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Seizure Sensitivity Is Ameliorated by Targeted Expression of K^+–Cl^- Cotransporter Function in the Mushroom Body of the Drosophila Brain

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ABSTRACT

The *kcc*^{DHS1} allele of *kazachoc (kcc)* was identified as a seizure-enhancer mutation exacerbating the bang-sensitive (BS) paralytic behavioral phenotypes of several seizure-sensitive Drosophila mutants. On their own, young *kcc*^{DHS1} flies also display seizure-like behavior and demonstrate a reduced threshold for seizures induced by electroconvulsive shock. The product of *kcc* shows substantial homology to KCC2, the mammalian neuronal K^+–Cl^- cotransporter. The *kcc*^{DHS1} allele is a hypomorph, and its seizure-like phenotype reflects reduced expression of the *kcc* gene. We report here that kcc functions as a K^+–Cl^- cotransporter when expressed heterologously in *Xenopus laevis* oocytes: under hypotonic conditions that induce oocyte swelling, oocytes that express Drosophila kcc display robust ion transport activity observed as a Cl^--dependent uptake of the K^+ congener $^{86}Rb^+$. Ectopic, spatially restricted expression of a UAS-*kcc*$^+$ transgene was used to determine where cotransporter function is required in order to rescue the *kcc*^{DHS1} BS paralytic phenotype. Interestingly, phenotypic rescue is largely accounted for by targeted, circumscribed expression in the mushroom bodies (MBs) and the ellipsoid body (EB) of the central complex. Intriguingly, we observed that MB induction of *kcc*$^+$ functioned as a general seizure suppressor in Drosophila. Drosophila MBs have generated considerable interest especially for their role as the neural substrate for olfactory learning and memory; they have not been previously implicated in seizure susceptibility. We show that *kcc*^{DHS1} seizure sensitivity in MB neurons acts via a weakening of chemical synaptic inhibition by GABAergic transmission and suggest that this is due to disruption of intracellular Cl^- gradients in MB neurons.

MUSHROOM body (MB) expression of the *kazachoc (kcc)* K^+–Cl^- cotransporter is shown here to rescue seizure-sensitive phenotypes in Drosophila through an effect on GABAergic fast synaptic inhibition. Heretofore, considerable interest has focused on the MB because of its essential role in olfactory learning and memory (Heisenberg 2003; Davis 2005; Keene and Waddell 2007; Berry *et al.* 2008). The MB occupies a central position in the fly nervous system, integrating incoming olfactory, mechanical, taste, and visual sensory signals and then sorting the distribution of outgoing motor signals (Heisenberg 2003). Short- and long-term alteration of individual nerve cell physiology in the MB is thought to form the basis of learning and memory (Davis 2005; Keene and Waddell 2007; Berry *et al.* 2008). A role for the MB in seizure susceptibility has not previously been suspected. Here we suggest that the orderly arrangements of axons and neuropile of MB Kenyon cells (KCs) not only facilitate learning and memory, but also provide the type of anatomical substrate in flies that is thought to be essential for seizure spread in the mammalian brain (Hauser and Hesdorffer 1990; Traub and Miles 1991).

Inhibitory synaptic transmission in Drosophila is thought to be mediated primarily by GABAergic neurons found throughout the CNS at all stages of development (Buchner *et al.* 1988; Jackson *et al.* 1990; Harrison *et al.* 1996; Yasuyama *et al.* 2002). γ-aminobutyric acid (GABA) is synthesized from glutamate via a conserved glutamic acid decarboxylase encoded by the Drosophila *Gad1* gene (Jackson *et al.* 1990; Buchner 1991). GABAergic activity is limited by sequestering extracellular GABA back into presynaptic neurons by GABA transporters that are sensitive to inhibition by DL-2,4-diaminobutyric acid, nipecotic acid, and valproic acid (Neckameyer and Cooper 1998; Leal *et al.* 2004). Three ionotropic $GABA_A$ receptor subunits have been identified in Drosophila

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and are encoded by the *Rdl*, *LCCH3*, and *GRD* loci (Hosie *et al.* 1997). When expressed heterotopically in Xenopus oocytes, the best studied of these, *Rdl,* forms GABA-gated Cl^- channels that are sensitive to block by picrotoxin (ffrench-Constant *et al.* 1991, 1993; Zhang *et al.* 1995). Inhibitory Cl^- currents are dependent on maintenance of Cl^- gradients, particularly in low intracellular Cl^- concentrations. In the fly, Cl^- gradients appear to be maintained by the kcc K^+–Cl^- cotransporter (Hekmat-Scafe *et al.* 2006).

Chemical synaptic transmission onto MB neurons has been examined in dissociated KCs in primary culture (Su and O'dowd 2003). Spontaneous miniature excitatory postsynaptic currents (EPSCs) are mediated mainly by nicotinic acetylcholine (ACh) receptors. Miniature inhibitory postsynaptic currents (IPSCs) appear to be mediated primarily by picrotoxin-sensitive $GABA_A$ receptors, probably encoded by *Rdl* (Su and O'dowd 2003; Harrison *et al.* 1996). *In vivo,* cholinergic inputs to the MB are thought to arise primarily from antennal lobe projection neurons (Yasuyama *et al.* 2002). Two antennal lobe neurons that project to the MB, the anterior paired lateral (APL) neurons, were recently shown to be GABAergic (Liu and Davis, 2009). Additional GABAergic inputs to the Drosophila MB seem likely; in locust they appear to come from a poorly understood region of the brain called the lateral horn, which is itself also driven by antennal lobe projection neurons (Perez-Orive *et al.* 2002).

Previously, we identified the kcc^{DHS1} *partial loss-of-function* mutation as a seizure enhancer that also causes increased seizure sensitivity in young flies (Hekmat-Scafe *et al.* 2006). The *kcc* product shows homology to the mammalian KCC2 K^+–Cl^- cotransporter, and we inferred that a decrease in inhibitory synaptic strength is responsible for causing the seizure phenotypes. In this study, we describe our search for identifying the source of these vulnerable inhibitory synapses and report that they appear to lie primarily in the MBs of the Drosophila brain. Further, we speculate on the possibility of their involvement in synaptic plasticity functions of the MB.

MATERIALS AND METHODS

Molecular biology: The *kcc* gene has two major alternative splice forms: the B form, which is found in adult heads, and the D form, which is found in embryos (Hekmat-Scafe *et al.* 2006). Plasmids GH09271 and LD02554, carrying cDNAs for *kcc-B* and *kcc-D*, respectively, were obtained from the Drosophila Genomics Resource Center. A 3.6-kb *Bgl*II/*Xho*I *kcc-B* cDNA fragment and 3.9-kb *Xba*I/*Hin*dIII *kcc-D* cDNA fragment were subcloned into the Xenopus expression vector pGEMHE (Mount *et al.* 1999) using standard methods (Sambrook and Russell 2001) to create expression plasmids pDH153 and pDH154, respectively. Expression constructs for human KCC2 (hKCC2) and mouse KCC4 in pGEMHE have been described previously (Song *et al.* 2002). To prepare cRNA for injection, Qiagen-purified cDNA constructs were linearized at the 3′-end using *Nhe*I, and cRNA was transcribed *in vitro,* using the T7 RNA polymerase mMESSAGE kit (Ambion). RNA integrity was confirmed on agarose gels, and concentration was determined by absorbance reading at 260 nm. cRNA was stored frozen in aliquots at −80° until used.

The P{w^+ UAS-kcc-B} Drosophila *P*-element transformation vector pDH156 was created by subcloning a 3.6-kb *Bgl*II/*Xho*I *kcc-B* cDNA fragment from GH09271 into pUAST (Brand and Perrimon 1993). The P{w^+ UAS-kcc-D} plasmid pDH157 was constructed by subcloning a 3.9-kb *Not*I/*Xho*I *kcc-D* cDNA fragment from LD02554 into pUAST. A P{w^+} plasmid carrying the human KCC2 gene (hKCC2) downstream of GAL4 UAS repeats (pDH159) was produced by subcloning a 3.4-kb *Eco*RI/*Xba*I fragment of hKCC2 cDNA (Song *et al.* 2002) into pUAST. Transgenic w^{1118} flies carrying either pDH156, pDH157, or pDH159 were created by standard *P*-element-mediated transformation procedures (Spradling and Rubin 1982) using Qiagen-purified DNA.

Measurement of K^+–Cl^- cotransport: Adult female *Xenopus laevis* were purchased from NASCO (Fort Atkinson, MI) and maintained in a Marine Biotech XR3 system (New Bedford, MA) under controlled light conditions at a water temperature of 16–18°. Oocytes were surgically collected from animals anesthetized by 0.17% tricaine immersion; after several such procedures, anesthetized frogs were killed by cardiac puncture. The use and care of the animals in these experiments were approved by the Institutional Animal Care and Use Committee at Harvard Medical School. After extraction, oocytes were incubated for 1 hr with vigorous shaking in a Ca^{2+}-free ND96 medium (96 mm NaCl, 2 mm KCl, 1 mm MgCl, and 5 mm HEPES/Tris, pH 7.4, plus 2 mg/ml collagenase A). Oocytes were then washed four times in regular ND96, defolliculated by hand, and incubated overnight in ND96 at 16°. Mature oocytes were injected with 50 μl of water or with water containing 0.5 μg/μl of cRNA transcribed *in vitro* from the various KCC constructs. Oocytes were incubated for 4–5 days prior to transport assays, in ND96 at 16° supplemented with 2.5 mm sodium pyruvate and 5 mg/100 ml of gentamicin.

K^+–Cl^- cotransport was assessed by measuring Cl^--dependent uptake of $^{86}Rb^+$ (Perkin-Elmer Life Sciences, Norwalk, CT), a congener of K^+, in *X. laevis* oocytes as described previously (Mount *et al.* 1999; Song *et al.* 2002; Mercado *et al.* 2006). Rubidium uptake was assessed 2–3 days after injection under both isotonic and hypotonic conditions, as noted. A 30-min incubation period in a Na^+- and Cl^--free medium (50 mm *N*-methyl-d-glucamine gluconate, 10 mm K^+ gluconate, 4.6 mm Ca^{2+} gluconate, 1 mm Mg^{2+} gluconate, 5 mm HEPES/Tris, pH 7.4) containing ouabain (1 mm) was followed by a 60-min uptake period in a Na^+-free medium (mm: 50 *N*-methyl-d-glucamine-Cl, 10 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES/Tris, pH 7.4) supplemented with ouabain and 2.5 μCi of $^{86}Rb^+$ per milliliter (PerkinElmer Life Sciences). Isotonic conditions were generated by supplementing the same solutions with 3.5 g/100 ml sucrose to reach isosmolar conditions for oocytes (∼210 mosmol/kg). Ouabain was added to inhibit $^{86}Rb^+$ uptake via Na^+–K^+–ATPase, and removal of extracellular Na^+ prevented $^{86}Rb^+$ uptake via the endogenous Na^+–K^+–$2Cl^-$ cotransporter. All uptakes were performed at 32°. At the end of the uptake period, oocytes were washed three times in ice-cold uptake solution without isotope to remove extracellular fluid tracer. Oocytes were dissolved in 10% SDS, and tracer activity was determined for each oocyte by beta-scintillation counting. The uptake experiments all included at least 15 oocytes in each experimental group; statistical significance was defined as two-tailed $P < 0.05$, and results were reported as means ±SE. All of the transport experiments shown were performed several times with the appropriate controls (water-, KCC2-, and/or KCC4-injected groups).

Fly stocks: A list of Drosophila stocks used is given in Table 1. Stocks were maintained on standard cornmeal–molasses medium at ~24°). The kcc^{DHS1} mutation is a 13-bp insertion in intron 11 of *kcc* leading to an approximate twofold reduction in transcript and an approximate fourfold reduction in protein; phenotypically it behaves as a hypomorph (Hekmat-Scafe *et al.* 2006). The X-linked *eas* gene encodes an ethanolamine kinase; the recessive eas^{PC80} allele carries a frameshift mutation and is believed to be a null (Pavlidis *et al.* 1994). The *bss* gene is located at 1-54.6 (corresponding to approximately cytological region 12F); the identity of its gene product has not been published (Ganetzky and Wu 1982). The bss^1 allele is a semidominant mutation.

The 1407, Rdl-GAL4-2-1, Gad1-GAL4 (Ng *et al.* 2002), 104Y and c232 (Renn *et al.* 1999), GH146 (Stocker *et al.* 1997), MB247 and c772, and c305a (Krashes *et al.* 2007) GAL4 drivers were obtained from Nara Muraro and Richard Baines (University of Manchester), Julie Simpson (Janelia Farms), Gero Miesenboeck (Yale University), Roland Strauss (University of Wuerzburg), Gautam Agarwal and Ehud Isacoff (University of California, Berkeley), Christopher Tabone and J. Steven de Belle (University of Nevada, Las Vegas), and Scott Waddell (University of Massachusetts Medical School), respectively; the remaining GAL4 drivers were obtained from the Bloomington Drosophila Stock Center. The kcc^{DHS1} stocks carrying a GAL4 driver on the second chromosome (D672, D590, D663, D695, D589, D689, D588, D643, and D658) were created as follows: D506 virgin females were crossed to males carrying the GAL4 driver. Non-CyO virgin female progeny were then crossed to D506 males and recombinant *w;* Driver-GAL4 kcc^{DHS1}/kcc^{DHS1} male progeny identified by orange eyes and bang sensitivity. A single recombinant male was crossed to D245 virgin females and orange-eyed *w;* Driver-GAL4 kcc^{DHS1}/*CyO* male and virgin female progeny used to establish a balanced stock. We created kcc^{DHS1} stocks carrying a GAL4 driver on the third chromosome (D586, D587, D670, D662, and D661) by crossing U036 virgin females to Driver-GAL4 males. Several *w; +/SM5 Cy;* Driver-GAL4/*TM3* males were then crossed to D579 virgin females. Finally, *w;* kcc^{DHS1}*/SM5 Cy;* Driver-GAL4/*TM6B* male and virgin female progeny from this cross were combined to establish a balanced stock; when possible, a homozygous Driver-GAL4 version of the stock lacking the TM6B balancer was also created. The kcc^{DHS1} stock carrying OK107 (D642) was constructed by first crossing D245 virgin females to a *w;;;OK107* stock, then crossing *w; +/CyO;;OK107/+* male progeny from this cross to homozygous *w;* kcc^{DHS1} D506 virgin females and finally combining *w;* kcc^{DHS1}*/CyO;;OK107/+* male and virgin female progeny from this cross to establish a stock. The MB-GAL80 flies were from Scott Waddell (University of Massachusetts Medical School); flies carrying the *Ncc69* RNAi (Dietzl *et al.* 2007) and *Rdl* RNAi (Liu *et al.* 2007) constructs were obtained from the Vienna Drosophila RNAi Center and from Xu Liu and Ron Davis (Baylor University Medical College), respectively; and UAS-TNT flies were obtained from Christopher Tabone and J. Steven de Belle (University of Nevada, Las Vegas). Stocks carrying kcc^{DHS1} along with each of these transgenes were created essentially as described for the kcc^{DHS1} + Driver-GAL4 lines above. The Rdl^1 (ffrench-Constant *et al.* 1991), $Rdl^{MD\text{-}RR}$ (ffrench-Constant *et al.* 1993), $Ncc69^{PL00618}$, and $Df(3L)eyg^{C1}$ were obtained from the Bloomington Drosophila Stock Center. Strains carrying kcc^{DHS1} along with one of these third chromosomal mutations were created as in the same manner as described for the third chromosomal GAL4 driver stocks.

BS behavioral testing: Testing for BS paralysis was performed on flies <1 day posteclosion except those involving the late-acting c772 driver (Yang *et al.* 1995; Armstrong *et al.* 1998), which were tested at 24–36 hr posteclosion. Flies were typically collected at <17 hr posteclosion, anesthetized with CO_2, transferred to fresh food vials in groups of ~15 for 6–17 hr, and then tested by vortexing at maximum speed for 10 sec. BS flies displayed a characteristic period of paralysis followed by a period of hyperactivity resembling seizure-like behavior.

Crosses were performed at 23° if kcc^{DHS1} progeny carrying a GAL4-driven transgene were being tested for BS behavior. This temperature is a compromise between 22°, which is ideal for discerning the BS phenotype of the cold-sensitive kcc^{DHS1} mutation (Hekmat-Scafe *et al.* 2006), and 25–29°, which is optimal for GAL4 function (Duffy 2002). Ordinarily, virgin females carrying the kcc^{DHS1} mutation, as well as a particular GAL4 driver, were crossed to males carrying the kcc^{DHS1} mutation and a UAS-*kcc* transgene over a TM6B balancer chromosome and the percentage bang sensitivity of ~200 kcc^{DHS1} + Driver-GAL4 + UAS-*kcc* test progeny compared to that of their kcc^{DHS1} + Driver-GAL4 control siblings. Initially, two to three independent third chromosomal insertions of each of the three transgenic plasmids (pDH156=UAS-kcc-B, pDH157=UAS-kcc-D and pDH159=UAS-hKCC2) were examined in this manner using the elav, REPO, and c739 drivers, and the insertion that gave the greatest degree of suppression (pDH156-3, pDH157-7, and pDH159-2, respectively) used in all subsequent experiments. Crosses used to assay suppression of kcc^{DHS1} by other chromosomal mutations (shown in Figure 7,

TABLE 1

Drosophila stocks

Stock no.	Genotype
CS-5	Wild type
D506	*w;* kcc^{DHS1}*/CyO*
D634	*w;* kcc^{DHS1}*/SM5 Cy; pDH156{*w^+ *UAS-kcc-B}-3/TM6B*
D635	*w;* kcc^{DHS1}*/SM5 Cy; pDH157{*w^+ *UAS-kcc-D}-7/TM6B*
D637	*w;* kcc^{DHS1}*/SM5 Cy; pDH159{*w^+ *UAS-hKCC2}-2/TM6B*
D696	*w; MB-GAL80* kcc^{DHS1}*/SM5 Cy; pDH156{*w^+ *UAS-kcc-B}-3/TM6B*
D586	*w;* kcc^{DHS1}*/SM5 Cy;* $elav\text{-}GAL4^{3A}$
D672	*w; 1407* kcc^{DHS1}*/CyO*
D587	*w;* kcc^{DHS1}*/SM5 Cy; REPO-GAL4/TM6B*
D670	*w;* kcc^{DHS1}*/SM5 Cy; Rdl-GAL4-2-1*
D590	*w; Gad1-GAL4* kcc^{DHS1}*/CyO*
D662	*w;* kcc^{DHS1}*/SM5 Cy; c232*
D663	*w; 104Y* kcc^{DHS1}*/CyO*
D695	*w; GH146* kcc^{DHS1}*/CyO*
D589	*w; Cha-GAL4* kcc^{DHS1}*/CyO*
D642	*w;* kcc^{DHS1}*/CyO;;OK107/+*
D661	*w;* kcc^{DHS1}*/SM5 Cy; MB247*
D689	*w; c772* kcc^{DHS1}*/CyO*
D588	*w; c739* kcc^{DHS1}*/CyO*
D643	*w; c305a* kcc^{DHS1}*/CyO*
D658	*w; 201Y* kcc^{DHS1}*/CyO*
5137	y^1 *w; P{w[+mC]=UAS-mCD8::GFP.L}LL5*
D629	*w; c739* kcc^{DHS1}*; +/TM6B*
D622	*w; c739* kcc^{DHS1}*; pDH156{*w^+ *UAS-kcc-B}-3/TM6B*
D623	*w; c739* kcc^{DHS1}*; pDH159{*w^+ *UAS-hKCC2}-2*
D690	*w;* kcc^{DHS1}*/SM5 Cy; P{GD14683=UAS-Ncc69-RNAi}v30000/TM6B*
D691	*w;* kcc^{DHS1}*/SM5 Cy; Rdl-RNAi-8-10G/TM6B*
D700	*w; UAS-TNT* kcc^{DHS1}*/CyO*
D610	*w; c739;; pDH156{*w^+ *UAS-kcc-B}-3/TM6B*
MR047	*w* eas^{PC80} *f*
MR068	*w* bss^1 *f*

B–C) were performed at 22°, and those used to monitor suppression of *eas* and *bss*/+ by ectopic mushroom body expression of kcc^+ (Figure 8) were performed at 25°. The chi-square test was used to determine the P-values for differences in percentage BS between test and sibling control flies. The relative percentage BS of test flies was determined by setting the level for control flies at 100%.

Electrophysiology: Flies were raised at 23° and collected less than 24 hr posteclosion for electrophysiological testing. Brain stimulation and recording of both giant fiber (GF)-driven muscle potentials and seizures was performed as described previously (Hekmat-Scafe *et al.* 2006). Electrical stimuli were delivered to the fly's brain using bipolar tungsten 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 muscle (DLM) using a tungsten recording electrode. Seizure-like spiking is observed in at least seven different muscle groups and over 30 muscle fibers in the thorax that reflect the HF firing of the innervating motoneurons (Kuebler and Tanouye 2000). Seizure-like activity was evoked 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. Seizure threshold was defined as the lowest intensity HF stimulus required to elicit seizure-like activity. A Student's two-tailed *t*-test was used to determine the P-values for differences in seizure threshold of the D622 and D623 test flies relative to that of the D629 control flies.

GFP monitoring of GAL4 expression patterns: Flies carrying each of the GAL4 drivers in combination with a UAS-mCD8::GFP reporter were obtained by crossing BL5137 virgin females to males bearing a particular GAL4 driver at 25°. For each Driver-GAL4/GFP combination, progeny were anesthetized with CO_2 <24 hr posteclosion and brains from several female progeny were harvested into HL3 buffer (Stewart *et al.* 1994). The brains were then imaged with an Andor IQ CCD camera (Andor, Belfast, Ireland) and a BX-50WI microscope with a 75-W Xenon lamp and a 10× 0.3 NA objective (Olympus). Excitation was done at 470 ± 20 nm with a 495LP dichroic and images (515 ± 15 nm emission) acquired for 0.2 sec. At least two brains were independently imaged for each GAL4 driver.

RESULTS

Drosophila *kcc* functions as a K^+–Cl^- cotransporter: Drosophila carry a single gene, *kcc* whose product displays structural features common to mammalian K^+–Cl^- cotransporters, KCC1-4 (Filippov *et al.* 2003). Alternative splicing of the *kcc* gene results in two major kcc isoforms: kcc-B, which displays widespread expression in the adult brain, and kcc-D, which is enriched in embryos and larvae (Hekmat-Scafe *et al.* 2006). The kcc-B and kcc-D isoforms differ in their N termini; kcc-D also encodes 31 extra amino acids near its C terminus in a region corresponding roughly to that of a 100-amino-acid insertion present in KCC2, but not the other mammalian KCCs (Hekmat-Scafe *et al.* 2006). To demonstrate that Drosophila *kcc* encodes functional cotransporters, *kcc* cRNAs were injected and their products heterologously expressed in Xenopus oocytes (Figure 1). Oocytes injected with *kcc-B* cRNA showed robust transport, as indicated by Cl^--dependent uptake of the K^+ congener, $^{86}Rb^+$ (Figure 1A). $^{86}Rb^+$ uptake by *kcc-B* is comparable to, although somewhat less than,

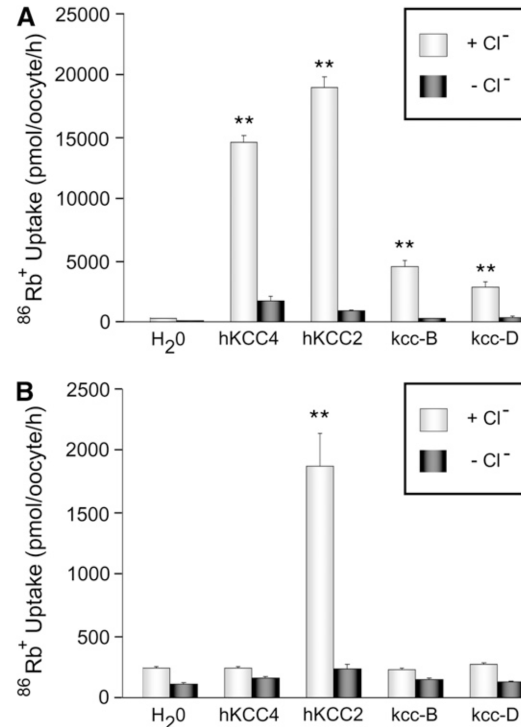

Figure 1.—Drosophila *kcc* mediates K^+–Cl^- cotransport when expressed in Xenopus oocytes. (A) Xenopus oocytes microinjected with *kcc-B*, *kcc-D*, or control (human KCC2 or KCC4) cRNA all demonstrate uptake of $^{86}Rb^+$ (a congener of K^+) under hypotonic conditions. This $^{86}Rb^+$ uptake is Cl^--dependent as it is observed in the presence (+Cl^-, lightly shaded bars), but not in the absence (−Cl^-, dark-shaded bars), of extracellular Cl^-. Control oocytes microinjected with H_20 (left) showed no Cl^--dependent uptake of $^{86}Rb^+$ under hypotonic conditions. (**) $P < 0.0001$ *vs.* water-injected control oocytes. (B) Xenopus oocytes microinjected with neither the *kcc-B* nor the *kcc-D* cRNA displayed uptake of $^{86}Rb^+$ under isotonic conditions that was significantly higher in the presence of Cl^- (+Cl^-, light-gray bar) than in its absence (−Cl^-, dark-shaded bar). Control oocytes microinjected with either H_20 or human KCC4 cRNA also showed no Cl^--dependent uptake of $^{86}Rb^+$. In contrast, Xenopus oocytes microinjected with cRNA for human KCC2 demonstrate a robust Cl^--dependent uptake of $^{86}Rb^+$ under isotonic conditions. (**) $P < 0.0001$ *vs.* water-injected control oocytes

that of human KCC2 and KCC4 positive controls (Figure 1A). As with the positive controls, kcc-B cotransport activity is induced under hypotonic conditions that induce oocyte swelling indicating stretch-activated activity. Cotransport activity is similarly observed by heterologous expression of the kcc-D isomer. Neither Drosophila kcc isoform conferred constitutive K^+–Cl^- cotransport activity on microinjected oocytes subjected to isotonic conditions (Figure 1B). As reported previously, control oocytes expressing human KCC2 exhibited considerable K^+–Cl^{--} cotransport activity under isotonic conditions (Figure 1B; Song *et al.* 2002).

Ectopic kcc^+ expression in neurons rescues BS phenotypes: GAL4 drivers (Brand and Perrimon 1993) were used to ectopically express UAS-kcc^+ to

determine where cotransporter function is required to rescue the BS paralytic phenotype of kcc^{DHS1} mutants. The BS phenotype is substantially reduced (<50% BS relative to their respective sibling control groups) by expression of *kcc-B* using a pan-neuronal driver, such as elav-GAL4[3A] or 1407, whereas expression of *kcc-B* in glia (REPO-GAL4) produces no apparent change in BS phenotype (Figure 2A). We interpret this to mean that *kcc-B* is required in neurons, but not in glia, to reduce BS behavior. Rescue of BS phenotypes by *kcc-D* expression was notably weaker than that of *kcc-B* (Figure 2B), and subsequent experiments described in this article focus only on the *kcc-B* splice form, utilizing "kcc^{+}" to refer to this product.

Expression of kcc^{+} in discrete neuronal subpopulations provides further insight into the neuronal circuitry underlying seizure susceptibility (Figures 2C and 3). Expression of kcc^{+} in either of two brain regions, the MB (OK107 driver) and the ellipsoid body (EB) of the central complex (c232 driver), produces marked reductions in BS phenotype (30% BS for OK107, 17% BS for c232, relative to sibling controls). Expression of kcc^{+} in the nearby fan-shaped body of the central complex (104Y driver) or in antennal neurons projecting to the MB (GH146 driver) produces only modest reductions in BS phenotype (76% BS for 104Y, 63% BS for GH146, relative to sibling controls). These results suggest that expression of kcc^{+}, and by extension, the maintenance of low intracellular Cl^{-} concentrations, in the MB and EB is especially important in reducing the overall seizure susceptibility of the Drosophila brain. The MBs are plastic structures associated with learning and memory (reviewed in, *e.g.,* Heisenberg 2003; Davis 2005; Keene and Waddell 2007; Berry *et al.* 2008), analogous to the mammalian hippocampus, a frequent site of epileptic foci (Hauser and Hesdorffer 1990). In subsequent experiments, we have examined more closely the link between expression of kcc^{+} in the Drosophila MB and seizure sensitivity.

kcc functions in MB neurons to reduce the BS phenotype: Much of the reduction in seizure susceptibility of kcc^{DHS1} flies observed when a kcc^{+} transgene is expressed in all neurons appears to be solely attributable to its effect in MB neurons (Figure 4). This is shown most clearly by utilizing the GAL4 inhibitor, GAL80, to exclude kcc^{+} expression from MB neurons, but to allow it in other neurons. When this is done in kcc^{DHS1} flies with kcc^{+} concurrently driven by elav-GAL4, suppression of BS behavioral paralysis is significantly compromised (35% BS without GAL80 inhibition, 53% BS with GAL80 inhibition, relative to sibling controls).

Each MB consists of ~2000 KC neurons with dendrites in the calyx region and axonal extensions into α/β, α'/β' or γ lobes (Berry *et al.* 2008; Aso *et al.* 2009). Ectopic expression of kcc^{+} in the entire MB using any of three GAL4 drivers OK107, MB247, or c772 markedly reduces the BS phenotype of kcc^{DHS1} mutant flies (30, 32,

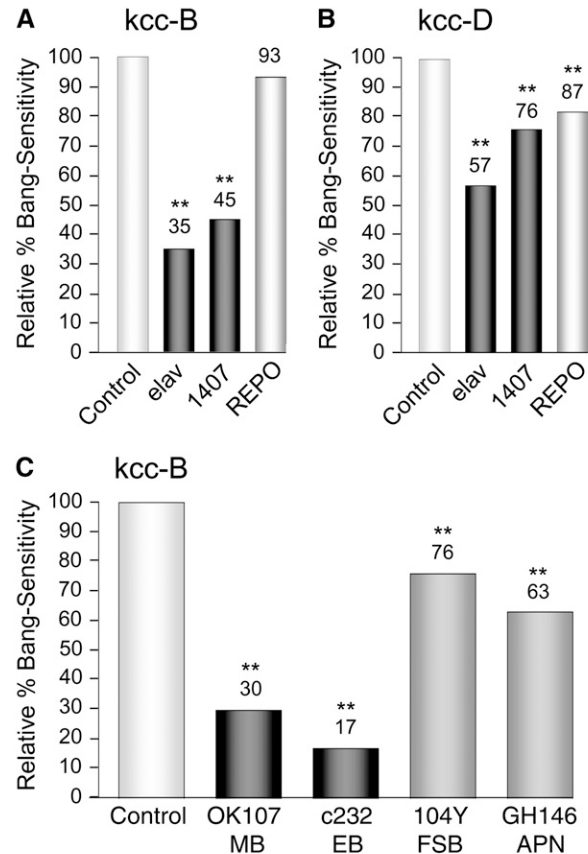

Figure 2.—Neuroanatomic mapping of *kcc* function in the Drosophila brain. (A) Expression of a UAS-*kcc-B* transgene in all neurons of an otherwise kcc^{DHS1} fly using either of two pan-neuronal GAL4 drivers (elav or 1407, dark-shaded bars) markedly reduces its bang-sensitive paralytic phenotype, a behavioral indicator of seizure susceptibility. On the other hand, expression of the UAS-*kcc-B* transgene in all gila using the REPO GAL4 driver (lightly shaded bar, right) does not produce any significant change in the degree of bang sensitivity. This suggests that *kcc* normally functions in neurons, rather than glia, to reduce susceptibility to seizures. (B) Expression of a UAS-*kcc-D* transgene in all neurons of a kcc^{DHS1} fly using a pan-neuronal GAL4 driver (dark-gray bars) produces modest rescue of its bang-sensitive phenotype; expression in glia (lightly shaded bar, right) produces slight, but statistically significant, suppression. (C) Expression of the UAS-*kcc-B* transgene in various subpopulations of brain neurons reduces their bang sensitivity. Expression of the wild-type *kcc-B* gene in the neurons targeted by either of two GAL4 drivers (OK107, mushroom body, MB, neurons; and c232, ellipsoid body, EB, neurons) produced marked reductions in the bang sensitivity (dark-shaded bars; 30 and 17% relative bang sensitivity, respectively). Expression of kcc^{+} in neurons targeted by two other GAL4 drivers (104Y, fan-shaped body, FSB; GH146, antennal projection neurons, APN) resulted in more modest reductions in bang sensitivity (right bars; 76 and 63% relative bang sensitivity, respectively). In all of these experiments, virgin females carrying the kcc^{DHS1} mutation as well as a particular GAL4 driver (*i.e.*, D586, D672, D587, D662, D663, D695, D642, D661, D689, D588, D643, or D658) were crossed to either D634 or D635 males carrying the kcc^{DHS1} mutation and a UAS-*kcc-B* or UAS-*kcc-D* transgene, respectively, over a TM6B balancer chromosome. The percentage bang sensitivity of the kcc^{DHS1} + Driver-GAL4 + UAS-kcc^{+} test progeny are given relative to that of their kcc^{DHS1} + Driver-GAL4 control siblings. $N > 180$; (**) $P < 0.001$.

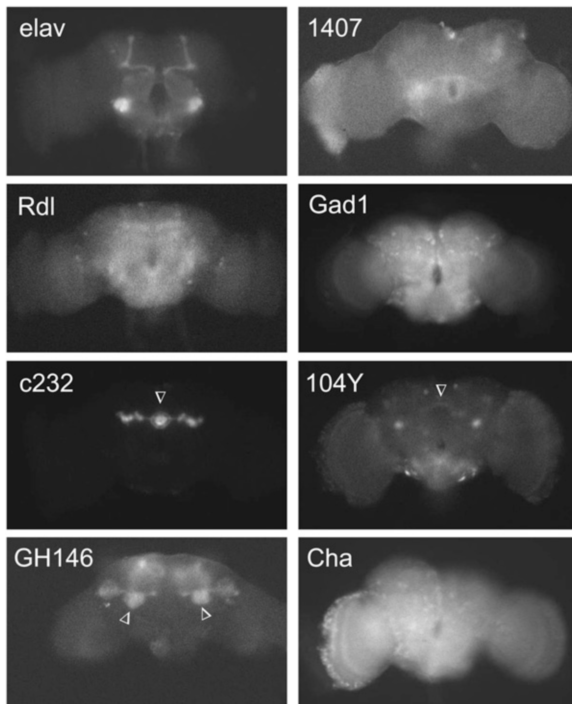

Figure 3.—Expression patterns of neuronal GAL4 drivers used for neuroanatomical mapping of *kcc* function. Shown is the fluorescence observed upon excitation at 470 nm when a UAS-mCD8::GFP reporter is combined with each of the GAL4 drivers indicated. Both elav and 1407 display pan-neuronal expression; the apparent enrichment of elav-GAL4 signal in mushroom bodies is an artifact of the mCD8-based membrane targeting and has been observed previously (Ito *et al.* 2003). The Rdl driver produces relatively widespread expression in neurons carrying the Rdl $GABA_A$ receptor. The Gad1 and Cha drivers display abundant expression in GABAergic and cholinergic interneurons, respectively (Salvaterra and Kitamoto 2001; Ng *et al.* 2002)). Three of the other GAL4 drivers produce relatively circumscribed expression patterns (arrowheads): c232 (ellipsoid body; Renn *et al.* 1999), 104Y (scatterned neurons, including those in the fan-shaped body; Renn *et al.* 1999) and GH146 (antennal lobe neurons, which project to the mushroom body) (Stocker *et al.* 1997). The expression pattern for the OK107 driver is shown with those of the other mushroom body drivers in Figure 5B. The representative brains are from 1- to 2-day-old female flies and are positioned such that the anterior side is facing the camera; dorsal is up and ventral down.

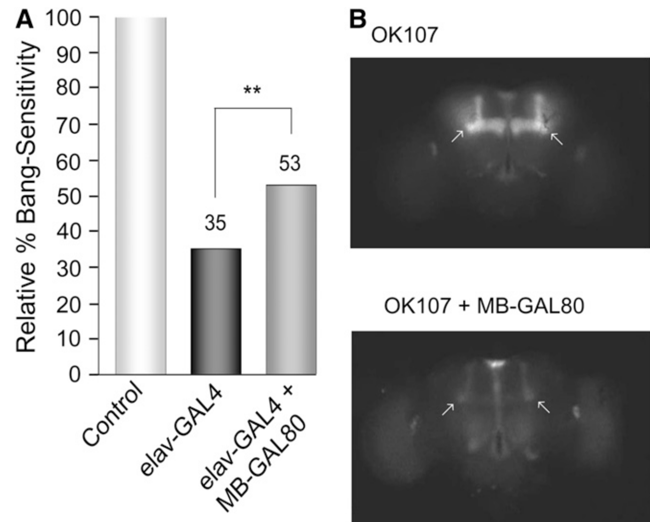

Figure 4.—*kcc*'s neuronal function reflects a significant contribution in mushroom bodies. (A) The reduction in seizure susceptibility of kcc^{DHS1} flies observed when a kcc^+ transgene is expressed in all neurons with an elav GAL4 driver (35% relative bang sensitivity, dark-shaded bar) is significantly reduced when kcc^+ induction in mushroom body is blocked due to the presence of a MB-GAL80 construct (53% relative bang sensitivity, medium-shaded bar). In these experiments virgin females carrying the kcc^{DHS1} mutation and elav-GAL4 +/− MB-GAL80 (D586 or D696, respectively) were crossed to D634 males carrying the kcc^{DHS1} mutation and a UAS-kcc^+ transgene over a TM6B balancer chromosome. The percentage bang sensitivity of the kcc^{DHS1} + elav-GAL4 +/− MB-GAL80 + UAS-kcc^+ test progeny is given relative to that of their kcc^{DHS1} + elav-GAL4 control +/− MB-GAL80 siblings. $n > 230$; (**) $P < 0.001$. (B) The MB-GAL4 construct (described in Krashes *et al.* 2007) largely eliminates GAL4-driven expression in the MB. Combination of the OK107 driver with the UAS-mCD8::GFP reporter (top) reveals robust expression in the MB (arrows); MB expression (arrows) is markedly reduced in the presence of MB-GAL4 (bottom), which represses GAL4 function specifically in the MB.

or 23% relative BS, respectively). Similar reductions in BS phenotype are observed when kcc^+ expression is restricted to KCs displaying specific axonal branches (c739—α/β lobes, 35% BS; c305a—α'/β' lobes, 21% BS; and 201Y—γ lobe, 51% BS, relative to sibling controls). The c772 driver is expressed later in development (late pupa) than the other MB drivers, which express in late larva–early pupa (Yang *et al.* 1995; Armstrong *et al.* 1998; Aso *et al.* 2009). The results for c772 are not substantially different from those of other drivers suggesting that kcc^+ is reducing the BS phenotype through an effect on MB function, rather than on MB development.

MB expression of either Drosophila kcc^+ or human KCC2 ameliorates the seizure susceptibility of kcc^{DHS1} flies behaviorally and electrophysiologically: Significant, albeit incomplete, rescue of the kcc^{DHS1} BS phenotype is observed after expressing one copy of UAS-kcc^+ and one copy of the MB GAL4 driver (Figure 5). By contrast, nearly complete rescue is observed in kcc^{DHS1} flies carrying a second copy of the MB GAL4 driving UAS-kcc^+ (1% BS for two copies of c739) (Figure 6A). Flies carrying two copies of the c739 MB GAL4 driver, but no UAS-kcc^+, still display significant bang sensitivity (61% BS). This indicates that the kcc^+ level in the kcc^{DHS1} fly's MB critically affects the BS phenotype. We infer that the somewhat higher residual BS behavior observed in experiments employing only one MB-GAL4 driver (Figure 5) reflects inadequate expression of the kcc^+ transgene at the lowered temperatures required to score the kcc^{DHS1} phenotype (Duffy 2002; Hekmat-Scafe *et al.* 2006), although other brain regions, such as the

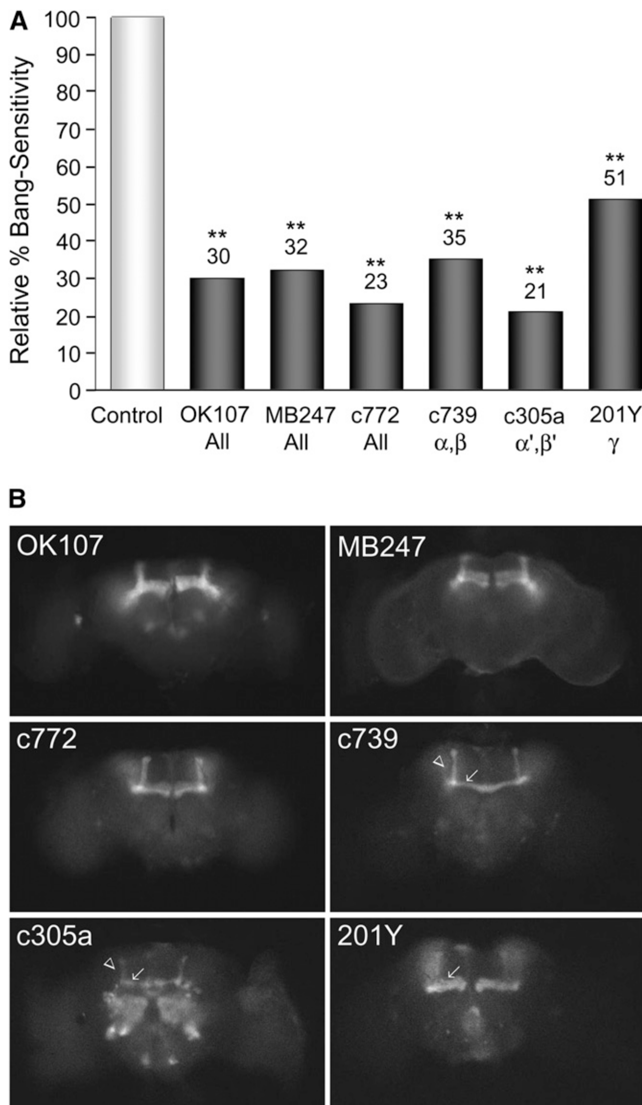

Figure 5.—Expression of *kcc* in all mushroom body subregions markedly reduces the behavioral seizure susceptibility of *kcc* mutant flies. (A) Expression of wild-type kcc^{+} in the entire MB using either of three GAL4 drivers markedly reduces the relative bang sensitivity of kcc^{DHS1} mutant flies (OK107 30%, MB247 32%, and c772 23%), as does more restricted induction of the UAS-kcc^{+} transgene in Kenyon cells with distinct axonal branches (c379—α/β lobes, 35%; c305a—α'/β' lobes, 21%; and 201Y—γ lobe, 51%). In these experiments, virgin females carrying the kcc^{DHS1} mutation and one of the MB GAL4 drivers (*i.e.*, D642, D661, D689, D588, D643, or D658) were crossed to D634 males, which carry the kcc^{DHS1} mutation and the UAS-kcc^{+} transgene over a TM6B balancer. The percentage bang sensitivity of the kcc^{DHS1} + MB-GAL4 + UAS-kcc^{+} test progeny is shown relative to that of their kcc^{DHS1} + MB-GAL4 control siblings lacking the UAS-kcc^{+} transgene. $n > 220$; (**) $P < 0.001$. (B) MB expression patterns of the varying MB-GAL4 drivers are revealed by examining the green fluorescence in brains of flies carrying a UAS-mCD8::GFP reporter in combination with each of the MB-GAL4 drivers. Three of the GAL4 drivers (OK107, MB247, and c772) are expressed in all five axonal branches of the MB, whereas three others show more restricted expression patterns: c739 in the α (arrow)/β (arrowhead) lobes, c305a in the α′ (arrow)/β′ (arrowhead) lobes, and 201Y in the γ lobe (arrow). Some GFP fluorescence is observed in regions outside of the MB; in particular, c305a is also expressed in the antennal lobe. The brains are positioned so that the anterior side is facing the camera; dorsal is up and ventral down.

ellipsoid body (Figure 2C) may also be contributing to the phenotype.

Interestingly, we find that human KCC2 can substitute for fly kcc^{+} and is surprisingly effective at rescuing the kcc^{DHS1} phenotypes. Flies mutant for kcc^{DHS1} and carrying two copies of a UAS-hKCC2 transgene plus two copies of c739 display only 10% BS (Figure 6A). This rescue suggests functional and structural conservation between human KCC2 and fly kcc and is consistent with the notion that kcc, like KCC2, functions as a neuronal K^{+}–Cl^{-} cotransporter (Figures 1 and 2).

Electrophysiology shows that MB expression of both Drosophila kcc and human KCC2 raise the seizure threshold of kcc^{DHS1} mutant flies, an indication that reduced seizure susceptibility is responsible for rescue of the BS phenotype (Figure 6B-C). Control kcc^{DHS1} flies show a seizure threshold of 20.8 ± 3.1 V, significantly lower than the 34.3 ± 1.9 V seizure threshold typically seen in wild-type CS-5 flies (Tan *et al.* 2004). By contrast, kcc^{DHS1} test flies expressing the UAS-kcc^{+} transgene in their MBs show a 36.7 ± 4.1 V seizure threshold, comparable to wild type. Thus, MB expression of kcc^{+} rescues the seizure susceptibility of kcc^{DHS1} mutant flies electrophysiologically as well as behaviorally. The kcc^{DHS1} flies expressing human KCC2 in their mushroom bodies display an intermediate seizure threshold (28.4 ± 2.6 V) indicating partial rescue of the seizure-susceptibility phenotype.

Disruption of GABAergic signaling or synaptic transmission in MB reduces the seizure susceptibilty of kcc^{DHS1} flies: The mammalian KCC2 cotransporter maintains intracellular Cl^{-} gradients, and changes in KCC2 expression or function alter the strength of GABAergic synaptic inhibition (Ben-Ari *et al.* 2007). Similarly, we find interaction between Drosophila *kcc* and the GABAergic system in determining the severity of the kcc^{DHS1} BS phenotype, supporting the notion that MB neurons, through GABAergic signaling, play an important role in the circuitry mediating seizure genesis or spread. The link between kcc function and GABAergic inhibition was initially examined by expressing UAS-kcc^{+} exclusively in neurons that contain the Rdl $GABA_A$ receptor (Figure 7A). The BS phenotype of kcc^{DHS1} is substantially reduced (43% relative BS) by driving kcc^{+} expression with Rdl-GAL4-2-1; in the same range as for pan-neuronal drivers (Figure 2A). Significant rescue of the BS phenotype is also observed with the Gad1-GAL4 driver suggesting that GABA transmitting cells may also play a role in the BS phenotype. By contrast, targeting kcc^{+} expression to all cholinergic neurons with Cha-GAL4 produces only a modest reduction in the BS phenotype (70% relative BS). The implication is that

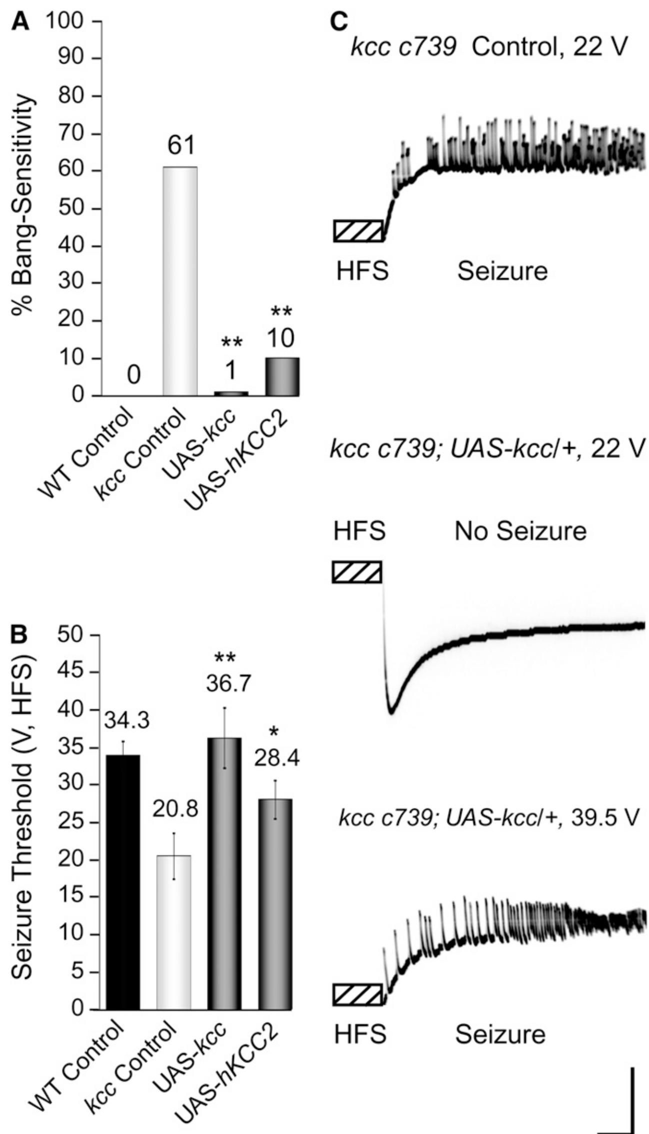

FIGURE 6.—Expression of either Drosophila *kcc* or human KCC2 in MB significantly reduces the seizure susceptibility of kcc^{DHS1} flies both behaviorally and electrophysiologically. (A) Expression of Drosophila kcc^{+} or human KCC2 (hKCC2) in mushroom bodies ameliorates the bang sensitivity of kcc^{DHS1} mutant flies. The kcc^{DHS1} control flies (D269, *w; c739* kcc^{DHS1}*; +/TM6B*) display significant bang sensitivity (61%) as compared to CS-5 controls, which are not bang sensitive. Expression of the UAS-kcc^{+} transgene in mushroom bodies with two copies of the c739 driver (D622, *w; c739* kcc^{DHS1}*; UAS-kcc/TM6B*) almost fully rescues the bang-sensitive phenotype (1% BS). Expression of human KCC2 in mushroom bodies (D623, *w; c739* kcc^{DHS1}*; UAS-hKCC2*) partially resues the bang-sensitive phenotype (10% BS). $n > 200$; (**) $P < 0.001$. (B) Mushroom body expression of either Drosophila kcc^{+} or human KCC2 raises the seizure threshold of kcc^{DHS1} mutant flies. Our kcc^{DHS1} control flies (D629) show a seizure threshold of 20.8 ± 3.1 V ($n = 7$), which is significantly lower than the 34.3 ± 1.9 seizure threshold typically seen in wild-type CS-5 flies (TAN *et al.* 2004). On the other hand, kcc^{DHS1} test flies that express the UAS-*kcc* transgene specifically in mushroom bodies (D622) have a seizure threshold of 36.7 ± 4.1 V ($n = 8$), which is comparable to that of wild-type CS-5 flies. Our kcc^{DHS1} flies that express human KCC2 in their mushroom bodies (D623) display an intermediate seizure threshold of 28.4 ± 2.6 V ($n = 5$). (*) $P < 0.05$, (**) $P < 0.001$. (C) Top: A seizure elicited in a kcc^{DHS1} control fly (D629) by a high-frequency stimulus of 22 V, which is above the fly's seizure threshold. The high-frequency stimulus (HFS) is a short wave train (0.5-ms pulses at 200 Hz for 300 ms) of electrical stimuli delivered to the brain. Middle: By contrast, a 22 V HFS fails to elicit a seizure in a kcc^{DHS1} test fly which express the UAS-kcc^{+} transgene specifically in mushroom bodies (D622) because it is below the fly's seizure threshold (32.7 +/− 0.23 V). Bottom: However, when this fly is given a higher intensity HFS of 39.5 V, which is above its seizure threshold, it sustains a seizure. The vertical calibration bar is 20 mV and the horizontal bar is 200 ms.

GABAergic inputs play a larger role than cholinergic ones in modulating the kcc^{DHS1} BS phenotype. Consistent with this notion, the kcc^{DHS1} BS paralytic phenotype is suppressed by a reduction in the level of the Rdl $GABA_A$ receptor either globally or specifically in the MB (Figure 7B). The kcc^{DHS1} BS paralytic phenotype is greatly reduced in heterozygous Rdl^{1}/+ or $Rdl^{MD\text{-}RR}$/+ flies (39% BS for Rdl^{1}/+, 35% BS for $Rdl^{MD\text{-}RR}$/+, relative to sibling controls). In addition, specifically reducing *Rdl* levels in the MB utilizing an *Rdl* RNAi construct reduces the kcc^{DHS1} BS paralytic phenotype to an even greater extent (25% BS, relative to sibling controls).

In mammals, the Na^{+}–K^{+}–Cl^{-} cotransporter, NKCC1, and the neuronal K^{+}–Cl^{-} cotransporter, KCC2, have opposing effects on intracellular Cl^{-} gradients (FARRANT and KAILA 2007). A BLASTp (ALTSCHUL *et al.* 1997) search of the Drosophila genome using human NKCC1 reveals four potential Na^{+}–K^{+}–Cl^{-} cotransporter genes (Ncc69, CG31547, CG10413, and CG12773). Ncc69 is the most likely NKCC1 homolog as it has significant brain expression (CHINTAPALLI *et al.* 2007) and is known to interact with fray (LEISERSON *et al.* 2000), a homolog of the SPAK kinase, which modulates NKCC1 (W. LEISERSON and H. KESHISHIAN, personal communication). Here we show that reducing the level of Ncc69 by mutation has only a modest effect on decreasing the BS of kcc^{DHS1} flies (Figure 7C). However, reducing *Ncc69* expression exclusively in the MB of kcc^{DHS1} flies by combining a MB driver and *Ncc69* RNAi construct produces a marked decrease in the BS phenotype: 31% BS, relative to sibling controls (Figure 7C).

Blocking synaptic transmission from MB neurons by directed expression of Tetanus toxin (TNT) dramatically reduces the bang sensitivity of kcc^{DHS1} flies (35% BS, relative to sibling controls). By contrast, blocking synaptic transmission in a control neuronal population (antennal neurons projecting to the MB targeted by the GH146 GAL4 driver) produces no significant difference in the bang sensitivity of kcc^{DHS1} flies (97% BS, relative to sibling controls).

MB expression of *kcc* functions as a global seizure suppressor: Expression of UAS-kcc^{+} not only rescues the BS phenotype of kcc^{DHS1} mutants, it also suppresses the

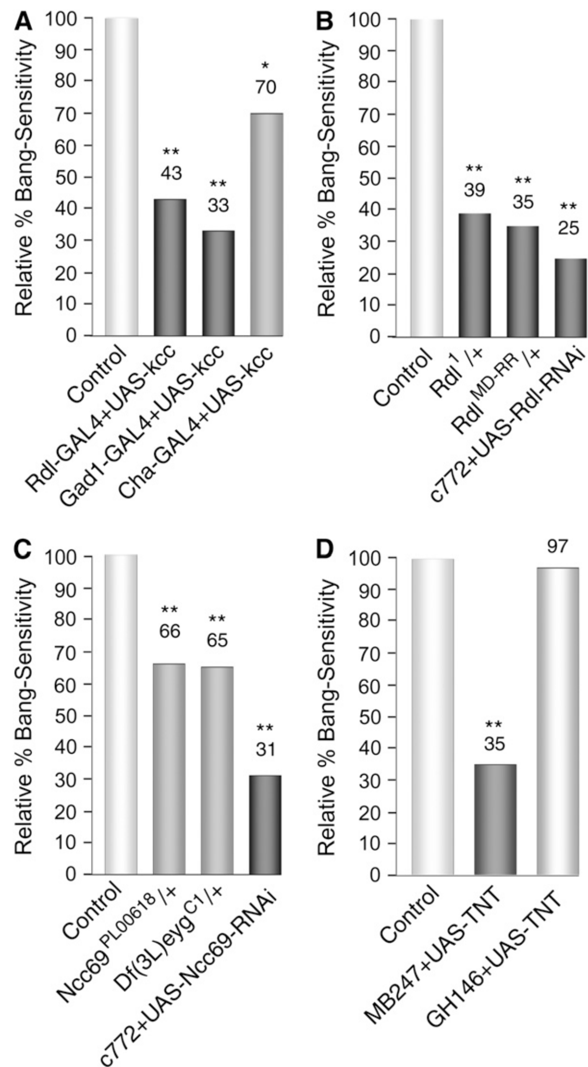

FIGURE 7.—Disruption of GABAergic signaling or synaptic transmission in MB reduces the bang sensitivity of kcc^{DHS1} flies. (A) Expression of the UAS-kcc^{+} transgene in GABAergic neurons reduces their bang sensitivity. Expression of the wild-type kcc^{+} gene in the neurons targeted by either of two GAL4 drivers (Rdl, those expressing the Rdl $GABA_A$ receptor and Gad1, those producing GABA) produced marked reductions in bang sensitivity. Expression of kcc^{+} in neurons targeted by the Cha GAL4 driver (neurons producing acetylcholine) resulted in more modest, albeit statistically significant, reductions in bang sensitivity. In these experiments, virgin females carrying the kcc^{DHS1} mutation as well as a particular GAL4 driver (*i.e.*, D670, D590, or D589) were crossed to D634 males carrying the kcc^{DHS1} mutation and a UAS-kcc^{+} transgene over a TM6B balancer chromosome. The percentage bang sensitivity of the kcc^{DHS1} + Driver-GAL4 + UAS-kcc^{+} test progeny is given relative to that of their kcc^{DHS1} + Driver-GAL4 control siblings. $n > 80$; (**) $P < 0.001$, (*) $P < 0.05$. (B) Reducing the dosage of the Rdl $GABA_A$ receptor by mutation or RNAi reduces the bang sensitivity of kcc^{DHS1} flies. kcc^{DHS1} flies heterozygous for either of two *Rdl* mutations (Rdl^{1} or Rdl^{MD-RR}) displayed almost one-third the bang sensitivity (39% or 35% relative BS, respectively) of their control siblings. Mushroom body expression of an RNAi construct that reduces the level of Rdl produces an even more marked reduction in the level of bang sensitivity of kcc^{DHS1} mutant flies (25% relative bang sensitivity). In the first set of experiments, D506 virgin females homozygous for kcc^{DHS1} were crossed to males carrying kcc^{DHS1} and the corresponding *Rdl* mutation over a TM6B balancer. The percentage bang sensitivity of the kcc^{DHS1}; Rdl/+ test progeny is presented relative to that of the corresponding kcc^{DHS1} control siblings. In the last experiment, D689 virgin females carrying kcc^{DHS1} and the MB GAL4 driver c772 were crossed to D691 males carrying kcc^{DHS1} as well as an *Rdl* RNAi construct over a TM6B balancer. The percentage bang sensitivity of the kcc^{DHS1} + c772 + Rdl-RNAi test progeny is presented relative to that of the corresponding kcc^{DHS1} + c772 control siblings. $n > 220$; (**) $P < 0.001$. (C) Reducing the level of the Ncc69 Na^{+}–K^{+}–Cl^{-} cotransporter specifically in MB reduces bang sensitivity. kcc^{DHS1} flies carrying either of two lesions in *Ncc69* [$Ncc69^{PL00618}$ or $Df(3L)eyg^{C1}$] displayed a modest reduction in bang sensitivity compared to their control siblings (lightly shaded bars; 65–66% relative bang sensitivity). By contrast, MB expression of an *Ncc69* RNAi construct produces a marked reduction in the level of bang sensitivity of kcc^{DHS1} mutant flies (dark-shaded bars; 31% relative bang sensitivity). In the first set of experiments, D506 virgin females homozygous for kcc^{DHS1} were crossed to males carrying kcc^{DHS1} and an *Ncc69* mutation over a TM6B balancer. The percentage bang sensitivity of the kcc^{DHS1}; Ncc69/+ test progeny is presented relative to that of the corresponding kcc^{DHS1} control siblings ($n > 75$). In the last experiment, D661 virgin females carrying kcc^{DHS1} and the MB GAL4 driver c772 was crossed to D690 males carrying kcc^{DHS1} as well as an RNAi construct for Ncc69 over a TM6B balance. The percentage bang sensitivity of the kcc^{DHS1} + c772 + Ncc69-RNAi test progeny is presented relative to that of the corresponding kcc^{DHS1} + c772 control siblings. $n > 360$; (**) $P < 0.001$. (D) Blocking synaptic transmission in MB ameliorates the bang sensitivity of kcc^{DHS1} flies. Tetanus toxin (TNT) blocks synaptic transmission by cleaving synoptobrevin, which is necessary for synaptic vesicle fusion (MARTIN *et al.* 2002). Flies that express tetanus toxin specifically in their mushroom bodies show a significant reduction in bang sensitivity (35% of control levels, dark-shaded bar); no such reduction was observed when synaptic transmission was instead blocked in the antennal neurons that project to the MB (97% of control, lightly shaded bar). In the first experiment, D661 flies carrying kcc^{DHS1} and the MB driver MB247 were crossed to either D700 males carrying kcc^{DHS1} and a UAS-TNT transgene or D506 males carrying only kcc^{DHS1}; percentage bang sensitivity of kcc^{DHS1} + MB247 + UAS-TNT progeny from the first cross is presented relative to that of kcc^{DHS1} + MB247 progeny from the second cross. In the second experiment, D695 flies carrying kcc^{DHS1} and the GH146 GAL4 driver were crossed to either D700 or D506; percentage bang sensitivity of kcc^{DHS1} + GH146 + UAS-TNT progeny from the first cross is presented relative to that of kcc^{DHS1} + GH146 progeny from the second cross. $n > 110$; (**) $P < 0.001$.

phenotype of other, unrelated BS mutants (Figure 8). Whereas all control flies carrying the *easily shocked* (*eas*) mutation are BS, only two-thirds of their siblings that express kcc^{+} ectopically in their MBs are BS. Similarly, although nearly half (49%) of control flies heterozygous for the semidominant *bang-senseless* (*bss*) mutation are BS, only 14% of their *bss*/+ test siblings that express kcc^{+} ectopically in their MBs show the BS phenotype. These observations suggest that GABAergic signaling in MB neurons is a fundamental determinant of seizure susceptibility in the Drosophila brain.

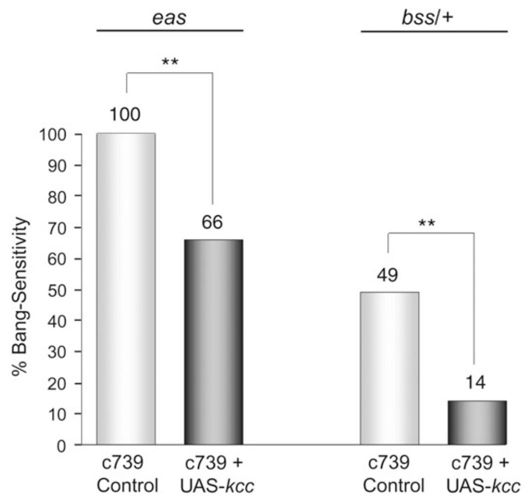

Figure 8.—Mushroom body expression of *kcc* functions as a global seizure suppressor. Expression of a UAS-kcc^+ transgene in the Drosophila mushroom bodies significantly reduces the behavioral seizure susceptibility of flies carrying either the *easily shocked* (*eas*) or *bang-senseless* (*bss*) bang-sensitive mutation. Whereas all of the *eas* + *c739* control flies are bang sensitive, only 66% of their *eas* + *c739* + *UAS*-kcc^+ siblings that express kcc^+ ectopically in their mushroom bodies display the bang-sensitive phenotype. Similarly, although nearly half (49%) of control *bss*/+ heterozygotes carrying c739 are bang sensitive, only 14% bang sensitivity is observed in their *bss*/+ test siblings carrying UAS-kcc^+ as well as c739. In the first experiment, *eas* virgin females (MR047) were crossed to D610 males carrying the c739 MB GAL4 driver and a UAS-kcc^+ transgene over a TM6B balancer chromosome, and the percentage bang sensitivity of *eas* + *c739* + *UAS*-kcc^+ male progeny compared to that of their *eas* + *c739* control brothers. In the second experiment, *bss* virgin females (MR068) were crossed to D610 males and the percentage bang sensitivity of the *bss*/+ female test progeny carrying *c739* and UAS-kcc^+ compared to that of their control sisters lacking the UAS-kcc^+ transgene. $n > 70$; (**) $P < 0.001$.

DISCUSSION

We reported previously that Drosophila kcc^{DHS1} mutants, with reduced levels of *kcc*, display an increased susceptibiilty to epileptic-like seizures in a manner similar to that seen in mice with reduced KCC2 levels (Woo *et al.* 2002; Hekmat-Scafe *et al.* 2006; Zhu *et al.* 2008). Here we confirm that Drosophila *kcc* and mammalian KCC2 are functional homologs. Furthermore, we show that expression specifically in the MB of either Drosophila kcc or human KCC2 largely alleviates kcc^{DHS1} seizure sensitivity, highlighting the importance of this brain structure in seizure genesis. Finally, we report a general role for MB kcc function in seizure susceptibility on the basis of observations that phenotypes of other BS mutants are ameliorated by MB expression of kcc^+. We propose a Drosophila model for investigating the roles of kcc and GABAergic inhibition in epileptogenesis.

Drosophila *kcc* is a functional homolog of mammalian KCC2: The KCC2 and NKCC1 ion cotransporters regulate mammalian nervous system excitability by modulating the strength of GABAergic synaptic inhibition (reviewed in Ben-Ari *et al.* 2007). Regulation occurs because the cotransporters exert opposing effects on the GABA reversal potential (E_{GABA}) by differentially affecting intracellular Cl^-, $[Cl^-]_{in}$. NKCC1 transports Cl^- into the neuron, thereby increasing $[Cl^-]_{in}$, whereas KCC2 transports Cl^- out of the neuron, thereby decreasing $[Cl^-]_{in}$. Thus, E_{GABA} varies according to the differential expression of these two cotransporters. Ordinarily, in most adult neurons, a relatively negative E_{GABA} arises from the presence of KCC2 and absence of NKCC1. Opening of the $GABA_A$ receptor Cl^- conductance then results in a typical inhibitory membrane hyperpolarization. In most fetal neurons, E_{GABA} is at a relatively more positive potential than for adult neurons due to the presence of NKCC1 and the absence of KCC2. Consequently, GABA activation can result in a depolarizing postsynaptic potential. This depolarization, sometimes referred to as "excitatory GABA," can overcome the inhibitory shunting effects of channel opening, initiate action potentials, and elicit Ca^{2+}-dependent synaptic transmitter release (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). During nervous system development, differential expression of NKCC1 and KCC2 underlies a so-called "GABA switch": a change in GABA synaptic transmission from the fetal excitatory form of transmission to the mature adult inhibitory form (Ben-Ari *et al.* 2007).

Mammals carry four KCC K^+–Cl^- cotransporters (KCC1–4) encoded by separate genes (Blaesse *et al.* 2009). Of these, KCC2 is neuron specific; the others display more widespread expression (Mercado *et al.* 2004). Neurons lacking KCC2 do not regulate $[Cl^-]_{in}$ despite co-expression of other KCCs (Zhu *et al.* 2008). The Drosophila genome includes only a single, alternatively spliced, K^+–Cl^- cotransporter gene, *kcc* (Hekmat-Scafe *et al.* 2006). Despite the absence of constitutive activity under isotonic conditions (Figure 1B), Drosophila kcc is evidently involved in GABAergic neuronal physiology (Figure 7), consistent with a role in regulating neuronal $[Cl^-]_{in}$. Our expression studies in Drosophila (Figure 6) as well as a heterologous system (Figure 1) indicate that the kcc-B isoform is a functional homolog of mammalian KCC2. Our ectopic expression experiments reveal that Drosophila kcc-B acts in neurons, rather than glia, to reduce BS (Figure 2A); the kcc-D isoform was less effective and specific (Figure 2B). Furthermore, human KCC2 functionally complements Drosophila kcc^{DHS1} (Figure 6).

Reduced *kcc* expression in MBs underlies seizure sensitivity in *kcc* mutants: Although *kcc*, like human KCC2, is normally expressed throughout the brain (Song *et al.* 2002; Hekmat-Scafe *et al.* 2006), expression of either wild-type kcc^+ or human KCC2 specifically in

the MBs of a kcc^{DHS1} fly's brain greatly reduces its seizure susceptibility both behaviorally and electrophysiologically (Figures 5 and 6). Ectopic expression of kcc^{+} in kcc^{DHS1} MB KCs, which extend their axons into either the α and β, α′ and β′, or γ lobes are all effective at rescuing the BS phenotype (Figure 5). This result, combined with our earlier observation that kcc protein is normally found at levels significantly higher in the MB calyx (dendritic region) than in the peduncle (axon tracts) (Hekmat-Scafe *et al.* 2006), suggests that kcc likely acts in the KC dendrites rather than in their axons. The degree of rescue may reflect some cell specificity, rather than simply the number of KCs expressing kcc^{+}, as we observe far greater suppression with the c739 than the 201Y driver, which targets the larger number of KCs (Wang *et al.* 2007). Expression of kcc^{+} in neuronal regions outside the MB, particularly the ellipsoid body (Figure 2C), can also modulate seizure susceptibility.

We suggest that kcc^{DHS1} causes seizure sensitivity because underexpression of its K^{+}–Cl^{-}-cotransporter results in a higher $[Cl^{-}]_{in}$ that compromises $GABA_A$ inhibitory synaptic strength. The decrease in inhibition would change the overall balance between excitation and inhibition making the kcc^{DHS1} brain hyperexcitable and thus, seizure sensitive. One possibility is that $[Cl^{-}]_{in}$ might be sufficiently high that some neurons, such as MB KCs, might display excitatory GABAergic responses. That is, these mutant neurons may come to resemble mammalian fetal neurons and depolarize in response to GABA transmission. Although E_{GABA} has not been measured in kcc^{DHS1} MB neurons to allow a direct test of this, observations on Rdl expression make us think that excitatory GABA may be a possibility. Reducing the level of the Rdl $GABA_A$ receptor in the MB by RNAi ameliorates seizure sensitivity in kcc^{DHS1} flies (Figure 7B) suggesting a reduction in excitability. Ordinarily, reduction of $GABA_A$ receptor is expected to decrease inhibition resulting in an overall increase in excitability. This resembles a previous finding (Hekmat-Scafe *et al.* 2006) that the $GABA_A$ blocker picrotoxin is a seizure suppressant in kcc^{DHS1} mutants, but is a convulsant for normal flies. Our results are most consistent with the notion that excitatory GABAergic signaling in the MBs influences the maintenance, rather than the development, of an epileptic state in the brain. We observed that kcc^{DHS1} flies in which kcc^{+} was induced in the MBs during the late pupal stage using the c772 driver (Armstrong *et al.* 1998) displayed identical levels of behavioral seizure susceptibility to control flies 24-hr postecclosion (A. Fajilan and D. Hekmat-Scafe, data not shown), but significantly reduced levels 12 hr later (Figure 5). This suggests that the effect of *kcc* in MB occurs during the late pupal–early adult period.

Perhaps it is the MB's neuronal plasticity that makes it particularly vulnerable to the development of epileptic-like seizures. The mammalian hippocampus, which is also critical for learning and memory, is a frequent site of epileptic foci (Hauser and Hesdorffer 1990). One possibility is that the plasticity of these brain regions reflects the type of excitatory GABAergic signaling normally observed during early neuronal development (Ben-Ari *et al.* 2007). Excitatory GABAergic signaling has been observed in the adult hippocampus, where rhythmic firing is believed to promote neuronal plasticity (Obrietan and van den Pol 1996; Ge *et al.* 2006), but may also make this structure particularly susceptible to epileptic seizures.

Inhibitory synaptic strength and Drosophila MB function: MB kcc^{+} rescues seizure sensitivity not only in kcc^{DHS1} mutants, but also in other BS mutants, such as *bss* and *eas*. These genes encode different products and appear to cause Drosophila seizure sensitivity through different mechanisms (Ganetzky and Wu 1982; Pavlidis *et al.* 1994). Nevertheless, a common feature must account for phenotypic suppression of these different mutations: we propose that this is a kcc^{+}-mediated increase in inhibitory synaptic strength. There are several implications arising from this proposition. The first is that inhibitory synaptic strength must normally be capable of being strengthened in MB neurons. We infer that this is the case because we believe that the mechanisms underlying *bss* and *eas* seizure sensitivity are not directly related to a damaged inhibitory signaling system; their synapses are more likely to reflect a fairly normal inhibitory synaptic situation in the MB. Nevertheless, synaptic inhibition is apparently strengthened by kcc^{+} expression in the MB of these mutants, producing seizure suppression. Second, this strengthening of inhibitory synaptic strength by kcc^{+} is likely generated by decreasing $[Cl^{-}]_{in}$, suggesting that normally, $[Cl^{-}]_{in}$ could be relatively high in MB neurons. The normally high $[Cl^{-}]_{in}$ might also be facilitated by the presence of substantial Ncc69, since a decrease in its level by RNAi can also markedly suppress seizures (Figure 7C).

Overall, these observations suggest that normally, inhibitory synaptic strength in the MB may have a relatively large working range due to the presence of both kcc and Ncc69: a working range that is apparently revealed by mutation or RNAi utilizing seizure sensitivity as an indirect assay. This could be an artifact of the experimental methodologies used especially given the vagaries of GAL4/UAS ectopic expression and exactly how to interpret seizure enhancement and seizure suppression as a measure of synaptic strength. Nevertheless, loss-of-function mutations and RNAi experiments indicate clearly the importance of kcc and Ncc69 functions in the MB. Taken together all of the observation are very suggestive of high $[Cl^{-}]_{in}$ in the MB. Finally, if, given these caveats, it remains true that inhibitory synaptic strength in the MB has a large working range, we wonder what is its normal function? It is tempting to speculate that inhibitory synaptic modulation might be critical in some aspect of fly learning or memory, the best studied and most interesting aspect of MB function

(Heisenberg 2003; Davis 2005; Keene and Waddell 2007; Berry *et al.* 2008). Inhibition and its modulation by upstream regulators such as fray, the fly homolog of SPAK (Leiserson *et al.* 2000), are generally not considered as important aspects of current models of learning and synaptic plasticity. However, they may ultimately play an important role in sprouting and connectivity, aspects that could contribute significantly to the importance of MB function.

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