Functional Coupling of $K^+–Cl^-$ Cotransporter (KCC) to GABA-Gated Cl$^-$ Channels in the Central Nervous System of Drosophila melanogaster Leads to Altered Drug Sensitivities

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ABSTRACT: GABAergic signaling is the cornerstone for fast synaptic inhibition of neural signaling in arthropods and mammals and is the molecular target for insecticides and pharmaceuticals, respectively. The $K^+–Cl^-$ cotransporter (KCC) is the primary mechanism by which mature neurons maintain low intracellular $Cl^-$ concentration, yet the fundamental physiology, comparative physiology, and toxicological relevance of insect KCC is understudied. Considering this, we employed electrophysiological, genetic, and pharmacological methods to characterize the physiological underpinnings of KCC function to the Drosophila CNS. Our data show that genetic ablation or pharmacological inhibition of KCC results in an increased spike discharge frequency and significantly ($P < 0.05$) reduces the CNS sensitivity to $\gamma$-aminobutyric acid (GABA). Further, simultaneous inhibition of KCC and ligand-gated chloride channel (LGCC) complex results in a significant ($P < 0.001$) increase in CNS spontaneous activity over baseline firing rates that supports functional coupling of KCC to LGCC function. Interestingly, 75% reduction in KCC mRNA did not alter basal neurotransmission levels indicating that only a fraction of the KCC population is required to maintain the $Cl^-$ ionic gradient when at rest, but prolonged synaptic activity increases the threshold for GABA-mediated inhibition and reduces nerve sensitivity to GABA. These data expand current knowledge regarding the physiological role of KCC in a model insect and provides the necessary foundation to develop KCC as a novel biochemical target of insecticides, as well as complements existing research to provide a holistic understanding of the plasticity in mammalian health and disease.

KEYWORDS: Target-site synergism, $K^+–Cl^-$ cotransporter, insecticide, potassium, insect nervous system, thallium-flux

INTRODUCTION

Inhibitory synaptic signaling through $\gamma$-aminobutyric acid (GABA) innervated neurons is responsible for shaping the neural activity patterns in the central nervous system of arthropods and mammals. Considering this, the GABA receptor (GABA-R) and the GABA-receptor-chloride-channel (GRCC) complex are well-established pharmaceutical targets for various mammalian diseases and disorders$^2–^4$ and also represents a long-standing target for insecticides, acaricides, and anthelmintics.$^5$ In mammals, the GRCC complex contains modulatory receptor sites for two classes of centrally acting drugs that are commonly used in pharmaceuticals; one for the benzodiazepines, and a second for barbiturates and picrotoxin-related convulsants.$^6,^7$ For insecticides, the GRCC contains drug binding sites for multiple chemical classes, such as polychlorocycloalkanes, cyclodienes, phenylpyrazoles, isoxazoline, and meta-di-amides, which are all established agrochemicals.$^8–^11$ However, mammalian sensitivity to benzodia-
zepines and arthropod sensitivity to GRCC-directed molecules has been significantly reduced through changes in the GABA<sub>A</sub> receptor<sup>1,2,5</sup> or target site mutations in rdl, the gene encoding GABA-R<sup>14</sup>, respectively.

Similar to the negative impact benzodiazepine tolerance has had on human medicine,<sup>15,16</sup> the development of resistance to insecticides targeting the function of GABA- or glutamate-gated Cl<sup>−</sup> channels is of significant concern for veterinary health and agricultural sustainability. The majority of molecules approved for control of insect ectoparasites and helminths of companion animals modulate the activity of LGCC in the nervous system.<sup>17</sup> Considering this, continued control of veterinary pests is reliant upon the identification of novel mechanisms of toxicity to circumvent cross-resistance to currently marketed chemical classes. For instance, the identification of novel chemical binding sites of GABA-R or the identification of proteins that are functionally coupled to LGCC may provide avenues for therapeutic development that help circumvent resistance mechanisms to currently commercialized molecules. One such physiological pathway that may be functionally coupled to LGCC and exploited for target-site synergism of GABAergic is the potassium (K<sup>+</sup>)-chloride (Cl<sup>−</sup>) cotransporter (KCC).

Inhibitory Cl<sup>−</sup> currents in mature neurons, such as those resulting from activation of GABA- or glutamate-gated chloride channels, are reliant upon a mechanism that persistently extrudes Cl<sup>−</sup> ions from the intracellular space to establish a low intracellular Cl<sup>−</sup> concentration ([Cl<sup>−</sup>]i).<sup>16,17</sup> Interestingly, the transporter responsible for establishing the Cl<sup>−</sup> gradient and the influence Cl<sup>−</sup> channels have to nerve polarity is mediated by developmental changes in the expression of neuronal cation-chloride cotransporters that regulate the chloride homeostasis.<sup>18,19</sup> In embryonic and early postnatal development of mammals, immature neurons have a high [Cl<sup>−</sup>]i, driven by the inward transport of Cl<sup>−</sup> ions through the sodium (Na<sup>+</sup>)–K<sup>+</sup>–2Cl<sup>−</sup> cotransporter-1 (NKCC1) that is highly expressed in immature neurons. On the contrary, mature neurons in later developmental stages have a reduced NKCC1 expression and increased KCC2 expression, which is the major Cl<sup>−</sup> exchanger in mature neurons and is responsible for maintaining a low [Cl<sup>−</sup>]i.<sup>18,19</sup> In mammals, the change in expression from NKCC1 to KCC2 results in a switch of GABAergic signaling from excitatory to inhibitory,<sup>18</sup> but unfortunately information pertaining to the GABAergic switch in insects is not defined.

KCC2 ultimately influences the efficacy and polarity of synaptic transmission mediated by LGCC in mature neurons, which suggests that inhibition of KCC2 would result in a reduced ability to hyperpolarize the nerve terminal during GABAergic neurotransmission. Importantly, the development of a small-molecule inhibitor of KCC2 that is highly selective for KCC2 over NKCC1 and other biochemical targets<sup>20</sup> termed VU0463271, has enabled the interrogation of KCC2's influence to GABAergic signaling. Electrophysiological studies performed in mammalian brain slices have shown that exposure to VU0463271 resulted in increased [Cl<sup>−</sup>]i and persistent central nervous system (CNS) discharge activity,<sup>21–24</sup> indicating that KCC2 is a critical component of mammalian neurotransmission.

Cation-chloride cotransporters have received relatively little attention within the field of insect science despite the known relevance in human systems.<sup>21,22,24,25</sup> Previous work has aimed to determine the physiological role of insect KCC within various tissue systems and has shown that pharmacological inhibition of KCC1 reduces the transepithelial fluid secretion in Rhodnius prolixus,<sup>26,27</sup> Drosophila melanogaster,<sup>28</sup> and Aedes aegypti,<sup>29,31</sup> suggesting KCC1 is critical for Malpighian tubule function. Further, immunohistochemical staining of the neural isofrom of KCC, which is analogous to human KCC2, has shown that the kazachoe gene products, which are homologous to mammalian KCC2, are expressed in the brain neuropil regions of adult Drosophila. Functional studies have shown that KCC is critical for proper insect neurotransmission as partial loss-of-function mutations to kazachoe resulted in an increased susceptibility to seizure-like activity in Drosophila<sup>30</sup> and exposure of adult and larval Aedes aegypti mosquitoes to VU0463271 resulted in hyperexcitatory behavior leading to mortality.<sup>31</sup> The increase in seizure-like susceptibility of adult Drosophila kazachoe mutants and the hyperexcitatory behavior of mosquitoes was speculated to be due to a decrease in inhibitory synaptic strength through reduced functional capacity of the GABA<sub>A</sub> receptor,<sup>30,31,32,33</sup> which mirrors interpretations from investigations of mammalian KCC2<sup>22,25,32,33</sup> and Caenorhabditis elegans.<sup>34</sup> Yet, conclusive evidence of this functional linkage in the Drosophila larval CNS has not been provided and is necessary to develop a holistic understanding of the physiology of synaptic signaling in the model insect that is used for human and insect studies.

There is a significant need to identify novel biochemical targets to prevent seizure disorders or increase benzodiazepine sensitivity in humans as well as mitigate the established resistance to GABAergics in insect pest species. Thus, we used a model insect to test the hypothesis that KCC is functionally coupled to GABA-gated Cl<sup>−</sup> channels and that pharmacological modulation of KCC in the Drosophila CNS will alter the sensitivity to LGCC-directed modulators. To test this hypothesis, we developed a robust, cell-based fluorescence assay that enabled in vitro characterization of small-molecules against Drosophila KCC and subsequently employed electrophysiological and pharmacological methods to characterize the influence of KCC to the neural activity of the Drosophila CNS, functional dependency of LGCC to KCC, and how KCC inhibition influences sensitivity to LGCC modulators. These data support and enhance previous characterizations of the physiological role of KCC in a model insect,<sup>30,35</sup> and provides the necessary foundation to develop KCC as a novel biochemical target of insecticides, and indicates KCC is a putative target to increase LGCC modulator sensitivity in mammals.

## Results and Discussion

We first sought to determine the inhibition potency of the mammalian KCC2 inhibitor VU0463271 and the structurally related analogue, 44BD (Figure 1) to Drosophila melanogaster KCC (DmKCC). To quantify the activity of small molecules on DmKCC, we developed a thallium (T<sup>+</sup>)-sensitive dye assay that measures DmKCC ion transport across the plasma membrane (Figure 2A). Our assay uses a T<sup>+</sup>-sensitive dye to measure the influx of T<sup>+</sup>, a K<sup>+</sup> congener, to quantify the amount of KCC substrate transport in cells stably expressing KCC. We expressed DmKCC isoform B, one of the most highly expressed KCC isoforms in the adult Drosophila nervous system,<sup>36</sup> in HEK293 cells. To increase the reproducibility of the assay, we isolated monoclonal cell lines stably expressing DmKCC. Of the dozens of clones isolated, clone 1D4 had the largest basal T<sup>+</sup> flux, consistent with strongest overexpression of KCC (Figure 2B). To verify that this T<sup>+</sup> influx was KCC-
mediated, we illustrated that the Tl⁺ flux was sensitive to the nonspecific cation-chloride cotransporter inhibitor bumetanide (Figure 2C) and verified KCC expression by Western blot (Figure 3A). Although bumetanide is significantly more potent to NKCC than KCC, it has been shown that bumetanide is a weak inhibitor of KCC, and thus, we performed additional experiments to ensure the reduced flux observed in Tl⁺ flux is indeed due to KCC inhibition. To do this, we performed ⁸⁶Rubidium (⁸⁶Rb⁺)-flux, and data show KCC activity was Na⁺-independent and active under both hypotonic and isotonic conditions (Figure 3B, C), which are characteristics that mirror K⁺−Cl⁻ coupled cotransport seen in mammalian neurally expressed KCCs and further indicate Tl⁺-flux shown in Figure 2 is KCC-mediated. After validation of the DmKCC expressing cell line, we sought to characterize the effect of the human KCC2-specific inhibitor VU0463271 and its structurally related control compound, 44BD on DmKCC.

Using the Tl⁺ flux assay, VU0463271 was shown to inhibit DmKCC in a concentration-dependent manner with curve fits to varying concentrations of VU0463271, resulting in an IC₅₀ value of 608 nM (95% CI: 457−809 nM) and a Hill coefficient value of −1.4 (Figure 2D). The potency of VU0463271 to Drosophila melanogaster KCC is similar to that of Aedes aegypti but significantly reduced when compared to human KCC. This is not surprising given that DmKCC and Aedes aegypti KCC share 88% amino acid sequence identity, but share approximately 50% amino acid identity with human KCC2. In an effort to identify a closely related inactive analogue of VU0463271 for use as a negative control in our toxicology studies, we assessed the potency of 44BD, which is a structural analogue to VU0463271 and was previously shown to possess very low inhibitory activity at human KCC2 and Aedes aegypti KCC. Indeed, in Tl⁺-flux assays, 44BD was significantly less potent to DmKCC when compared to VU0463271 with only a 15 ± 10% inhibition at 10 μM, which was the highest concentration tested (Figure 2D). These data indicate that VU0463271 and 44BD are suitable pharmacological probes to test the physiological role of KCC in the Drosophila CNS.

After validation that VU0463271 is potent against DmKCC and 44BD is a suitable “inactive” analogue, we performed Drosophila larval CNS recordings to test the initial hypothesis that DmKCC is an essential ion transport system that mediates proper neurotransmission in Drosophila. This is not an entirely

Figure 1. Chemical structures of pharmacological agents used in this study.

Figure 2. Development and use of an in vitro assay to quantify pharmacological inhibition of D. melanogaster KCC. (A) Scheme depicting assay design and data processing workflow. (B) Screening of monoclonal cell lines for increased Tl⁺ flux identifies cell line 1D4 (red highlighted data point). Tl⁺ flux slopes for individual cell lines are represented as points. The dotted line represents the Tl⁺-flux slope for the polyclonal cell line. (C) Bumetanide-sensitive response of monoclonal DmKCC-expressing cell line 1D4, polyclonal DmKCC cell line, and wild type HEK293 cells as detected by Tl⁺-flux (n = 3). *Asterisks represent statistical significance in each clone group at P < 0.05 as determined through a one-way ANOVA with Tukey’s post-test. (D) Activity of specific KCC2 inhibitor VU0463271 (open circle) and structurally related, but less efficacious, analogue 44BD (closed circle) to DmKCC as quantified by Tl⁺-flux (n = 3).
new hypothesis as Drosophila KCC, encoded by khazachoc, has been speculated to contribute to inhibitory synaptic signaling of Drosophila central nervous systems, because it is related to mammalian KCC2, which is the principal extruder of Cl− ions in mature neurons of mammals. To begin testing this hypothesis, VU0463271 was applied to the transected CNS preparation and resulted in a significant increase in firing rates of the Drosophila CNS with 1 μM VU0463271 resulting in a peak firing rate that was a 2.7- and 2.5-fold increase over baseline firing rate for OR and rdl strains, respectively (Figure 4A, B). Importantly, 44BD did not alter firing rates of the Drosophila CNS at concentrations ranging up to 1 mM (Figure 4C, E). For VU0463271, the concentration required to increase firing rate by 50% (EC50) for OR was found to be 479 nM (95% CI, 281−679 nM; Hill slope, 1.6; r2, 0.83) and the rdl strain was found to be near identical with an EC50 of 485 nM (95% CI, 313−695 nM; Hill slope, 1.4; r2, 0.87). It is important to note that 10−100 nM VU0463271 resulted in approximately 20% reduction of CNS firing frequency within a small percentage of preparations. This inhibition was not concentration dependent and was considered to be an artifact of the dissection or recording, yet this observation warrants notation due to the dual action of dieldrin to cockroach neurons. These data mirror previous work in mammalian systems that have shown pharmacological inhibition of KCC2 increased spontaneous firing rates of cultured neurons and rat brain slices as well as increased membrane potential and input resistance, which were attributed to an increase in [Cl−].

Importantly, the CNS recordings were performed on larval Drosophila preparations and it is currently unknown when the GABAergic switch occurs in this organism; thus, it was necessary to ensure the increased spike discharge frequency observed in Figure 4A, B was not due to inhibition of NKCC prior to the developmental switch. We studied the influence of bumetanide, which is a validated NKCC inhibitor, to the firing rate of the Drosophila CNS. Bumetanide was found to have slight inhibitory actions to the firing rate of the Drosophila CNS with 250 μM (solubility limits in saline) resulting in 26 ± 9% reduced firing when compared to baseline (Figure 4D). An EC50 of bumetanide was not able to be determined due to the low inhibition at maximal concentrations (Figure 4F). These data indicate that (1) the developmental switch from NKCC to KCC has occurred in the L3 lifestage because NKCC inhibition did not result in excitation and (2) VU0463271 is likely targeting KCC to induce the observed phenotype.

The data presented in Figure 4 indicate KCC provides an essential ion transport system in the fly central nervous system. Although the selectivity of VU0463271 has been shown to be highly specific for KCC2 and our in vitro data (Figures 2 and 3) clearly indicate DmKCC is the target of VU0463271, we reduced kazachoc mRNA levels specifically in the larval CNS by RNA-interference by using the GAL4-UAS system to ensure a combination of proteins were not responsible for altered neuronal activity after VU0463271 exposure. Data show the CNS of the F1 progeny expressed 69 ± 14% less kazachoc mRNA relative to the wild type (OR) and GFP dsRNA knockdown controls (Figure 5A). Furthermore, kazachoc mRNA levels were not different from the carcass of control flies, indicating knockdown was CNS specific (Figure 5A).

Due to the tight regulation of the nervous system, we tested if genetic ablation of kazachoc mRNA altered the baseline firing frequency of the Drosophila CNS. No significant changes in baseline firing frequencies were found between OR flies, GFP knockdown, or kazachoc knockdown with baseline firing frequency of 28 ± 11 Hz, 30 ± 9 Hz, and 34 ± 7 Hz, respectively (Figure 5B). The lack of influence to baseline firing is potentially due to compensatory mechanisms through other cation-chloride cotransporters that are sufficient for maintaining a Cl− gradient at rest or poor KCC protein reduction in the mRNA knockdown line that reduces the observed phenotype.

Next, we constructed concentration−response curves against OR, GFP knockdown, and kazachoc knockdown flies (Figure 5C) and data show a 3.2- and 2.7-fold reduction in VU0463271 potency in knockdown flies when compared to OR and GFP knockdown flies, respectively, with an EC50 value of 1237 nM (95% CI: 699−2189 nM; r2, 0.83). A representative trace showing the loss of potency is shown in Figure 5D. Interestingly, kazachoc knockdown did not result in any obvious impact to the behavior of the third instar maggots. The loss of VU0463271 potency after CNS-specific knockdown of KCC mRNA supports our, and others’, hypothesis that KCC is required for proper neuronal function.
that neuroexcitation after VU0463271 exposure (Figure 4) is due to inhibition of KCC. However, the influence to CNS firing after genetic knockdown of KCC mRNA was significantly less than that of pharmacological inhibition, which may be due to compensatory mechanisms that negate the impact of KCC mRNA depletion, nontarget effects of VU0463271 on other Cl⁻ cotransporters, or that protein reduction was not correlative to mRNA reduction. If the latter is true, then it is important to note that the significant impact of VU0463271 to CNS firing may be due to nontarget inhibition and thus, must be interpreted cautiously.

Next, we aimed to provide empirical evidence that KCC is functionally coupled to LGCC in Drosophila CNS because selective inhibition of KCC2 has been shown to cause a depolarizing shift of $E_{\text{Gly}}$ and $E_{\text{GABA}}$ within mammalian cultured neurons, which validates functional coupling of KCC proteins to LGCC in an indirect manner within mammalian systems. To do this, we studied changes in GABA-mediated inhibition of Drosophila CNS firing after exposure to VU0463271 and knockdown of kazachoc, which would alter [Cl⁻]. As expected, exposure of the CNS to 5 mM GABA resulted in an immediate and complete cessation of spike activity (Figure 6A). However, pre-exposure of the CNS to 3 μM VU0463271 followed by a cotreatment of 3 μM VU0463271 + 5 mM GABA resulted in partial inhibition of CNS spike activity, but the sensitivity of the CNS to 5 mM GABA was not altered by pretreatment of 500 μM 44BD. Representative recordings of these treatments are shown in Figure 6A-C. GABA (5 mM) reduced spike discharge frequencies to an average of 0.5 ± 0.2 Hz from a baseline firing frequency of 28 ± 11 Hz, which was a statistically significant ($P < 0.001$) reduction (Figure 6D). The inhibitory effect of GABA was reduced when the CNS was pretreated with VU0463271 with an average firing rate 16 ± 5 Hz, which was significantly ($P < 0.01$) greater than GABA alone but was significantly ($P < 0.05$) reduced when compared to baseline firing rates. Importantly, the mean spike discharge frequency of CNS pretreated with 44BD followed by GABA was not significantly different when compared to baseline, suggesting that the reduced GABA sensitivity after pretreatment with VU0463271 is indeed due to KCC inhibition (Figure 6D).

Despite the relatively weak effect of KCC mRNA knockdown, genetic inhibition of KCC resulted in a significant reduction in GABA potency, which supports our pharmacological data collected in Figure 6 and the speculation that LGCC are functionally coupled to KCC in Drosophila CNS. The influence of kazachoc knockdown to GABA potency and CNS spike discharge frequency was studied through extracellular recordings of Drosophila CNS and GABA was shown to have an IC₅₀ value of 1.1 mM (95% CI, 0.8–1.5 mM; Hill slope, −1.5; $r^2$, 0.96) to OR flies, which was nearly identical to the IC₅₀ value found for GFP knockdown flies.
The functional relationship of KCC to GABA-gated chloride channels indicated by our data is somewhat unsurprising considering the large amount of data supporting the critical role of KCC2 in affecting Cl⁻ homeostasis in human systems and the exclusive role played by synaptic Cl⁻ channels in mammalian and insect nervous systems. However, the ability to interact with GABA-mediated inhibitory signaling pathways suggests perturbing the function of KCC could synergize the activity of LGCC directed insecticides. Thus, we aimed to further test the hypothesis that KCC and GABA are functionally coupled proteins by quantifying the influence of partial inhibition of KCC and LGCC to the spike discharge frequency of the Drosophila CNS. In theory, if LGCC function is dependent on KCC function, then neural activity should be enhanced in a multiplicative fashion and not additive when an EC₁₀ of each inhibitor is applied. Indeed, cotreatment of an EC₁₀ of dieldrin, a known GABA-R inhibitor, and an EC₁₀ of VU0463271 significantly (P < 0.001) increased the firing rate with a 70% and 110% increase over baseline at 0−5 min and 5−10 min postexposure, respectively (Figure 8). These firing rates are significantly (P < 0.001) greater than the expected 20% increase over baseline if the relationship was additive (dotted line; Figure 8D). It is important to note that approximately 10% of the preparations did not respond to the cotreatment of dieldrin and VU0463271. These recordings were excluded from the data analysis and we speculate this lack of effect was due to high baseline firing rates that approached maximal firing frequencies (~60 Hz), increased rate of nerve mortality, or variability within the fly population. These data suggest that increased potency of Cl⁻ channel blockers, such as dieldrin, can be obtained through simultaneous inhibition of KCC, but this was somewhat surprising due to previous work that showed the bang-sensitivity of KCC⁻/⁻ adult Drosophila is suppressed when they carry one null allele of the rdl GABA-R gene or are fed chemical inhibitors of GABA-R. These data are opposite of those shown in Figure 8 and could be due to unknown differences between third instar and adult CNS neural networks.

(Figure 7A). Kazachoc knockdown flies were shown to have a 2.2-fold reduction in GABA potency with an IC₅₀ value of 2.3 mM (95% CI, 1.8−2.9 mM; Hill slope, −2.1; r², 0.94). Interestingly, complete inhibition of CNS firing was never achieved in kazachoc knockdown flies with the greatest GABA-mediated inhibition reaching approximately 70% at concentrations ranging from 5 to 30 mM (Figure 7A). These concentrations were found to completely eliminate all CNS activity in the OR and GFP-knockdown flies. All recordings were terminated with ejection of the descending nerves from the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode to ensure the electrode 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Lastly, we aimed to study the role of KCC in the peripheral nervous system of Drosophila. Disruption of mammalian KCC2 function has been primarily attributed to symptomology related to diseases of the central nervous system, such as epilepsy, whereas disruption of KCC3 and KCC4 function alters the activity of the central and peripheral nervous systems, resulting in psychosis and peripheral neuropathy. VU0463271 was found to have an excitatory influence on the peripheral nervous system of Drosophila with an EC$_{50}$ of 103 μM (95% CI, 63–169 μM; Hill slope, 2.1, r$^2$, 0.84), which is 215-fold less potent when compared to the CNS (Figure 9). Reduction of maximal nerve firing was observed at a VU0463271 concentration of 1 mM, but firing was still over 2-fold greater than that of baseline rates (Figure 9B). As with the CNS response, 44BD was found to have marginal influence to the firing rate of the sensory nerves with a maximal increase of firing rate being approximately 30% higher than baseline at 700 μM (Figure 9B). The significant difference in VU0463271 potency to central and peripheral nerves suggests that the KCC isoforms between the two nervous systems are different or the excitation observed in the peripheral nervous system is due to modulation of a protein different than KCC.

In conclusion, our previous work indicates that the K$^+$/Cl$^-$ cotransporter represents a toxicologically relevant biochemical
target in mosquitoes$^{31}$ and the data presented in this study support this notion by describing a fundamental role of KCC in neurotransmission and regulation of GABAergic signaling. Further, these data potentially have toxicological significance as we show altered sensitivity of LGCC to small-molecules after KCC inhibition in a model insect, raising the intriguing possibility that KCC can be exploited for “target-site synergism”, similar to inhibition of multidrug resistance proteins $^{43,44}$.

Although the potential of KCC directed modulators to mitigate rdl phenotypes is yet to be determined, the ability to alter GABA and dieldrin sensitivity is noteworthy and of significance for the future direction of insecticide science. Similarly, understanding the physiological role of KCC in a model organism is critical for developing a holistic

Figure 8. Evidence of functional coupling of KCC and GABA-gated Cl⁻ channels in the D. melanogaster CNS. Representative traces of the effect of EC_{10} of VU0463271 (A), dieldrin (B), and VU0463271 + dieldrin (C) to the firing of Drosophila CNS. (D) Mean (n = 5–8) CNS firing rates after exposure to VU0463271 alone, dieldrin, and in combination with error bars representing SEM. Dotted line represents 20% increase over baseline, which is the expected additive firing rate. *Asterisks represent statistical significance at P < 0.001 as determined by an unpaired students t-test.

Figure 9. Sensory nervous system firing frequency recordings from D. melanogaster. (A) Representative traces of VU0463271 on the sensory nerves of third instar Dm. (B) Concentration—response curve for VU0463271 and 44BD on D. melanogaster PNS firing frequency where each data point represents mean (n = 5–8) PNS firing rates with error bars representing SEM.
understanding of how modulation of GABAergic signaling influences epileptogenesis, mammalian neural diseases, and general neurotransmission. Future work should be directed to characterize the influence of invertebrate KCC to the maintenance of the nerve physiology (e.g., membrane equilibria and resistance), toxicological relevance, and rd1 mitigating potential in helminths and arthropods of medical, veterinary, or agricultural relevance.

**METHODS**

**Compounds and Compound Synthesis.** VU0463271 was acquired from Tocris Bioscience, and 44BD was synthesized as described previously\(^\text{29}\) and solubilized in dimethyl sulfoxide (DMSO) that was purchased from Sigma-Aldrich (St. Louis, MO). Molecular structures of VU0463271 and 44BD are shown in Figure 1.

**Insect Stocks and Rearing Conditions.** Five strains of *D. melanogaster* were used in this study. The wild type Oregon-R (OR) strain was provided by Dr. Jeffrey Bloomquist at the University of Florida, Gainesville, FL, and was originally donated by Doug Knipple, Cornell University, Ithaca NY. The cyclodiene-resistant (rdl-1675) strain was purchased from the Bloomington Drosophila Stock Center at Indiana University, Bloomington, IN. All GAL4-UAS fly strains were purchased from Bloomington Drosophila Stock Center. The GAL4-UAS strain 3739 expresses the Gal-4 pattern in the brain of third instars with strong expression throughout the CNS, but not in the disks. The strain 34854 expresses dsRNA for RNAi of KCC (FBgn0025698) under UAS control. The strain 41554 expresses hairpin RNA (hpRNA) under the control of UAS for RNAi of GFP and was used as a negative knockdown control. The genotypes of each hairpin RNA (hpRNA) under the control of UAS for RNAi of GFP (FBgn0025698) under UAS control. The strain 41554 expresses the Gal-4 pattern in the brain of third instars with strong expression throughout the CNS, but not in the disks. The strain 34854 expresses dsRNA for RNAi of KCC (FBgn0025698) under UAS control. The strain 41554 expresses hairpin RNA (hpRNA) under the control of UAS for RNAi of GFP and was used as a negative knockdown control. The genotypes of each strain are as follows: 3739, P[w+;mWls]=GawB/c698a, w[1118]; 34854, y[1] sc[\*] v[1]; P[y[+];7.7] v[+t1.8]=TRIP.HSM01058-attP2; 41554, y[1] sc[\*] v[1]; P[y[+];7.7] v[+t1.8]=VALIUM20-EFGP.shRNA.2attP2.

All fly strains were maintained in culture at Louisiana State University Agricultural Center (Department of Entomology) and were reared on standard medium in *Drosophila* tubes at 25 °C, 12 h/12 h photoperiod, and 55% relative humidity. For dissection, flies were anesthetized by chilling on ice and decapitated before dissecting out CNS in Schneider's medium (Invitrogen, Paisley, Scotland, UK).

**Drosophila melanogaster KCC (DmKCC)-Overexpressing Cell Line Generation.** We created a vector suitable for stable expression of DmKCC in human embryonic kidney 293 (HEK293) cells by cloning the ORF of *D. melanogaster* KCC variant B (NCBI Reference Sequence: NM_166632.2) into pcDNA3.1/Zeo(+) (ThermoFisher, Waltham MA). A clone containing the KCC ORF was purchased from the Drosophila Genomics Resource Center (Bloomington IN; stock number: 4659). Polymerase Chain Reaction was used to amplify the ORF while adding 5′ XbaI and 3′ HindIII restriction sites for cloning into the pcDNA3.1/Zeo(+) backbone. The backbone and the ORF PCR product were digested with XbaI and HindIII (New England Biolabs, Ipswich MA) and the ORF was ligated into the backbone using New England Biolabs Quick Ligation kit. The ligated DNA was transformed into chemically competent DH5α E. coli. The resulting plasmid DNA was purified (Qiagen, Venlo, Netherlands) and the sequence was confirmed by Sanger sequencing (GenHunter, Nashville TN).

**Monoclonal DmKCC Cell Line Generation.** HEK293 cells (ATCC, Manassas, VA) were plated in a TC-treated T75 flask at 40% confluence and incubated in α-MEM (Corning, Corning, NY) supplemented with 10% (v/v) fetal bovine serum (ThermoFisher, Waltham MA) and 1% glutagone (Corning, Corning, NY medium). The HEK293 cells were transfected with 7 μg of DmKCC-pcDNA3.1/Zeo(+) DNA using Fugene6 (Promega, Madison WI) following the manufacturer’s instructions. At 48 h after transfection, the cells were put under selection using 250 μg/mL Zeocin. After 2 weeks of Zeocin selection, the polyclonal cells were plated in 96 well plates at ~1 cell per well. The most promising monoclonal cell lines were selected based on the magnitude of KCC inhibitor-sensitive Tl flux and proliferative capacity relative to untransfected HEK293 cells. These cell lines were expanded into TC-treated T25 flasks and retested for activity using Tl′ flux and KCC inhibitors. The monoclonal cell line used in this paper (1D4) was selected based on its large KCC inhibitor-sensitive Tl′ flux and robust growth characteristics. KCC expression in the 1D4 monoclonal cell line was confirmed by Western blotting. The 1D4 monoclonal cell line was used for heterologous characterization of KCC activity in this manuscript.

**Cell Culture of HEK293 Cell Lines Expressing KCC.** Monoclonal, HEK293 cells stably expressing DmKCC and wild type KCC293 cells were grown to 80%–90% confluence in TC-treated T75 flasks containing cell culture medium. DmKCC-expressing cells were grown in the presence of 250 μg/mL Zeocin. Cells were passaged before they reached >90% confluence, for a maximum of 20 passages.

**Thallium (Tl′)-Flux Assays.** Tl′ flux assays were performed as described previously.\(^\text{35}\) The day before the experiment, the cells were plated at 20,000 cells/well in black-walled, clear-bottom, 384-well BD PureCoat amine-coated plates (BD, Bedford, MA). On the day of the experiment, the cell culture medium was evacuated and replaced with Hank’s buffered salt solution plus 20 mM HEPES-NaOH, pH 7.3 (assay buffer) containing 1.25 μg/mL Thallos-AM plus 0.02% final Pluronic F-127 (Millipore Sigma, St. Louis, MO) (dye loading solution) at 20 μL/well. Cells were incubated in dye loading solution for approximately 1 h at room temperature in the dark. After incubation, the dye loading solution was evaporated and replaced with 20 μL/well assay buffer. Compound serial dilutions were made in dimethyl sulfoxide and subsequently transferred to assay buffer at 2x concentration. Tl′ flux was recorded using the Panoptic (Wavefront Biosciences, Franklin, TN) kinetic imaging plate reader (1 Hz, excitation 480/40, emission 538/40). Following the collection of 10 frames of baseline images, 20 μL/well of 2x compound solution was added to the cells with continued imaging. After 4 min of continuous imaging while the cells incubated with compound, 10 μL/well of 125 mM sodium bicarbonate, 12 mM thallium sulfate, 1 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, and 10 HEPES-NaOH (pH 7.3) was added to the cells and images were collected for two additional minutes.

**Rubidium (86Rb)-Flux Experiments.** DmKCC-expressing cells (1D4) were grown to confluence in 10 cm dishes with 10 mL of DMEM:F12 (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 180 U/mL penicillin + 180 U/mL penicillin + 250 μg/mL zeocin, at 37 °C 95% air, 5% CO₂. The day of the flux experiment, cells from 3–10 cm dishes were detached with trypsin and resuspended into 50 mL of complete medium, and 2 mL of homogeneous suspension was added to 24 × 35 mm dishes precoated with 0.1 mg/mL poly-l-lysine (Sigma). The cells were allowed to attach for 2 h in the incubator. For the rubidium uptake experiment, the medium was aspirated and replaced with 1 mL isosmotic saline containing 140 mM NaCl (or N-methylglucamine Cl for Na−free solution), 5 mM KCl, 2 mM CaCl₂, 0.8 mM MgSO₄, 2 mM glucose, 200 μg/μL, 2.5 mM HEPES, pH 7.4, 310 μM O₂, for a 15 min preincubation period. For the hypotonic saline, the concentration of NaCl (or NMDG-Cl) was reduced to 100 mM. After preincubation, the saline was aspirated and replaced with 1 mL of an identical solution containing 1 μCi/ml 86Rb (PerkinElmer) for 15 min. After the uptake period, the medium was aspirated and the cells were washed three times with 1 mL of ice-cold saline. Following the rinse, the cells were lysed with 500 μL of 0.25 N NaOH for 1 h and then neutralized with 250 μL of glacial acetic acid. Aliquots of 300 μL in 5 mL of BioSafe-II liquid were utilized for β-scintillation counting using a Tri Carb 2910-TR, (PerkinElmer), and 20 mL for Bradford protein assay, respectively. Two aliquots of 5 mL of uptake solution were also counted per condition to transform the measured cpm into pmol K⁺. The K⁺ influx was expressed as pmol K⁺/mg protein x min⁻¹.

**Western Blotting.** HEK293, DmKCC-expressing 1D4 monoclonal HEK293 cells, and *Aedes aegypti* KCC-expressing HEK293 cells\(^\text{35}\) were grown to 90% confluence in 150 mm TC-treated dishes (Corning, Corning, NY) in cell culture medium (see the Monoclonal
DmKCC Cell Line Generation (section above). Cells were washed in ice-cold phosphate buffered saline (Corning, Corning, NY), mechanically dissociated from the culture dishes, and transferred to prechilled conical tubes on ice. Cells were pelleted at 500 g for 2 min and resuspended in extraction buffer containing 50 mM HEPES, 10 mM dithiothreitol, 5 mM EDTA, 150 mM KCl, 1× Halt Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA). Cells were triturated with a p1000 pipet to achieve a single-cell suspension and lysed by vortexing and sonication. The lysates were then clarified by centrifugation for 8 min at 3800 g at 4 °C. To isolate membranes, the supernatants from the previous step were ultracentrifuged at 132,000g for 1 h at 4 °C. The pellets from the ultracentrifugation step were resuspended in ice-cold extraction buffer (membrane fraction) and stored at −80 °C until use. Membrane fractions were diluted in Laemmli sample buffer (BIO RAD, Hercules, CA) at room temperature to prevent protein aggregation and separated by SDS-PAGE on a NuPAGE Novex 4–12% Bis-Tris protein gel using the XCell SureLock Mini-Cell system (ThermoFisher Scientific, Waltham, MA) according to the manufacturer’s instructions. Separated proteins were transferred to an Odyssey nitrocellulose membrane (LI-COR, Lincoln, NE) using the XCell II Blot Module at 25 V for 1 h at RT, according to the manufacturer’s instructions. Membranes were then rinsed in Tris buffered saline (TBS) (Corning, Corning, NY) and blocked in 5% dry milk (BIO RAD, Hercules, CA) in TBS. Membranes were then rocked in a 1:2000 dilution of a rabbit anti-KCC antisera in 5% milk in TBS + 0.1% Tween20 (TBS-T) (Millipore Sigma, St. Louis, MO) at 4 °C overnight. Membranes were then washed 3× times in TBS-T, rocked in a 1:15,000 dilution of donkey anti-rabbit IRDYE800CW secondary antibody (LI-COR, Lincoln, NE) for 1 h at room temperature and then washed 3× additional times in TBST. Finally, membranes were then rinsed in TBS and visualized on the Odyssey CLx near-infrared fluorescence imaging system (LI-COR, Lincoln, NE). The anti-KCC antisera was derived against a C-terminal KCC epitope conserved from D. melanogaster KCC to human KCC1–4 as in ref 30.

Electrophysiological Studies of Drosophila Melanogaster Neural Systems. Electrophysiological recordings were performed on the CNS of third instar D. melanogaster as described previously.46 Glass pipet electrodes were pulled from borosilicate glass capillaries on a P-1000 Flaming/Brown micropipet puller (Sutter Instrument, Novato, CA). The CNS was excised from the larvae and placed in a separate dish with physiological saline (200 μL) containing 157 mM NaCl, 3 mM KCl, 2 mM CaCl₂, and 4 mM HEPES, pH = 7.25. The CNS was manually transected posterior to the cerebral lobes to disrupt the blood-brain barrier and enhance chemical penetration into the CNS.47–49 Peripheral nerve trunks were drawn into a recording suction electrode and electrical activity was monitored from descending nerves originating from the CNS, with amplification by an AC/DC amplifier (Model 1700, A-M Systems, Inc., Carlsborg, WA). Descending electrical activity was subjected to window amplitude discrimination and converted online into a rate plot, expressed in hertz (Hz), using LabChart7 Pro (ADInstruments, Colorado Springs, CO). Noise (60 Hz) was eliminated using Hum Bug (A-M Systems, Sequim, WA). Activity was monitored for a 5 min time period to establish a constant baseline and each concentration prior to the addition of the next drug. After a baseline was established, the CNS preparation was directly exposed to test compounds by adding 200 μL of saline. The inhibitors were applied directly to the larval body cavity and the final concentration of DMSO never exceeded 0.1%. Each concentration was recorded for 3 min or until the spike frequency became constant. Mean spike frequencies for each concentration were analyzed identically to those of the CNS recordings.

Genetic Knockdown of CNS Specific KCC. The GAL4-UAS system was used for tissue specific genetic ablation of KCC. The GAL4-UAS construct binds next to the gene of interest, which in this case is hairpin RNA (hpRNA) for KCC, to genetically enhance or decrease mRNA expression.49,50 The two components, GAL4 and UAS, are carried in separate Drosophila stocks that allow for hundreds of combinatorial possibilities after a simple parental cross. In this study, we utilized a strain of fly that expressed the GAL4-UAS promoter only in the CNS of third instars, which is the lifestyle analyzed using electrophysiological methods. These methods enabled the CNS-specific knockdown of KCC.

A schematic representation of the cross that enabled CNS specific knockdown of KCC is described by St. Johnston.51 Knockdown was achieved by crossing virgin females from the respective KCC RNAi strain (Bloomington stock 34584) with males from the CNS expressing GAL4-UAS strain (Bloomington stock 6870). The flies were given 96 h to mate and oviposit prior to removal from the growing medium. F1 offspring were allowed to reach the wandering stage and these maggots were used in electrophysiological recordings. RNA isolation, cDNA Synthesis, and Quantitative-PCR. RNA isolation, cDNA synthesis and qRT-PCR was performed as described in our previous studies.46 Briefly, total RNA was isolated and extracted from 30 Drosophila larvae CNS using TRIzol Reagent (Life Technologies, Carlsbad, CA) and purified using the RNEasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from poly(A) RNA using the SuperScript III First-Strand Synthesis system for real-time quantitative PCR (qRT-PCR) (Life Technologies) according to manufacturer instructions. qRT-PCR was then performed on a Qiagen Rotor Gene Q 2Plex Real-Time PCR system using the operating instructions. Relative quantification was carried out using the 2 ΔΔCT method,52 and β-actin was used as the reference gene. Appropriate controls, such as DNase and removal of reverse transcriptase, were performed to ensure the sample was not contaminated with genomic DNA. The kcc primers used in this study were identical to previous work30 and were as follows: forward: 5′- CCGAGAGCATACCCATCG and reverse: 5′- AATGATGGGCTTCCCCATAG. β-Actin was the housekeeping gene used as a standard, and actin primers were purchased from Life Technologies with primer reference numbers of Dm02361909.1s1. Five biological replicates were conducted and each was analyzed in triplicate. The graphed output displays average fold-change in mRNA levels relative to the wild type Oregon-R control CNS.

Statistical Analyses. For T1-flux studies, fluorescence values were normalized on a well-to-well basis by dividing the fluorescence values at each time point to the average of the baseline fluorescence values (F0) for a given well. The slope of the fluorescence increase between 1 and 86 s after T1’ addition was used as a measure of T1’ flux. The slopes of the change in fluorescence in the absence of inhibitor were designated as 100% KCC activity and slopes obtained in the presence of a maximally effective concentration of 10 μM were designated as 0% KCC activity. To compare the effect of DMSO on DmKCC-mediated T1’ flux, a one-way ANOVA was performed with a Tukey’s multiple comparison test. The potency of test compounds was determined by fitting slopes obtained at compound concentrations varying over a range of 10 μM to 0.03 nM using a four-parameter logistic equation with GraphPad Prism. For a given experiment, all conditions were tested in six wells per plate. Values reported are averages of those obtained from three independent experiments ± standard error of the mean (SEM).
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