

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

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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., 1994; 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-

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Table 1. *Drosophila* stocks

Stock no.	Genotype
CS-5	Wild type
D505	$w^{1118}; kcc^{DHS1}/SM5\ Cy; P\{w^+ LacZ\}CG9924'$
D506	$w^{1118}; kcc^{DHS1}/CyO$
U036	$w; SM5\ Cy; TM3/ap^{Xa}$
D225	$w; TM3/TM6B$
D245	$w^{1118}; nub\ b\ noc^{Sco}\ It\ stw^3/CyO$
2(A)	$y\ ac\ w; P\{YES:YBB = y^+ CG9925^+\}$
D572	$w; kcc^{DHS1}/CyO; sda^{iso7.8}/TM6B$
MR047	$w\ eas^{PC80}\ f$
MR068	$w\ bss^1\ f$
D547	$w; SM5\ Cy; sda^{iso7.8}/ap^{Xa}$
D548	$w\ eas^{PC80}\ f; +/CyO$
D508	$w\ bss^1\ f; CyO; +/ap^{Xa}$
D250	$al\ dp\ b\ pr\ c\ px\ sp$
D253	$w; Sp/CyO; Dr\ \Delta 2-3/TM3$
D254	$y; cn\ bw\ sp$
5206	$dp^{ov1}\ b^1\ cn^1\ l(2)60A-C^{20-180}/SM6a\ Cy$
5207	$dp^{ov1}\ b^1\ cn^1\ l(2)60A-C^{Ad-4}/SM6a\ Cy$
D562	$w; dp^{ov1}\ b^1\ cn^1\ l(2)60A-C^{Ad-4}/CyO$
5224	$Df(2R)106/SM5\ Cy$
16887	$y^1\ w^{67c23}; P\{w^+ = EPgy2\}CG5594^{EY08304}$
EP2164	$w^{1118}; P\{w^+ = EP\}CG10413^{EP2164}$
13216	$y^1\ w^{67c23}; P\{y^+ w^+ = SUPor-P\}KG02390$
D577	$w; kcc^{ML1}/CyO$
D569	$w; kcc^{DHS1}/SM5\ Cy; Rdl^1/TM6B$
D582	$w; kcc^{DHS1}/SM5\ Cy; Gad1^{L352F}\ e^s/TM6B$

citation rather than inhibition (Luhmann and Prince, 1991; Yuste and Katz, 1991; Wang et al., 1994; Obrietan and van den Pol, 1995; Chen et al., 1996; Owens et al., 1996). Both reduced KCC2 expression and excitatory GABAergic signaling have been linked to temporal lobe epilepsy (Köhling et al., 1998; Cohen et al., 2002; Shinnar and Glauser, 2002; Baulac et al., 2004; Palma et al., 2006).

This paper describes a K^+/Cl^- cotransporter gene from *Drosophila* called *kazachoc* (*kcc*) that is homologous to mammalian KCC2. Complete loss-of-function *kcc* mutations are lethal, indicating that *kcc* is an essential gene. Phenotypic characterization of partial loss-of-function mutants that reduce *kcc* level reveals neurological excitability defects that render the *kcc* flies especially susceptible to epileptic-like seizures. The seizure susceptibility of *kcc* flies shows a pronounced age dependence that mirrors a developmental increase in *kcc* level. The seizure sensitivity of *kcc* mutants is mediated by *Rdl* GABA_A receptors, thereby linking *kcc* seizures with dysfunction of the GABAergic inhibitory system. The *kcc* mutation is a potent seizure enhancer, interacting with a series of other neurological mutations to exacerbate seizure disorder phenotypes. We discuss a model in which K^+/Cl^- cotransporters play a central role in polygenic inheritance of seizure phenotypes, the most common mode of inheritance of idiopathic generalized epilepsies in humans. The K^+/Cl^- cotransporter may provide an important link between idiopathic epilepsy and perturbation of GABAergic inhibition.

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 (~24°C) (Ashburner, 1989). 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*, *bss*, and *sda*. The *eas* gene is located at cytological region 14B and encodes an

ethanolamine kinase (Pavlidis et al., 1994). The recessive *eas*^{PC80} allele carries a frameshift mutation and probably constitutes a null allele. The *bss* gene is located at 1–54.6 (corresponding to approximately cytological region 12F); its gene product has not been described (Ganetzky and Wu, 1982). The *bss*¹ allele is a semidominant mutation. The *sda* gene is located at 97D and encodes an aminopeptidase (Zhang et al., 2002). The recessive *sda*^{iso7.8} mutation occurs in the 5' noncoding region and greatly diminishes the level of *sda* transcript. The genotypes $w\ eas^{PC80}\ f$, $w\ bss^1\ f$, and $w\ sda^{iso7.8}$ are abbreviated as *eas*, *bss*, and *sda*, respectively, in the text. The deficiency stocks carrying *Df(2R)OV1* (59F5;60A1), *Df(2R)b23* (59F8; 60A3), and *Df(2R)bw^{DRa}* (59E1;60A4–5) were kindly provided by Kristi Wharton (Brown University, Providence, RI). Deficiency stocks carrying *Df(2R)tid* (59F4;59F5), *Df(2R)egl2* (59E;60A1), *Df(2R)bw^{S46}* (59D8–11; 60A7), *Df(2R)Ch^{ig230}* (60A3–7;60B4), *Df(2R)106* (60A3;60A7), and *Df(2R)PX1* (60B8–10;60D1) were obtained from the Bloomington *Drosophila* Stock Center, as was the *Dp(2;Y)bw⁺* (58F1–3;60E11–12) duplication stock. Lethal alleles of *Nap1* [*Nap1*^{KO1} (Lankenau et al., 2003)] and CG4882 [*l(2)SH2263* (Oh et al., 2003)] were provided by Dirk Lankenau (University of Heidelberg, Heidelberg, Germany) and Steven Hou (National Institutes of Health, Bethesda, MD), respectively.

Behavioral testing. Testing for BS paralysis was performed on flies reared at 22°C and 1–2 d after eclosion unless otherwise specified. Flies were anesthetized with CO₂ and collected <1 d after eclosion, held at 18°C overnight, and tested the following day unless otherwise specified. Approximately 15 flies were transferred to a clean vial (Applied Scientific, Eugene, OR) without agitation and immediately vortexed at maximum speed for 10 s. Bang-sensitive flies displayed a period of paralysis followed by a period of hyperactivity. The χ^2 test was used to determine the *p* values for differences in percent bang sensitivity between test flies and their sibling controls.

Identification of the initial *kazachoc* (*kcc*) mutation and construction of a *kcc* stock. We initially identified *kcc* as a spontaneous mutation present on the second chromosome of one of our *sda*-enhancer stocks, D505 (Zhang et al., 2002), which also carries an unlinked P-element insertion, $P\{w^+ LacZ\}CG9925'$, on its third chromosome. To determine whether the P-element insertion upstream of the CG9925 gene was responsible for the strain's recessive BS phenotype, we asked whether this phenotype could be rescued by a P-element construct carrying the complete CG9925 gene inserted into the second chromosome. Accordingly, we first crossed non-Cy virgin females from our D505 stock to U036 males. We then crossed what we presumed to be $w^{1118}; +/SM5\ Cy; P\{w^+\}CG9925/TM3$ male progeny from this cross to virgin 2(A) females. The 2(A) stock, kindly provided by Gary Landis (University of Southern California, Los Angeles, CA), has a P-element carrying the CG9925 gene inserted into the second chromosome (Landis et al., 1997). Finally, we crossed what we assumed to be $P\{CG9925^+\}/SM5\ Cy; P\{w^+\}CG9925/TM3$ males from this cross to virgin females from our original D505 stock and analyzed the progeny for bang sensitivity. To our surprise, we observed no bang sensitivity in any of the homozygous $P\{w^+\}CG9925$ progeny: neither the $P\{CG9925^+\}$ ones that carried the rescue construct nor the $+/SM5\ Cy; P\{w^+\}CG9925$ ones that lacked it were BS. We postulated that the original D505 strain must carry an unmarked mutation on its second chromosome that was necessary for its recessive BS phenotype. The presence of a deleterious second chromosomal mutation in D505 would also be consistent with the observation that most of the flies in our D505 stock retained their SM5 balancer chromosome. We termed this new BS mutation *kazachoc* (*kcc*) and the spontaneous allele *kcc*^{DHS1}. The “*kazachoc*” is a Slavic dance that involves squatting with the alternate kicking out of legs, a process that to some extent resembles a BS fly experiencing a bang-induced seizure. The *kcc*^{DHS1} mutation apparently arose as a spontaneous mutation in the w^{1118} stock used in a screen for enhancers of *sda*⁺.

We obtained a balanced *kcc* stock, D506, as follows: non-Cy D505 males ($w^{1118}; kcc^{DHS1}; P\{w^+\}CG9925$) were crossed to U036 virgin females. Virgin female progeny of the genotype $w^{1118}; kcc^{DHS1}/SM5\ Cy; P\{w^+\}CG9925/TM3$ were then crossed to D245 males. Male and virgin female progeny of the genotype $w^{1118}; kcc^{DHS1}/CyO; +/TM3$ were then crossed and their homozygous $w^{1118}; kcc^{DHS1}$ progeny mated to create an initial stock. Several bang-sensitive males from this initial $w^{1118}; kcc^{DHS1}$

stock were then crossed to D245 virgin females. Finally, male and virgin female progeny of the genotype $w^{1118}; kcc^{DHS1}/CyO$ were crossed to create our D506 balanced kcc^{DHS1} 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 $+/w^{1118}; al dp b pr c px sp/kcc^{DHS1}$ 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 $kcc^{DHS1}/cn bw sp$ 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 (kcc^{DHS1}/kcc^{DHS1}) male progeny were then individually crossed to D254 virgin females, and the progeny were scored for the *bw* and *sp* 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 *white*⁺ P-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 $w^-; P(w+)/CyO; \Delta 2-3/+$ 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(w+)* 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 produced 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 $kcc^{DHS1}/Df(2R)$ progeny were tested for bang-sensitive paralysis. We concluded that a deficiency uncovered *kcc* if a significant fraction of the $kcc^{DHS1}/Df(2R)$ progeny were bang sensitive. In our duplication mapping experiment, D506 virgin females were first crossed to $y^1 w^a/Dp(2:Y)bw^+; E(w^a)/CyO$ males (Bloomington stock no. 1024). Individual pairs of the resulting $w^{1118}/y w^a; kcc^{DHS1}/CyO$ virgin females and $w^{1118}/Dp(2:Y)bw^+; kcc^{DHS1}/CyO$ (or $w^{1118}/Dp(2:Y)bw^+; E(w^a)/CyO$) males were then crossed. For those crosses that yielded bang-sensitive progeny, the percent bang sensitivity of the duplication-bearing males was significantly higher than that of their female siblings, indicating that *kcc* is covered by *Dp(2:Y)bw*⁺.

We determined that kcc^{DHS1} enhances *sda*, *bss*, and *eas* by comparing the percent bang sensitivity at 22°C of at least 60 of the indicated flies: (*sda*): kcc^{DHS1}/kcc^{DHS1} ; *sda/TM6B* and kcc^{DHS1}/CyO ; *sda/TM6B* D572 flies, as well as the $+/CyO$; *sda/+* progeny of a cross of CS-5 females × D547 males; (*bss*): *bss/+*; kcc^{DHS1}/kcc^{DHS1} and *bss/+*; kcc^{DHS1}/CyO female progeny of D506 females × *bss*; kcc^{DHS1}/CyO males as well as *bss/+*; $+/CyO$ females progeny of a cross of CS-5 females × D508 males; and (*eas*): *eas/+*; kcc^{DHS1}/kcc^{DHS1} and *eas/+*; kcc^{DHS1}/CyO female progeny of a cross of D506 females × D548 males as well as *eas/+*; $+/CyO$ females progeny of a cross of CS-5 females × D548 males.

To show that *Rdl*¹ suppressed kcc^{DHS1} , we crossed D506 virgin females to D569 males and compared the fraction of bang-sensitive flies among the resulting kcc^{DHS1}/kcc^{DHS1} ; *Rdl*¹/*+* and kcc^{DHS1}/kcc^{DHS1} ; $+/TM6B$ progeny. We assayed suppression of *eas*, *sda*, or *bss* by *Rdl*¹ by crossing D506 virgin females to MR047, MR063, or MR068 males, respectively. We then compared the fraction of BS flies among the resultant *eas/+*; *Rdl*¹/*+* versus *eas*; $+/TM6B$ female progeny, *Rdl*¹/*sda* versus *sda/TM6B*

progeny, or *bss*; *Rdl*¹/*+* versus *bss*; $+/TM6B$ female progeny, respectively. Similarly, to show that *Gad1*^{L352F} suppressed kcc^{DHS1} , we crossed D506 virgin females to D582 males and compared the fraction of bang-sensitive flies among the resulting kcc^{DHS1}/kcc^{DHS1} ; *Gad1*^{L352F}/*+* and kcc^{DHS1}/kcc^{DHS1} ; $+/TM6B$ progeny. We assayed suppression of *eas* or *bss* by *Rdl*¹ by crossing D506 virgin females to MR047 or MR068 males, respectively, and compared the fraction of BS flies among the resultant *eas/+*; *Rdl*¹/*+* versus *eas*; $+/TM6B$ or *bss*; *Rdl*¹/*+* versus *bss*; $+/TM6B$ female progeny, respectively.

Picrotoxin feeding. Homozygous kcc^{DHS1}/kcc^{DHS1} flies from D506 bottles grown at 22°C were collected <24 h after eclosion. Flies were then placed in groups of 10 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 mM picrotoxin (PTX) (Sigma, St. Louis, MO). The flies were allowed to ingest either the control or picrotoxin-containing sucrose for ~24 h at 22°C and then tested for bang-sensitive paralysis. Green-colored abdomens indicated that the flies had ingested the corresponding picrotoxin-laden or control sucrose solution.

DNA molecular biology. Genomic DNA was prepared from aliquots of ~30 flies essentially as described in the Berkeley *Drosophila* Genome Project Methods (<http://www.fruitfly.org/about/methods>) except that the buffer used to resuspend the final DNA pellets contained 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 kcc^{DHS1}/kcc^{DHS1} D506 flies. (The ~1 kb intronic regions flanking each of the alternative exons were not examined.) Two primers, CG14-5 (5'-CAATTGTAGACCACCATTATGACGC) and Pwh1 (5'-GTAACGCTAATCACTCCGAACAGGTCACA), were used to amplify the *kcc* gene/P-element junction in homozygous kcc^{ML1}/kcc^{ML1} D577 flies. CG14-5' is in an alternative exon ~1 kb from the 3' end of *kcc*, whereas Pwh1 occurs in SUPor-P ~10 kb downstream of the *kcc* gene. As anticipated, no CG14-5/Pwh1 PCR product was obtained using DNA from the original no. 13216 stock. However, a 1.5 kb CG14-5/Pwh1 PCR product was obtained using D577 DNA, indicating an imprecise excision that removed the 3' 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 kcc^{DHS1} D506 and CS-5 control flies were reared at 22°C, tested for bang sensitivity at <2 d after eclosion, collected using CO₂, and frozen at -80°C. Fly heads were detached by vigorous shaking of the frozen flies. For each strain, equivalent numbers of male and female heads were collected in the presence of liquid nitrogen, and total RNA was prepared using the Trizol Reagent (Invitrogen Life Technologies, San Diego, CA) according to the manufacturer's instructions. RT-PCR was performed using the Titan One Tube RT-PCR System (Roche) essentially according to the manufacturer's instructions. The *kcc* primers were *kcc*-15-5 (5'-CCAGAAGAGCATACCCATCG) and *kcc*-15-3 (5'-AATGATGGCCATCCCATAG), and the *Act79B* primers were *act*-rtf2 (5'-ATGGAGAAGTCTGGCACCA) and *act*-rtf2 (5'-TCTGCTGGAAGGTGGACAAC). The *kcc*-15-5 and 15-3 primers are located in the *kcc* exons (11 and 13, respectively) that span intron 11, where the 13 bp kcc^{DHS1} insertion is located. The thermocycling program used was as follows: 50°C for 30 min; followed by 94°C for 2 min; and then 33 cycles of 94°C for 10 s, 50°C for 30 s, and 68°C for 45 s, with the extension time increased by 5 s/cycle starting with the ninth cycle; and finally 68°C for 7 min. To determine the linear range for both the *kcc* and control RT-PCRs, we varied the total RNA concentration from 10 ng to 1 μg and the number of PCR amplification cycles from 33 to 35. Ultimately, we found that RT-PCR using a total RNA concentration of 50 ng and 33 PCR cycles fell within the linear range, and these were the conditions used for the quantitation. An inverse image of the ethidium bromide-stained gel of the CS-5 and kcc^{DHS1} RT-PCR products shown in

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 *Act79B* band. A separate set of RT-PCRs was performed using 1 μ g 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 ($\sim 24^{\circ}\text{C}$), collected using CO_2 , and frozen at -80°C . For the developmental Westerns (see Fig. 9), flies were reared at 22°C , collected using $\text{CO}_2 < 4$ h after eclosion, 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 PMSF) 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 d 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 -SA24 (Su et al., 1999), directed against a peptide (RGGGREGVITIIYS) found at the C terminus of mammalian KCC1, as well as *Drosophila kcc* (Payne et al., 1996; Filippov 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 reblocked 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*^{DHS1}/*kcc*^{DHS1} and heterozygous *kcc*^{DHS1}/*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 test 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

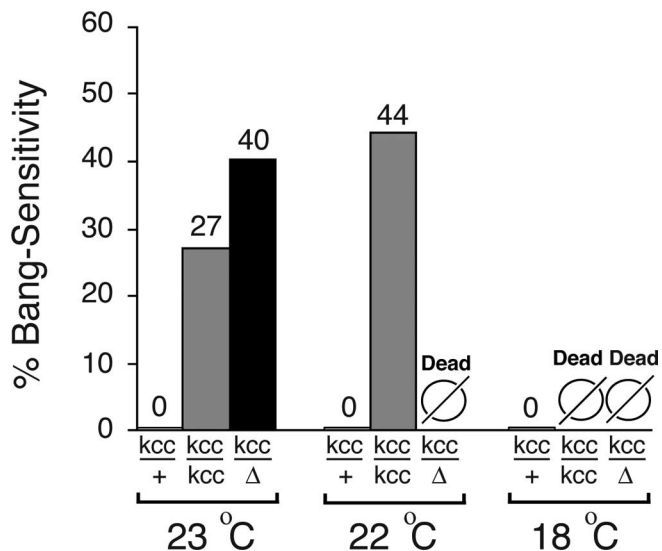


Figure 1. The kazachoc (*kcc*) mutation produces bang-sensitive behavioral phenotype. The *kcc*^{DHS1} mutation produces an incompletely penetrant, recessive bang-sensitive paralytic phenotype. The phenotype is more penetrant if the flies are grown at a lower temperature, and if the temperature is dropped to 18°C , homozygous *kcc*^{DHS1}/*kcc*^{DHS1} flies are inviable. Flies heterozygous for *kcc*^{DHS1} and a deficiency, *Df(2R)106*, which completely removes the *kcc* gene, show a more severe phenotype than do the homozygous *kcc*^{DHS1}/*kcc*^{DHS1} flies, indicating that the *kcc*^{DHS1} mutation produces a partial loss of function. The “*kcc*/*kcc*” and “*kcc*/+” flies were *kcc*^{DHS1}/*kcc*^{DHS1} and *kcc*^{DHS1}/*CyO* siblings, respectively, from our D506 stock ($n > 200$). The “*kcc*/Δ” flies were unbalanced *kcc*^{DHS1}/*Df(2R)106* progeny produced by a cross of D506 virgin females to 5224 males ($n > 80$). At temperatures at which no unbalanced progeny were observed (“Dead”), > 300 balanced progeny were counted.

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*^{DHS1}/*kcc*^{DHS1} flies and their *kcc*^{DHS1}/*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*^{DHS1}). The *kcc*^{DHS1} mutation confers an incompletely penetrant BS paralytic phenotype, a behavioral indication of seizure sensitivity. At room temperature (23°C), 27% of homozygous *kcc*^{DHS1}/*kcc*^{DHS1} flies display BS paralysis (Fig. 1). The incomplete penetrance of the *kcc*^{DHS1}/*kcc*^{DHS1} 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-

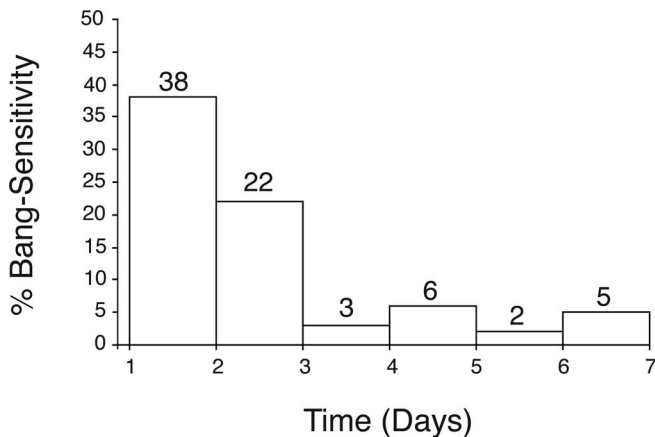


Figure 2. The *kazachoc* (*kcc*) mutation displays an age-dependent decline in bang sensitivity. The fraction of *kcc^{DHS1}* flies that display the BS phenotype decreases after 2–3 d (from 38 to 22% BS) and then falls precipitously (to 3% BS) 1 d later. Homozygous *kcc^{DHS1}* flies were collected 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

quently found to be BS when retested the next day (data not shown). The recovery time for homozygous *kcc^{DHS1}* flies is relatively short: 19.8 ± 5.9 s (14 s of paralysis followed by 5.8 s of recovery seizure). In contrast, the BS mutants *sda*, *eas*, and *bss* have significantly longer recovery times of 37, 52, and 198 s, respectively; they show 100% penetrance of the BS phenotype (Tan et al., 2004). Heterozygous *kcc^{DHS1}/+* flies are not BS, indicating that the *kcc^{DHS1}* mutation is recessive (Fig. 1). Heterozygotes carrying both *kcc^{DHS1}* and a deletion (Δ) that uncovers it (described later) display 40% bang sensitivity (Fig. 1). Because the phenotype of the *kcc^{DHS1}/\Delta* flies is more severe than that of the homozygous *kcc^{DHS1}/kcc^{DHS1}* flies, the *kcc^{DHS1}* mutation likely represents a partial loss-of-function (hypomorph).

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}* flies 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}/\Delta* 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 sensitivity 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}/+* sib-

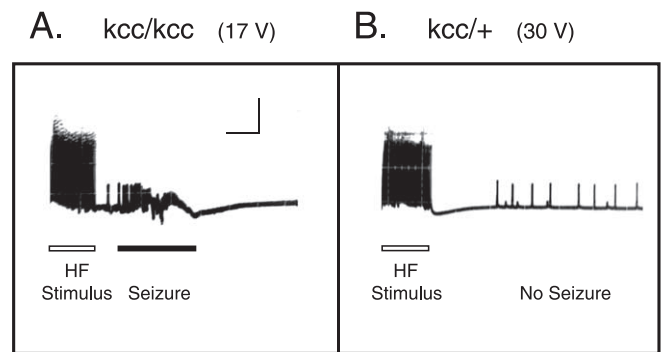


Figure 3. *kcc* reduces seizure threshold. **A**, A seizure is elicited in a homozygous *kcc^{DHS1}/kcc^{DHS1}* fly by a high-frequency stimulus at its seizure threshold (17.0 ± 1.4 V). This seizure activity is similar in appearance and time course to those previously observed in other BS mutants (Pavlidis 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. **B**, 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 homozygous *kcc^{DHS1}/kcc^{DHS1}* mutation produces almost a threefold reduction in seizure threshold.

ling 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 homozygous *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}/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-senseless* (*bss*) and *easily shocked* (*eas*) (Fig. 4). Both heterozygous and homozygous *kcc^{DHS1}* mutations produce significant enhancement of *bss*. Whereas 0% of either heterozygotes

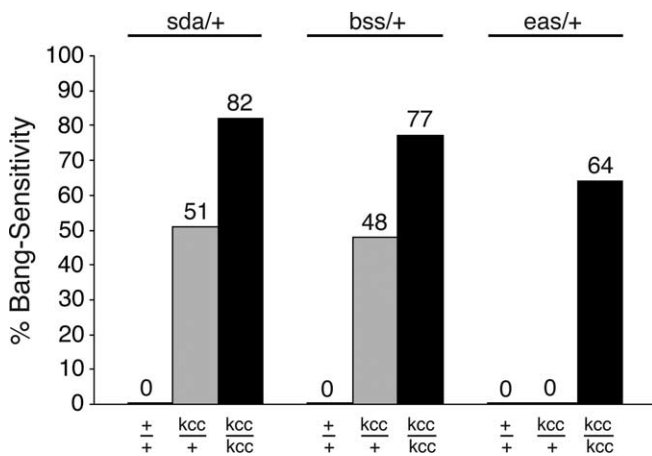


Figure 4. The *kcc* mutation enhances a variety of other bang-sensitive mutations. Flies of the indicated different genotypes were examined for the bang-sensitive paralytic behavioral phenotype in response to a 10 s mechanical bang. The data reveal that the *kcc*^{DHS1} mutation produces significant enhancement of the BS mutations *sda*, *bss*, and *eas*. The *kcc*^{DHS1} 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*/+ or *bss*/+; *kcc*/+) display significant bang sensitivity (51 and 48%, respectively). If the *sda*/+ or *bss*/+ heterozygotes are instead homozygous for the *kcc*^{DHS1} mutation (*kcc*/*kcc*; *sda*/+ or *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*/*kcc* mutations alone (0 or 44%, respectively), indicating moderate enhancement.

kcc^{DHS1}/+ or *bss*/+ flies are BS, almost one-half (48%) of the double heterozygotes (*bss*/+; *kcc*^{DHS1}/+) are BS. An even greater fraction (77%) of *bss*/+; *kcc*^{DHS1}/*kcc*^{DHS1} flies are BS. The *kcc*^{DHS1} enhancement of the BS phenotype for *eas* is modest. None of the double heterozygotes (*eas*/+; *kcc*^{DHS1}/+) are BS. However, there is genetic interaction because 64% of *eas*/+; *kcc*^{DHS1}/*kcc*^{DHS1} flies are BS (Fig. 4). This proportion is higher than that of *kcc*^{DHS1}/*kcc*^{DHS1} flies at this temperature (40%) (Fig. 1). The percent bang sensitivity of homozygous *kcc*^{DHS1}/*kcc*^{DHS1} 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)bw^{Dra}*, 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 mutations *kcc*^{DHS1}, *kcc*^{ML1}, CG5594^{EY08304}, *l(2)60A-C^{Ad4}*, and *l(2)60A-C^{P20-180}*. 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 *kcc*^{EY08304}, *kcc*^{Ad4}, and *kcc*^{P20-180}.

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 *kcc*^{EY08304}, *kcc*^{ML1}, and *kcc*^{DHS1} mutants (Fig. 6). The lethal *kcc*^{EY08304} 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 *kcc*^{EY08304} product also lacks a conserved tyrosine known to be essential in KCC2, KCC1, and KCC4 (Strange et al., 2000). The *kcc*^{ML1} allele is a deletion that removes 83 bp of 3'-UTR from the last *kcc* exon. The *kcc*^{DHS1} 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 *kcc*^{DHS1} 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 *kcc*^{DHS1} and *kcc*^{ML1} 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 *kcc*^{DHS1} and *kcc*^{ML1} mutants, respectively. Heterozygotes carrying one copy of the lethal *kcc*^{Ad4} allele and one wild-type allele also

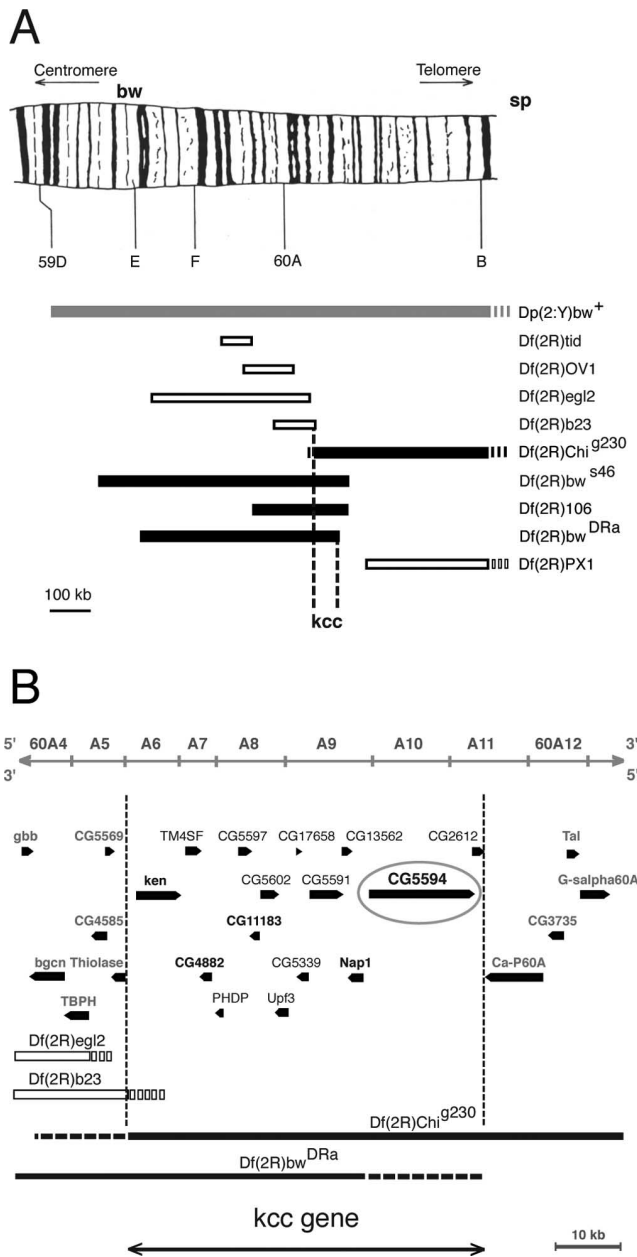


Figure 5. Genetic mapping of the *kazachoc* (*kcc*) gene. **A**, Initial recombination mapping experiments revealed that the *kazachoc* gene is located between the markers *bw* and *sp* on the distal tip of chromosome 2R. Subsequent duplication and deficiency mapping confirmed this result and further narrowed down the location of the *kcc* gene to a small cytological region (60A). A duplication of genomic DNA containing this region onto the Y chromosome (*Dp*(2; *Y*)*bw*⁺; 58F1-3; 60E11-12) produced a significant reduction in the bang sensitivity of homozygous *kcc*^{DHS1} flies, indicating the presence of the *kcc* gene on the duplicated segment (gray box). Four of the deficiencies examined failed to complement *kcc*^{DHS1} and therefore included the *kcc* gene (black boxes) (*Df*(2R)*bw*^{S46}, *Df*(2R)*Chi*^{g230}, *Df*(2R)*106*, and *Df*(2R)*DRa*), whereas five others fully complemented *kcc*^{DHS1} and therefore lacked the *kcc* gene (open boxes) (*Df*(2R)*tid*, *Df*(2R)*OV1*, *Df*(2R)*egl2*, *Df*(2R)*b23*, and *Df*(2R)*PX1*). The schematic drawing of chromosomal region 59D–60A has been reproduced with permission from Kurzik-Dumke et al. (1992). **B**, Superposition of the results of our deficiency mapping experiments onto a molecular map of the 60A cytological region (from Flybase) is shown. Deficiencies that uncover *kcc* are depicted as black boxes, and those that do not uncover *kcc* are depicted as white boxes; regions of uncertainty are shown as broken lines. The location of the *kcc* gene is bounded by the distal break points of *Df*(2R)*b23*, which does not uncover *kcc*, and those of *Df*(2R)*bw*^{DRa}, which uncovers *kcc*. Hence, the *kcc* gene maps to a 57 kb region of 60A, which carries 15 genes. The distal break points shown for *Df*(2R)*egl2* and *Df*(2R)*b23*, as well as the proximal break point for *Df*(2R)*Chi*^{g230}, are from Wharton et al. (1999). The position of the distal *Df*(2R)*bw*^{DRa} break point takes into account the observations that this deficiency fails to uncover *Ca-P60A* (Periz and Fortini, 1999) but does uncover lethal mutations in *CG4882*, *Nap1*, or *CG5594* (data not shown).

Table 2. Complementation tests reveal *kcc* is allelic to *CG5594* and *I(2)60A-C*

	<i>kcc</i> ^{DHS1}	<i>kcc</i> ^{ML1}	<i>CG5594</i> ^{EY08304}	<i>I(2)60A-C</i> ^{Ad4}	<i>I(2)60A-C</i> ^{P20-180}	+
<i>kcc</i> ^{DHS1}	44%	13%	42%	34%	12%	0%
<i>kcc</i> ^{ML1}		8%	41%	15%	0%	0%
<i>CG5594</i> ^{EY08304}			Lethal	Lethal	Lethal	0%
<i>I(2)60A-C</i> ^{Ad4}				Lethal	Lethal	0%
<i>I(2)60A-C</i> ^{P20-180}					Lethal	0%
+						0%

Heterozygous flies (*n* > 100) carrying the corresponding pair of mutations listed on the horizontal and vertical axes were examined for a bang-sensitive paralytic phenotype. The data reveal that mutations in *kcc*, *CG5594*, and *I(2)60A-C* fail to complement each other, indicating that they are alleles of the same gene. We propose calling this gene *kazachoc* (*kcc*) and renaming the *CG5594*^{EY08304}, *I(2)60A-C*^{Ad4}, and *I(2)60A-C*^{P20-180} alleles *kcc*^{EY08304}, *kcc*^{Ad4}, and *kcc*^{P20-180}, respectively.

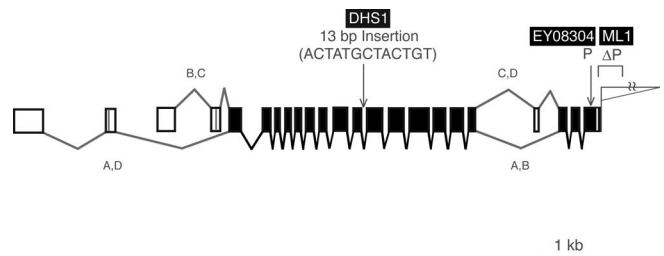


Figure 6. The *kcc* K⁺/Cl[−] cotransporter gene. Shown is a diagram of the *kcc* (*CG5594*) transcription unit. The gene is alternatively spliced at two points generating four possible transcripts. A database search revealed a large number of cDNAs corresponding to two of the alternative splice forms, A and B, in libraries prepared from *Drosophila* embryos or adult heads, respectively (data not shown). Positions of the *kcc*^{EY08304}, *kcc*^{ML1}, and *kcc*^{DHS1} mutations are indicated. The lethal *kcc*^{EY08304} mutation results from a P-element insertion into the last exon of the *CG5594* gene. The *kcc*^{ML1} mutation was produced by the imprecise excision of a nearby P(w⁺) element, which resulted in the deletion of 88 bp of genomic DNA. This deletion truncated the *kcc* 3′-UTR by 83 bp. The original *kcc*^{DHS1} mutation carries a 13 bp insertion (ACTATGCTACTGT) after the seventh base pair in the 11th intron of the *kcc* gene. Note that our diagram of the *kcc* gene lacks the large 412 transposable element within the 13th intron included in the Flybase annotation of *CG5594*, because sequencing of the genomic PCR products revealed no 412 element in any of the strains we examined.

show a threefold reduction in *kcc* protein relative to wild-type flies.

The level of *kcc* protein in the heads of *kcc*^{DHS1} flies progressively increases with developmental age (Fig. 9A). By 4 d after eclosion, the level of *kcc* in the heads of *kcc*^{DHS1} flies has risen ~13-fold. This rise in *kcc* level likely explains the progressive decrease in bang sensitivity of the *kcc*^{DHS1} 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_A receptor except that Rdl also displays strong expression in mushroom body (Aronstein and ffrench-Constant, 1995; Harrison et al., 1996).

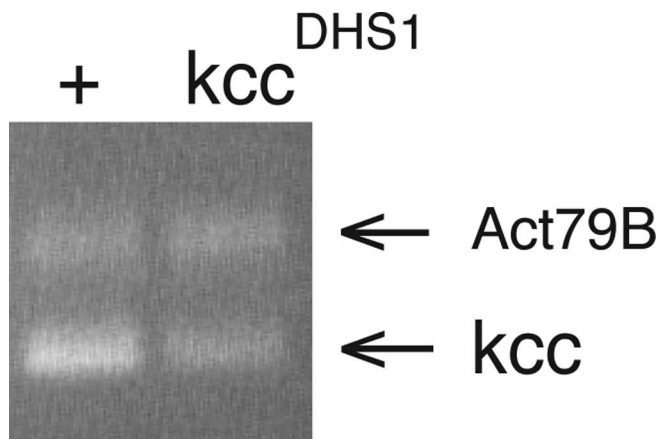


Figure 7. RT-PCR reveals a reduction in *kcc* transcript level in the *kcc^{DHS1}* mutant. Shown are the results of a RT-PCR analysis of RNA prepared from the heads of *kcc^{DHS1}* and control flies that used *kcc* primers located in exons that span the intron carrying the *kcc^{DHS1}* insertion. Positions of both the *kcc* and *Act79B* control bands (at 0.6 and 0.82 kb, respectively) are indicated with arrows. The heads of *kcc^{DHS1}* flies display a significant (2.3-fold) reduction in the level of *kcc* transcript. Sequence analysis of *kcc^{DHS1}* and wild-type RT-PCR products revealed no alteration in the splicing of intron 11 in the *kcc^{DHS1}* mutant (data not shown).

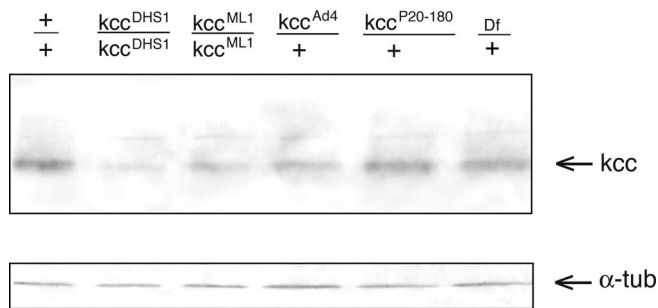


Figure 8. The *kcc* protein level is reduced in heads of *kcc* mutants. Shown is a Western blot of protein homogenates from the heads of wild-type (+/+) flies and various *kcc* mutants. The positions of both *kcc* and α -tubulin (α -tub) control bands are indicated by arrows. All of the strains produce an \sim 125 kDa *kcc* protein. However, the heads of flies homozygous for the seizure-sensitive *kcc^{DHS1}* mutation show a significant (\sim 4-fold) reduction in *kcc* protein. The heads of flies homozygous for the weaker *kcc^{ML1}* mutation also display a more modest (\sim 1.9-fold) reduction in *kcc* level. Flies heterozygous for the strong, lethal *kcc^{Ad4}* mutation show a threefold reduction in the level of *kcc* protein; those heterozygous for *kcc^{P20-180}* show a more modest (1.6-fold) reduction in *kcc* protein. Flies carrying one copy of *Df(2R)106*, which removes the *kcc* gene, and one wild-type *kcc* allele (lane 6) display the expected twofold reduction in *kcc* level, suggesting that the protein levels used for quantitation are within the linear range.

kcc-mediated seizure susceptibility requires GABAergic signaling

The *kcc* seizure phenotype depends on signaling via the GABA_A receptor suggesting that dysfunction is attributable to a disruption of the normal Cl[−] gradients that underlie GABAergic inhibition in the CNS. GABA_A receptor involvement was initially tested using PTX. PTX is a GABA_A receptor blocker and also a convulsant drug in mammals (Usunoff et al., 1969). PTX is also found here to act as a convulsant in wild-type *Drosophila*. Feeding wild-type flies PTX (1 mM) produces a low level of seizure susceptibility (1% BS) (Fig. 11A). This dose of PTX is ultimately lethal for wild-type flies with death occurring in 1.5 d ($t_{1/2}$). Feeding PTX to *kcc* flies produces a surprising result: PTX acts as an anticonvulsant rather than a convulsant. PTX (1 mM) reduces the level of BS paralysis of *kcc* flies: 16% of PTX-fed *kcc* flies are BS, whereas 38% of control-fed *kcc* siblings are BS. PTX feeding is less

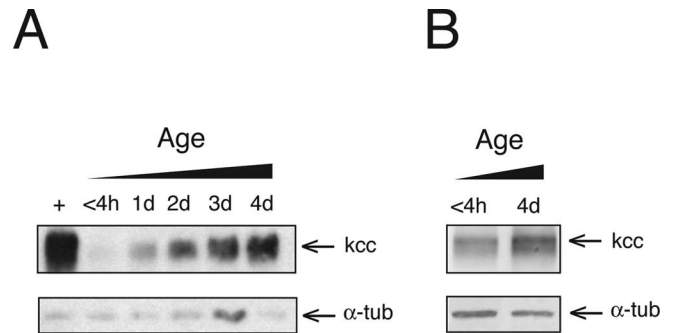


Figure 9. Both wild-type and *kcc^{DHS1}* mutant flies display an age-dependent increase in *kcc* protein. **A**, Western analysis of protein extracts from *kcc^{DHS1}* heads (lanes 2–6) reveals a progressive increase in *kcc* level in aging flies. After 4 d (lane 6), the level of *kcc* in *kcc^{DHS1}* heads is \sim 13-fold higher than that observed in the heads of newly eclosed flies (lane 2). Nonetheless, it is still significantly lower than that seen in 4-d-old wild-type flies (+; lane 1). **B**, Wild-type flies also show an increase (1.9-fold) in the level of *kcc* protein after the first 4 d of life. The loading control is again α -tubulin (α -tub).

toxic for *kcc* flies than for their wild-type counterparts: *kcc* flies die with a longer $t_{1/2}$ 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* (*Rdl¹*) 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 *Rdl¹* mutation (Fig. 11B). Here, 26% of homozygous *kcc^{DHS1}/kcc^{DHS1}* flies are BS. However, only \sim 1% of their *kcc^{DHS1}/kcc^{DHS1}; Rdl¹/+* siblings show the BS phenotype ($p < 0.001$). Reducing the dosage of the GABA biosynthetic enzyme glutamic acid decarboxylase via the *Gad1^{L352F}* null mutation similarly reduces the seizure sensitivity of *kcc* flies (Table 3). Whereas 28% of homozygous *kcc^{DHS1}/kcc^{DHS1}* flies are BS, only 3% of their *kcc^{DHS1}/kcc^{DHS1}; Gad1^{L352F}/+* 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,

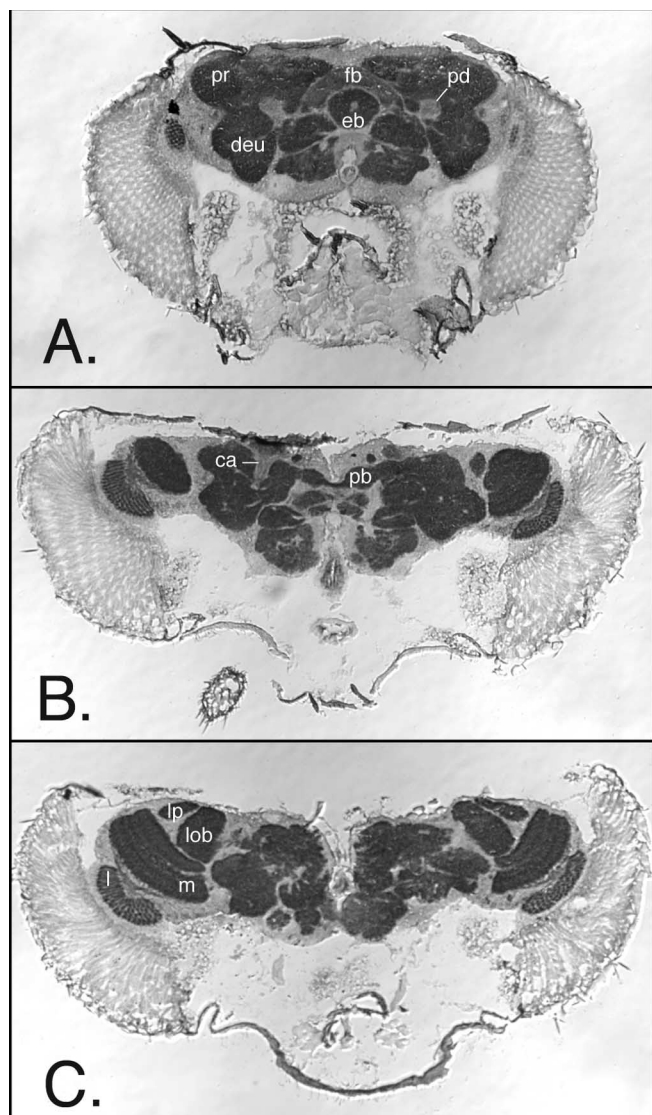


Figure 10. Immunohistochemical staining reveals *kcc* expression in brain neuropil. **A**, In this frontal section, *kcc* immunostaining is observed in much of the brain neuropil regions including the protocerebrum (pr) and deutocerebrum (deu), as well as the ellipsoid body (eb) and fan-shaped body (fb) of the central brain. In contrast, little staining is observed in the mushroom body peduncle (pd). **B**, This frontal section shows strong *kcc* immunostaining in the protocerebral bridge (pb) of the central brain. A low, but discernible, level of staining is observed in the mushroom body calyx (ca), particularly in the upper, dendritic region. **C**, This horizontal section reveals that *kcc* is expressed in all four optic neuropils of the optic lobe: lamina (l), medulla (m), lobula (lob), and lobula plate (lp), as well as the optic chiasma connecting the lamina and medulla. Distinct neuronal layers of immunostaining are particularly evident in the medulla. Magnification, 125 \times in all sections.

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^+/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

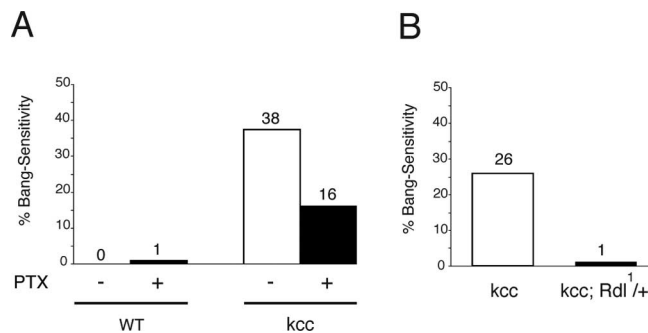


Figure 11. Reduced levels of GABA_A receptor suppress the *kcc* mutation. **A**, Feeding wild-type (WT) flies ($n = 120$) the convulsant PTX, which is a competitive inhibitor of the GABA_A receptor, produces a low-level bang sensitivity (1% BS). In contrast, PTX-fed *kcc*^{DHS1} flies ($n = 74$) displayed 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*^{1/+} 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*^{1/+} siblings ($n = 301$) were BS ($p < 0.05$).

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

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_A 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 sensitivity and enhance the seizure susceptibility of other seizure-sensitive mutations. If so, then PTX could act as an anticonvulsant by blocking GABA_A 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_A 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_A 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 Glauser, 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 $\alpha 1$ subunit of the GABA_A receptor (Wallace et al., 2001; Cossette et al., 2002), CICN2, a voltage-gated Cl⁻ channel primarily expressed in cerebral neurons inhibited by GABA (D'Agostino et al., 2004), and the KCNQ3 K⁺ 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 epilepsies (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 confirma-

Table 3. Reduced dosage of GABA_A receptor gene *Rdl* or GABA biosynthetic gene *Gad1* suppresses *kcc*^{DHS1} and some other BS mutations

Genotype	% Bang sensitivity				<i>p</i> value
	Test	(<i>n</i>)	Control	(<i>n</i>)	
<i>kcc</i> ^{DHS1} ; <i>Rdl</i> ^{1/+}	1	(301)	26	(231)	3.7 × 10 ⁻¹⁹
<i>bss</i> ^{1/+} ; <i>Rdl</i> ^{1/+}	53	(146)	78	(137)	1.0 × 10 ⁻⁵
<i>sda</i> ^{507.8/+} ; <i>Rdl</i> ^{1/+}	1	(144)	2	(80)	1.9 × 10 ⁻⁹
<i>eas</i> ^{PC20/+} ; <i>Rdl</i> ^{1/+}	0	(104)	0	(94)	
<i>kcc</i> ^{DHS1} ; <i>Gad1</i> ^{L352F/+}	3	(72)	28	(47)	1.0 × 10 ⁻⁴
<i>bss</i> ^{1/f/+} ; <i>Gad1</i> ^{L352F/+}	19	(145)	53	(110)	1.9 × 10 ⁻⁷
<i>eas</i> ^{PC20/+} ; <i>Gad1</i> ^{L352F/+}	0	(85)	0	(49)	

Genotypes for *Rdl*^{1/+} and *Gad1*^{L352F/+} test flies (labeled "Test") are indicated; control siblings (labeled "Control") carried the TM6B balancer chromosome rather than the *Rdl*¹ null allele of *Rdl*. Results are highly significant (*p* < 0.001) for all BS genotypes listed, except *eas*^{+/+}. *Rdl*^{1/+} produced slight (4%) suppression of hemizygous *bss* (*p* = 0.06).

tion (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*^{DHS1} and *kcc*^{ML1} 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 2). 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*^{DHS1} 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 *jbug* 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⁻. This could have the net effect of acting to sharpen or refine inhibitory GABA_A 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.

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