sup&gt;</td>
<td>19 (145)</td>
<td>53 (110)</td>
<td>1.9 × 10&lt;sup&gt;−7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
| GABA<sub>A</sub> biosynthetic gene Gad1 suppresses kcc<sup>−/−</sup> and some other BS mutations

Genotypes for Rdl<sup>/+</sup> and Gad1<sup>L352F</sup>/+ test flies (labeled “Test”) are indicated; control siblings (labeled “Control”) carried the TM6B-barrier chromosome rather than the Rdl<sup>−/−</sup> null allele of Rdl. Results are highly significant (p < 0.001) for all BS genotypes listed, except eas<sup>/−</sup>; Rdl<sup>/+</sup> produced slight (4%) suppression of hemisynaptic bis (p = 0.06).
Ben-Ary Y (2002) Excitatory actions of GABA during development: the
Buchner E, Bader R, Buchner S, Cox J, Emson PC, Flory E, Heizmann CW,
for the brain of normal and mutant Drosophila melanogaster. Cell Tissue
Buckingham SD, Biggin PC, Sattelle BM, Brown LA, Sattelle DB (2005)
Insect GABA receptors: splicing, editing, and targeting by antiparasitics and
Casula S, Shmukler BE, Wilhelm S, Stuart-Tilley AK, Su W, Chernova MN,
Buckingham SD, Biggin PC, Sattelle BM, Brown LA, Sattelle DB (2005) In-
sectants with altered nerve excitability in Drosophila: both N- and C-
terminal cytoplasmic domains are required for K-Cl cotransport activity.
intercalar activity in human temporal lobe epilepsy in vitro. Science
298:1418–1421.
Cossette P, Liu L, Brisebois K, Dong H, Lortie A, Vanasse M, Saint-Hilaire JM,
GABRA1 in an autosomal dominant form of juvenile myoclonic epilep-
D’Agostino D, Bertelli M, Gallo S, Cecchin S, Albiero E, Garofalo PG, Gamba-
expression of an anti-GABA receptor antibody in the brain. J Neurophysiol
26:803–843.
Drosophila melanogaster
• kcc
Mutations Produce Seizures in
Drosophila
D DLC1 is sufficient to switch the polarity of GABAergic synapses in the brain. Trends Neurosci 17:517–525.
Drosophila melanogaster
• kcc
Mutations Produce Seizures in
Drosophila
Drosophila melanogaster
• kcc
Mutations Produce Seizures in
Drosophila
Drosophila melanogaster
• kcc
Mutations Produce Seizures in
Drosophila
Drosophila melanogaster
• kcc
Mutations Produce Seizures in
Drosophila
Drosophila melanogaster
• kcc
Mutations Produce Seizures in


Obrietan K, van den Pol AN (1995) GABA neurotransmission in the hypothalamus: developmental reversal from Ca\(^2\+)


Periz G, Fortini ME (1999) Ca\(^2\+)
-ATPase function is required for intracellular trafficking of the Notch receptor in Drosophila. EMBO J 18:5983–5993.


Yuste R, Katz LC (1991) Control of postsynaptic Ca\(^2\+)


