RETT SYNDROME

Pharmacological enhancement of *KCC2* gene expression exerts therapeutic effects on human Rett syndrome neurons and *Mecp2* mutant mice

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Rett syndrome (RTT) is a neurodevelopmental disorder caused by mutations in the methyl CpG binding protein 2 (*MECP2*) gene. There are currently no approved treatments for RTT. The expression of K^+/Cl^- cotransporter 2 (KCC2), a neuron-specific protein, has been found to be reduced in human RTT neurons and in RTT mouse models, suggesting that KCC2 might play a role in the pathophysiology of RTT. To develop neuron-based high-throughput screening (HTS) assays to identify chemical compounds that enhance the expression of the *KCC2* gene, we report the generation of a robust high-throughput drug screening platform that allows for the rapid assessment of *KCC2* gene expression in genome-edited human reporter neurons. From an unbiased screen of more than 900 small-molecule chemicals, we have identified a group of compounds that enhance KCC2 expression termed KCC2 expression–enhancing compounds (KEECs). The identified KEECs include U.S. Food and Drug Administration–approved drugs that are inhibitors of the fms-like tyrosine kinase 3 (FLT3) or glycogen synthase kinase 3β (GSK3β) pathways and activators of the sirtuin 1 (SIRT1) and transient receptor potential cation channel subfamily V member 1 (TRPV1) pathways. Treatment with hit compounds increased KCC2 expression in human wild-type (WT) and isogenic *MECP2* mutant RTT neurons, and rescued electrophysiological and morphological abnormalities of RTT neurons. Injection of KEEC KW-2449 or piperine in *Mecp2* mutant mice ameliorated disease-associated respiratory and locomotion phenotypes. The small-molecule compounds described in our study may have therapeutic effects not only in RTT but also in other neurological disorders involving dysregulation of KCC2.

INTRODUCTION

Disruptions in the intricate balance between excitatory and inhibitory neural activities (E/I imbalance) have emerged as a potential common underlying cause of brain disorders including epilepsy (*1*, *2*), schizophrenia (*3*, *4*), autism (*5*, *6*), fragile X syndrome (*7*–*9*), and Rett syndrome (RTT) (*10*). The neuron-specific K^+/Cl^- cotransporter 2 (KCC2) plays a pivotal role in the maintenance of E/I balance in the brain. KCC2 is a major membrane Cl^- transporter that maintains neuronal chloride homeostasis and determines the polarity and efficacy of γ-aminobutyric acid (GABA) inhibition (*11*, *12*) and is also a critical regulator of dendritic spine morphogenesis and excitatory synapse development (*13*–*15*). Reduction in KCC2 expression and/or function has been reported in human brain disorders such as epilepsy (*16*–*18*), schizophrenia (*19*, *20*), and spinal cord injury (*21*, *22*), as well as in the senile brain characterized by synaptic plasticity deficits (*23*). RTT is a genetically defined neurodevelopmental disorder caused by mutations in the X-linked gene *MECP2*, a transcriptional factor that binds to methylated DNA and regulates gene expression (*24*–*26*). Our previous work has shown that the expression of KCC2 is reduced in human neurons derived from patients with RTT and in the brains of a *Mecp2* mutant mouse model of RTT (*27*, *28*). Identification of drugs that increase KCC2 expression could provide a novel therapeutic approach to treat a diverse array of nervous system diseases.

Unlike other Cl^- transporters, KCC2 is expressed exclusively in neurons (*29*). This tissue-restricted expression pattern confers target specificity and reduces the likelihood of side effects that KCC2-targeting drugs may exert on non-neuronal tissues (*30*), highlighting KCC2 as a prime drug target to regulate intracellular $[Cl^-]$ in neurons (*31*–*33*). Previous efforts to identify small-molecule modulators of KCC2 relied on ectopic expression of KCC2 in non-neuronal cell lines, which lack endogenous KCC2 expression (*34*, *35*) and have failed to identify compounds that increase KCC2 expression (*36*), possibly because these cells lacked the complex cellular and genomic regulatory context of KCC2. We hypothesize that enhancing KCC2 expression will not only increase the efficacy of GABAergic inhibition but also boost dendritic spine and excitatory synapse development, both of which are abnormal in many brain diseases including RTT (*37*–*39*) and may be caused by aberrant interaction between KCC2 and the dendritic cytoskeleton (*13*, *15*, *40*). In this study, we focused on identifying small-molecule compounds that are capable of enhancing *KCC2* gene expression in neurons.

We have used human embryonic stem (ES) cells in combination with CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9) gene editing and high-throughput screening (HTS) technologies to identify small molecules that enhance KCC2 expression in neurons. We developed a robust HTS assay by inserting a 2A-luciferase reporter gene into the endogenous *KCC2* locus in human ES cells, providing a convenient readout of *KCC2* gene expression from its endogenous genetic locus. By screening these KCC2 reporter human neurons, we identified a number of hit KCC2 expression–enhancing compounds (KEECs) from ~900

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small-molecule compounds. Identified KEECs were validated by Western blot and quantitative reverse transcription polymerase chain reaction (RT-PCR) experiments on cultured human wild-type (WT) and isogenic RTT neurons, as well as on organotypic mouse brain slices. Pharmacological and molecular biology experiments showed that identified KEECs act through inhibition of the fms-like tyrosine kinase 3 (FLT3) or glycogen synthase kinase 3β (GSK3β) kinases, or activation of the sirtuin 1 (SIRT1) or transient receptor potential cation channel subfamily V member 1 (TRPV1) pathways. Treatment of RTT neurons with KEECs rescued disease-related deficits in GABA functional switch, excitatory synapses, and neuronal morphological development. Last, injection of the identified KEEC KW-2449 or piperine into a *Mecp2* mutant mice ameliorated behavioral phenotypes including breathing pauses and reduced locomotion, which represent important preclinical data, suggesting that the KEECs identified in this study may be effective in restoring impaired E/I balance in the RTT brain and provide symptomatic treatment for patients with RTT.

RESULTS

CRISPR-Cas9 genome-edited stem cells were used to generate KCC2 expression reporter neurons

To develop a robust platform to screen for KEECs in neurons, we used CRISPR-Cas9 genome editing technology to insert a 2A-luciferase reporter gene directly before the stop codon of the endogenous *KCC2* locus in human ES cells. KCC2 expression reporter human neurons were differentiated from the gene-targeted ES cells and used as the substrate for unbiased compound screening (Fig. 1A). When the KCC2 gene is transcribed from its endogenous locus and translated into proteins, the self-cleaving 2A peptide–mediated ribosomal skipping mechanism mandates the expression of an equal number of firefly luciferase enzymes, which can be conveniently detected with a sensitive luciferase assay (*41*). The small V5 and hemagglutinin (HA) epitope tags were fused to the C terminus of KCC2 to facilitate protein detection (*12*). We used Sanger sequencing and Southern blot analyses to confirm correct gene editing (fig. S1A), and conducted assay development experiments to optimize the parameters for screening (fig. S1, B to D). These gene-targeted KCC2 reporter cells were used in an HTS pipeline to screen for small-molecule compounds that enhance KCC2 gene expression (fig. S1E).

Small-molecule screen identifies compounds that increase KCC2 expression in neurons

Using neurons derived from the KCC2 reporter ES cells, we conducted an unbiased screen with several drug libraries including the Library of Integrated Network-Based Cellular Signatures (LINCS) kinase inhibitor library, the Selected Molecular Agents for Rett Therapy (SMART) compound library from International Rett Syndrome Foundation, and the Institute of Chemistry and Cell Biology (ICCB) known bioactive library (Fig. 1B). From six replicate screenings, a total of 14 compounds were identified as hit KEECs (*B* score >3), including KW-2449, BIO (6-bromoindirubin-3′-oxime), and resveratrol (Fig. 1C; a complete list of hit compounds generated from WT neuron screening is provided in table S1). Further analysis showed that the hit KEECs increased KCC2 reporter activity by more than twofold (Fig. 1C). To validate the KEECs, we treated human neurons derived from WT ES cells (WIBR1 male ES cell line) with KEECs and conducted Western blot experiments to measure changes in KCC2 expression. Treatment of cultured WT human neurons with KW-2449, a potent inhibitor of FLT3 (*42*), induced a significant increase in KCC2 expression in a dose-dependent manner (1 μM concentration; P = 0.002; Fig. 1D). BIO, an inhibitor of the GSK3β pathway (*43*), significantly increased KCC2 expression (Fig. 1E).

To elucidate the molecular mechanisms through which hit KEECs regulate KCC2 expression, we tested additional chemical compounds that are structurally different but functionally analogous to the primary hit compounds KW-2449 and BIO. Our results demonstrated significant enhancement of KCC2 expression in human neurons treated with a number of structurally diverse FLT3 kinase inhibitors including crenolanib, XL-184, and sunitinib (P = 0.031 for crenolanib, P = 0.002 for XL-184, and P = 0.009 for sunitinib, drugs applied at 1 μM concentration; Fig. 1, F to H). TWS-119 is a GSK3β inhibitor that is structurally unrelated to the primary hit compound BIO. Our results show that treatment of WT neurons with TWS-119 robustly increased KCC2 expression by more than twofold even at the low concentration of 0.03 μM (P < 0.001; Fig. 1I).

KEECs increase KCC2 mRNA and protein expression and induce hyperpolarizing shift in E_{GABA} in immature neurons

Organotypic mouse brain slices preserve the cellular architecture and cell-cell contact environment of the brain and, accordingly, are considered a suitable model system to investigate drug-induced changes in brain-specific gene expression (*44*). We treated organotypic brain slices with KEECs and analyzed KCC2 protein expression by Western blot analysis (Fig. 2A). Treatment of mouse brain slices with the FLT3 kinase inhibitor KW-2449 (2 μM; P = 0.010; Fig. 2B) or the GSK3β inhibitor BIO (1 μM; P = 0.002; Fig. 2C) enhanced KCC2 expression as compared to a dimethyl sulfoxide (DMSO)–treated control and an inactive analog of BIO (MeBIO), respectively.

We further treated brain slices with the FLT3 inhibitors crenolanib and XL-184. Both compounds significantly increased the KCC2 protein expression as compared to culture medium–only control slices (P < 0.001 for crenolanib and P = 0.017 for XL-184; drugs applied at 2 μM concentration; Fig. 2D). To assess whether increased KCC2 protein expression was due to increased gene transcription or enhanced protein stability, we performed quantitative RT-PCR experiments to measure the KCC2 mRNA expression in KEEC-treated brain slices. As shown in Fig. 2E, treating the brain slices with the FLT3 inhibitor sunitinib, XL-184, or crenolanib or with the GSK3β inhibitor indirubin monoxime significantly increased KCC2 expression (P = 0.018 for sunitinib, P = 0.001 for XL-184, P = 0.045 for crenolanib, and P < 0.001 for indirubin monoxime; drugs applied at 2 μM concentration). In contrast, the expression of NKCC1, a chloride transporter that antagonizes KCC2 activity by shuttling Cl^- into the cell (*45*), was decreased by treatment with KEEC compounds (2 μM; Fig. 2F). As a result, the KCC2/NKCC1 ratio was significantly increased in the sunitinib-treated (P = 0.011), XL-184–treated (P = 0.014), and indirubin monoxime–treated (P = 0.003) brain slices. When primary mouse astrocyte cultures were treated with KEECs, no detectable ectopic KCC2 expression or change in NKCC1 expression was observed (fig. S2). We conclude that KCC2 is expressed in neurons but not in astrocytes, and regulates the expression of KCC2 and NKCC1 in response to KEECs.

Because notable increases in the ratio of KCC2/NKCC1 expression were detected in KEEC-treated organotypic brain slices, we investigated

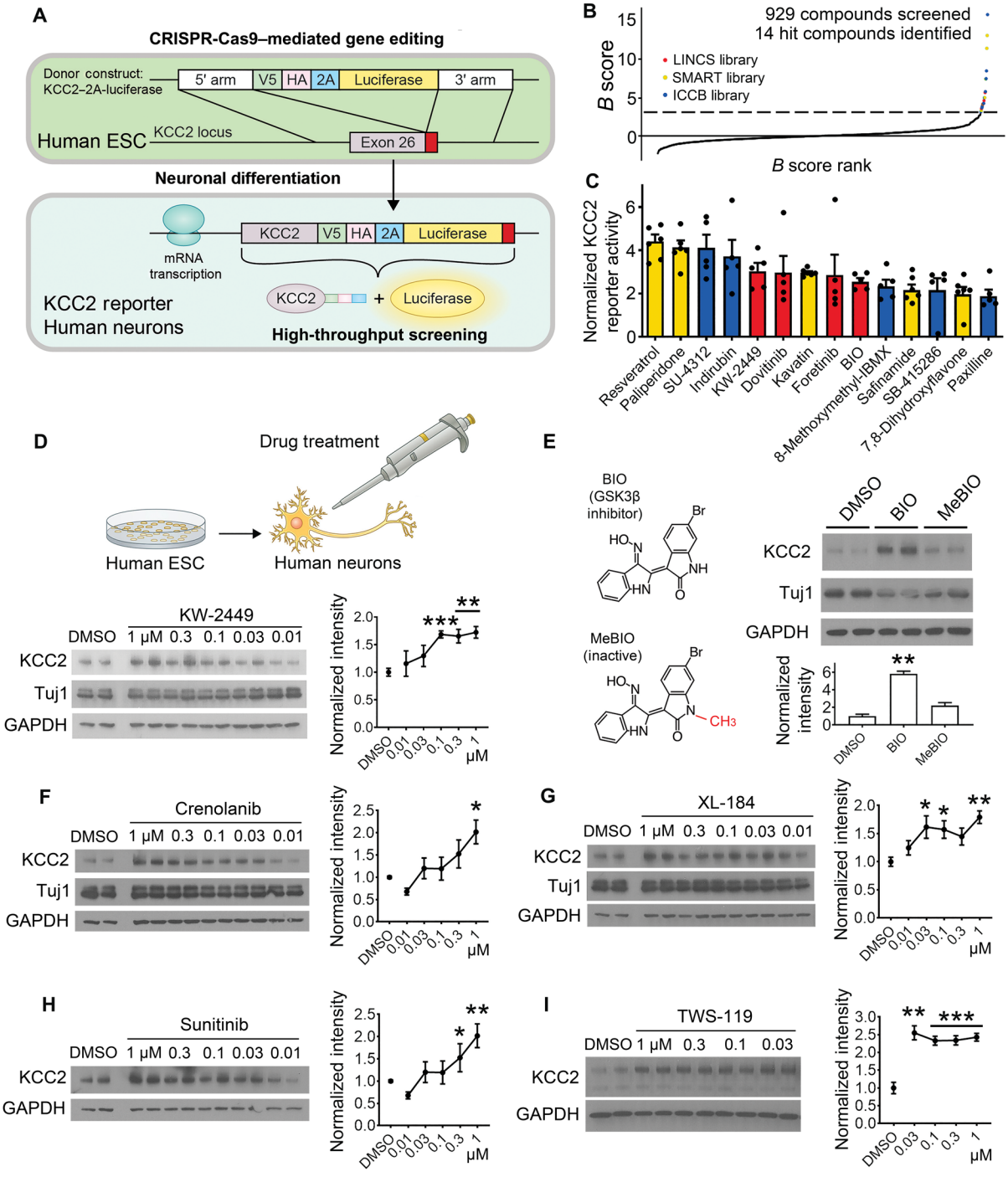

Fig. 1. Development of an HTS platform with human KCC2 reporter neurons to identify a group of KEECs. (**A**) Diagram depicting the compound screening platform: A 2A-luciferase gene expression reporter was inserted in-frame immediately before the endogenous stop codon of KCC2 gene in human ES cells. Luciferase activity faithfully reflects the expression amount of KCC2 in the KCC2 reporter human neurons differentiated from the gene-targeted ES cells. (**B**) *B* score rank of 929 small-molecule compounds from LINCS, SMART, and ICCB drug libraries; *B* score >3 indicates compounds potentially increasing KCC2 expression. (**C**) KCC2 reporter activity induced by the most significant compounds identified from the WT neuron screening. Compound data were color-coded according to their library of origin: LINCS (red), SMART (yellow), and ICCB (blue). (**D**) Drug treatment of cultured WT human ES cell (ESC)–derived neurons (shown in the diagram) and the KCC2 protein expression induced by KEEC KW-2449 (an inhibitor of FLT3 kinase, $n = 4$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**E**) KCC2 protein expression induced by KEEC BIO (6-bromoindirubin-3′-oxime; $n = 2$) and MeBIO (methylated BIO; $n = 2$) treatments in cultured human WT neurons. (**F** to **I**) KCC2 expression induced by crenolanib (F), XL-184 (G), sunitinib (H), and TWS-119 (I) in cultured human WT neurons ($n = 4$ for each group). Data are means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, Student's *t* test.

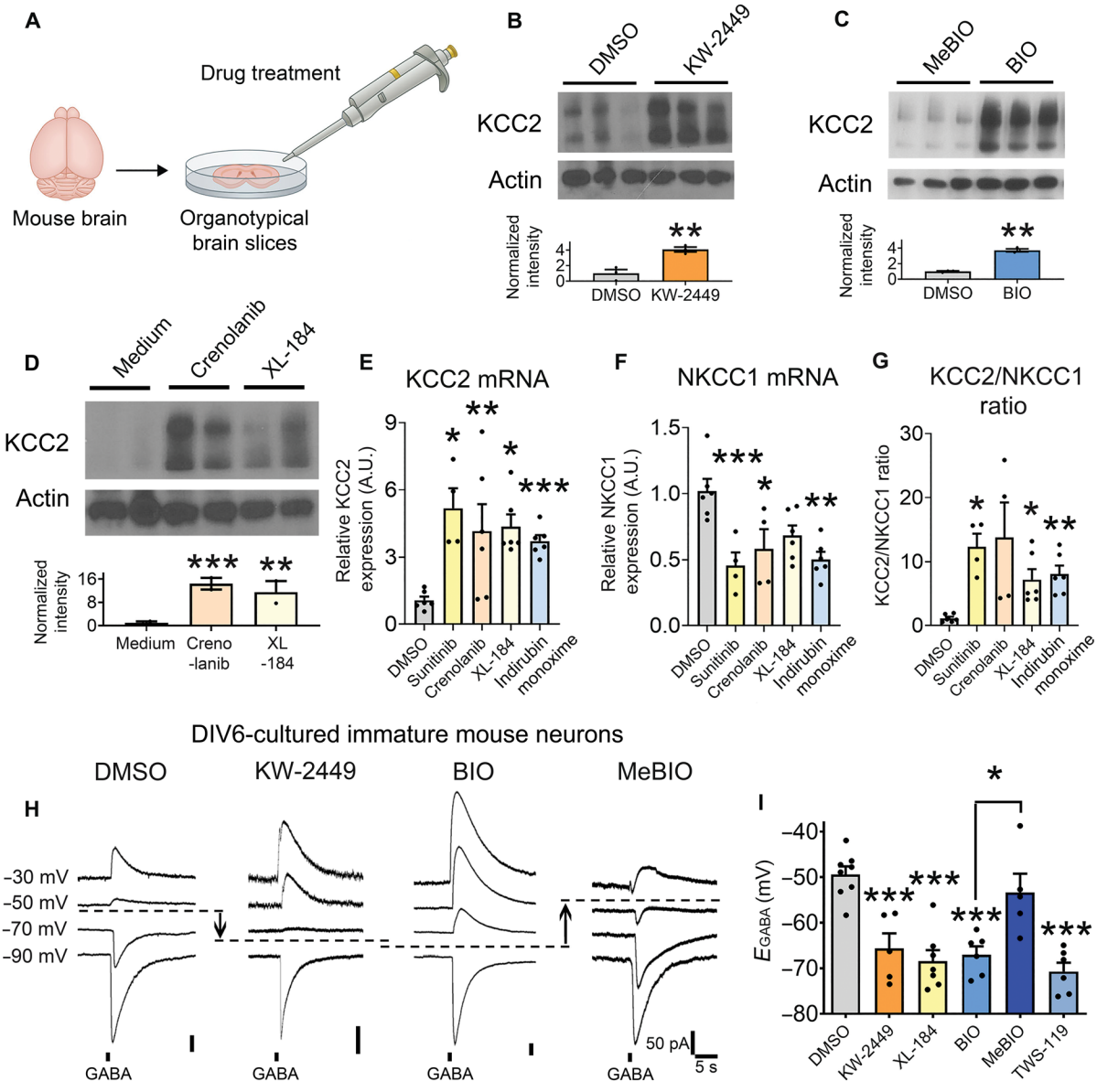

Fig. 2. KEEC treatment–induced enhancement of KCC2 protein and mRNA expression in cultured organotypic mouse brain slices and a hyperpolarizing E_{GABA} shift in cultured immature neurons. (**A**) Diagram depicting the experimental scheme: Organotypic brain slices were prepared from P3 neonatal mouse brain and treated with vehicle or KEECs for 7 days before analysis. (**B**) KCC2 protein expression induced by KW-2449 in organotypic mouse brain slices ($n = 3$). (**C**) KCC2 protein expression induced by BIO in organotypic mouse brain slices ($n = 3$). (**D**) KCC2 protein expression induced by crenolanib or XL-184 in organotypic mouse brain slices ($n = 2$). (**E** to **G**) KCC2 and NKCC1 mRNA expression induced by FLT3 inhibitors including sunitinib ($n = 4$), XL-184 ($n = 6$), crenolanib ($n = 4$), or a structural analog of BIO termed indirubin monoxime ($n = 6$). The calculated ratios of KCC2/NKCC1 mRNA expression are shown in (G). A.U., arbitrary units. (**H**) Representative gramicidin-perforated patch recording results showing the responses to GABA recorded from neonatal mouse neurons cultured for 6 days in vitro (DIV6) and treated with KEECs or controls. Dashed lines indicate GABA reversal potential (E_{GABA}) in each condition. (**I**) Quantified E_{GABA} recorded from DIV6-cultured immature mouse neurons treated with KEEC KW-2449 ($n = 5$), XL-184 ($n = 7$), BIO ($n = 6$), MeBIO ($n = 5$), or TWS-119 ($n = 6$). Data are means ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, one-way ANOVA with Bonferroni correction.

whether treatment with KEECs would result in a hyperpolarizing shift in the GABA reversal potential (E_{GABA}), an indicator of the efficacy of GABAergic inhibition in neurons (*46*). To determine the developmental timeline of the GABA functional switch in cultured mouse neurons, we conducted gramicidin-perforated patch recording experiments in mouse cortical neurons cultured for different periods of time to estimate E_{GABA}. Figure S3 shows that in neurons cultured for 6 days in vitro (DIV6), E_{GABA} remained at the depolarized numbers of about −50 mV, indicating that the cells have not yet undergone the GABA functional switch consistent with previous reports (*47*). When E_{GABA} was recorded from DIV6–7 immature mouse neurons treated with KEECs or controls, we found that treatment with KEEC KW-2449, XL-184, TWS-119, or BIO (drugs applied at 1 μM concentration), but not DMSO or the inactive compound MeBIO, induced a significant hyperpolarizing shift in neurons to an E_{GABA} of about −70 mV ($P < 0.001$ for KW-2449, XL-184, BIO, and TWS-119 groups; drugs applied at 1 μM concentration; Fig. 2, G and H). To validate the GABA response recording results,

we conducted pharmacology experiments and showed that both the inward and outward currents induced by administrating GABA onto neuronal cell body were blocked by the $GABA_A$ receptor antagonist picrotoxin (50 μM; $P < 0.001$) but not by the AMPA or NMDA (*N*-methyl-D-aspartate) receptor blocker NBQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline) or AP-V [(2R)-amino-5-phosphonopentanoate] (blockers applied at 20 and 50 μM, respectively; $P > 0.5$; fig. S4). We conclude that treatment of immature mouse neurons with KEECs facilitates a GABA functional switch.

KEECs enhance KCC2 expression in human RTT neurons

A reduction in KCC2 protein expression has been detected in *MECP2*-deficient human RTT neurons and in the brains of *Mecp2* mutant mouse model of RTT (*27*, *28*), in autopsy brain samples (*48*), and in the cerebrospinal fluid of patients with RTT (*49*). To assess whether the KEECs identified above would enhance KCC2 expression in RTT neurons, we screened *MECP2*-null RTT human KCC2 reporter neurons (isogenic to the WT reporter cells used in Fig. 1) with the same compound libraries for reporter activation. Most KEECs that enhanced KCC2 expression in WT neurons, including KW-2449, BIO, and resveratrol, also induced a robust increase of KCC2 reporter activity in RTT neurons (Fig. 3, A and B; a complete list of hit compounds is provided in table S2). The increase in KCC2 signal induced by KEECs was higher in RTT neurons than in WT neurons, consistent with baseline KCC2 expression being lower in RTT neurons (*28*, *49*). Figure S5 shows the dose-response curves of a number of hit KEECs and their derivatives to induce luciferase reporter activity. Confirming the KCC2 luciferase reporter assay, a significant enhancement of KCC2 protein expression was detected by Western blot in human female RTT neurons treated with KEECs. This was seen with FLT3 kinase inhibitors, including KW-2449 (applied at 1 μM concentration; $P = 0.010$; Fig. 3C), crenolanib (1 μM concentration; $P = 0.007$; Fig. 3E), XL-184 (1 μM concentration; $P = 0.008$; Fig. 3G), and FLT3 inhibitor-1 (10 μM concentration; $P = 0.001$; Fig. 3H), as well as with the GSK3β inhibitor BIO (0.3 μM concentration; $P = 0.018$; Fig. 3D) and a structural analog of BIO, indirubin monoxime (1 μM concentration; $P = 0.007$; Fig. 3F).

To further corroborate that inhibition of the FLT3 or GSK3β pathway leads to increased KCC2 expression, we used small interfering RNA (siRNA) to directly silence the expression of target genes. Transfection of siRNA against the mouse *Flt3* or *Gsk*β gene into immature mouse neurons significantly increased KCC2 gene expression ($P = 0.010$ for flt3 siRNA group and $P = 0.031$ for gsk3β siRNA group; fig. S6, A to C). Similarly, transfection of siRNA against the human *FLT3* or *GSK3*β genes significantly increased KCC2 gene expression in cultured human RTT neurons ($P = 0.005$ for FLT3 siRNA group and $P = 0.042$ for GSK3β siRNA group; fig. S6, D to F). Our results establish a causal relationship between reduced FLT3 or GSK3β signaling activity and increased KCC2 expression.

Two hit compounds, resveratrol and piperine, act on different pathways than the kinase inhibitors, activating the SIRT1 signaling pathway (*50*) and the TRPV1 (*51*), respectively. A study using a glioblastoma cell line has shown that resveratrol activates the SIRT1 pathway, reducing expression of the neuronal gene repressor neuron-restrictive silencer factor (NRSF)/RE1-silencing transcription factor (REST) (*50*), an inhibitor of KCC2 expression (*52*). When resveratrol (10 μM) was applied to cultured neurons in the presence of a high concentration of the SIRT1 pathway blocker EX-527 (10 μM), the expression of neither KCC2 nor REST was changed as compared to the DMSO control ($P = 0.271$ for KCC2 and $P = 0.664$ for REST; Fig. 3I). However, when resveratrol was co-applied with 1 μM EX-527 that is insufficient to block SIRT1 pathway signaling, a substantial increase in KCC2 expression and a concurrent reduction in the expression of REST were observed ($P = 0.019$ for KCC2 and $P = 0.034$ for REST; Fig. 3I). Our results support the notion that SIRT1-mediated reduction in REST expression is a likely link between resveratrol treatment and increased KCC2 expression. Treatment with piperine (10 μM), an activator of the TRPV1 channel (*51*), induced a significant rise in KCC2 expression in cultured human neurons ($P = 0.040$; Fig. 3J), whereas blocking the TRPV1 channel with the TRPV1 antagonist A784168 (10 μM) eliminated the KCC2-inducing effect of piperine ($P = 0.277$; Fig. 3K). Thus, our data demonstrate that activation of the SIRT1 pathway or the TRPV1 channel enhances KCC2 expression in RTT human neurons.

Treatment with KEECs rescues the functional and morphological deficits in RTT neurons

Our previous work demonstrated that reduced KCC2 expression in the RTT neurons causes a depolarizing shift in E_{GABA} reflecting impaired GABAergic inhibition (*27, 28*). Moreover, a reduction in the number or the strength of excitatory glutamatergic synapses in RTT neurons has been observed in postmortem brain samples obtained from patients with RTT (*31*), mouse models of RTT (*53*), and human stem cell–derived RTT neurons (*54*). We used electrophysiological recording to assess whether KEECs could rescue the deficits in E_{GABA} and excitatory synaptic transmission of RTT neurons.

Results from gramicidin-perforated patch recording experiments indicated that the cultured human RTT neurons treated with DMSO had E_{GABA} values of about −50 mV, which represents a significant depolarizing shift compared to the average value of −70 mV in WT neurons ($P < 0.001$; Fig. 4, A and B). Treatment of RTT neurons with KEECs including KW-2449 and BIO (1 μM), but not the inactive compound MeBIO (1 μM), induced a significant hyperpolarizing shift in E_{GABA} to numbers comparable to that in WT neurons ($P < 0.001$ for all groups; Fig. 4B). We further conducted intracellular [Cl^-] imaging experiments by transfecting the chloride indicator Super-Clomeleon into human RTT neurons treated with DMSO or KW-2449 (1 μM). Our results showed a significant increase in the chloride extrusion rate in RTT neurons treated with KW-2449, as demonstrated by the reduction of the decay time constant of fluorescence resonance energy transfer (FRET) signal recovery to be equivalent to the time constant observed in isogenic WT neurons ($P = 0.030$; fig. S7, D and E).

Compared to WT human neurons, DMSO-treated RTT neurons showed a severe reduction in the frequency of miniature excitatory postsynaptic currents (mEPSCs), indicating reduction in the number of excitatory synapses (Fig. 4, C, D, and H). Because KCC2 promotes the development of excitatory synapses through its interaction with actin cytoskeleton and AMPA receptors (*13*, *15*), restoration of KCC2 expression in RTT neurons through KEEC treatment may rescue mEPSC deficits. Treatment of RTT neurons with KW-2449 (Fig. 4E) or BIO (Fig. 4F) significantly increased the frequency of mEPSCs to numbers equivalent to WT control, whereas treatment with the inactive compound MeBIO (Fig. 4G) failed to rescue synaptic deficits in RTT neurons ($P > 0.5$ for MeBIO group and $P < 0.001$ for all other groups; quantified data shown in Fig. 4H). Statistical analyses revealed that treatment with KW-2449 or BIO (1 μM) significantly

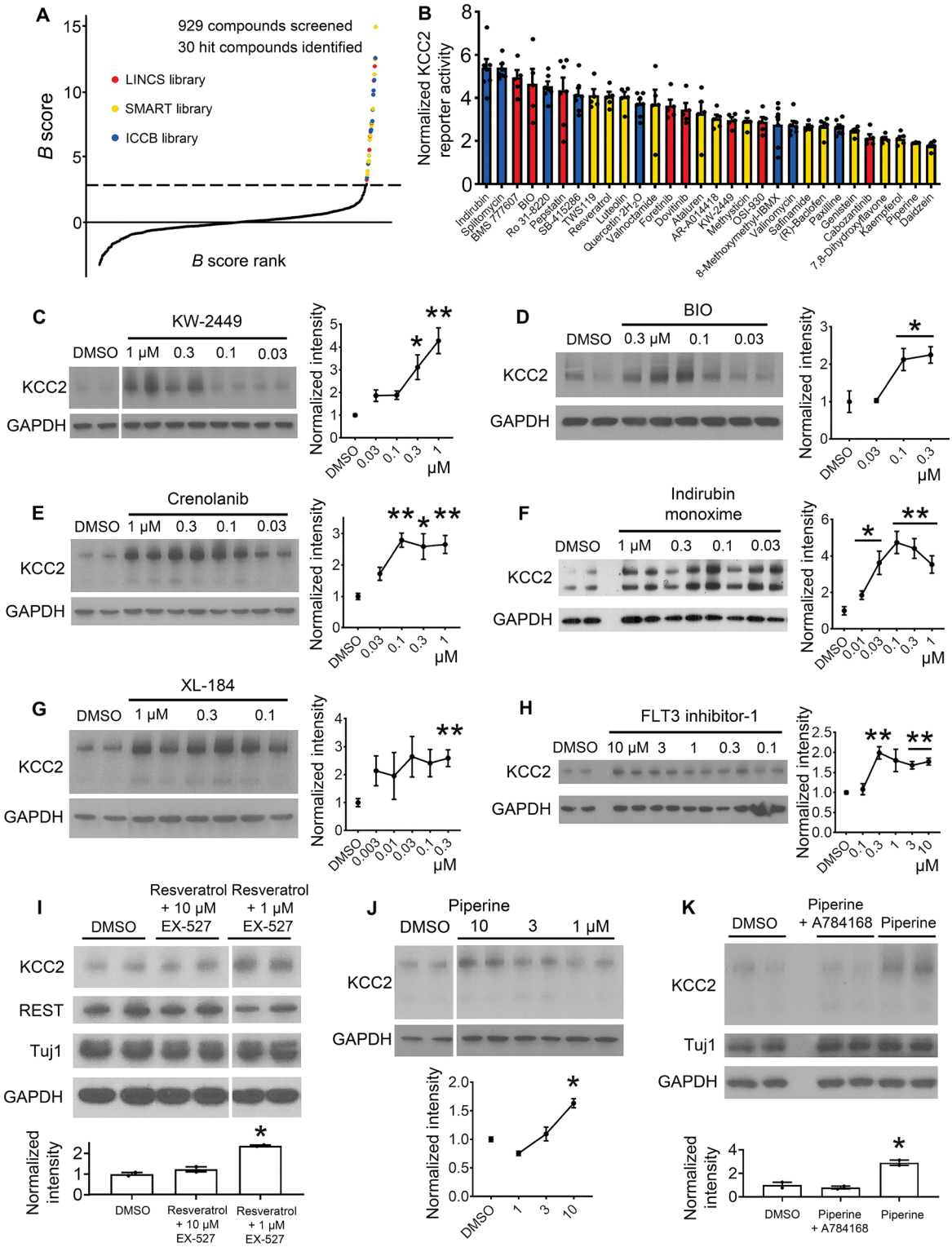

Fig. 3. Identification of KEECs that increase KCC2 expression in human RTT neurons. (**A**) *B* score rank of 929 small-molecule compounds from LINCS, SMART, and ICCB drug libraries; *B* score >3 indicates compounds potentially increasing KCC2 expression. (**B**) KCC2 reporter activity induced by the most significant compounds identified from the RTT neuron screening. Compound data were color-coded according to their library of origin: LINCS (red), SMART (yellow), and ICCB (blue). (**C** to **H**) KCC2 protein expression induced by KEEC KW-2449 ($n = 4$), BIO ($n = 3$), crenolanib ($n = 4$), indirubin monoxime ($n = 4$), XL-184 ($n = 4$), and FLT3 inhibitor-1 ($n = 4$) in cultured human RTT neurons. (**I**) KCC2 protein expression induced by treatment of RTT neurons with resveratrol, a SIRT1 activator ($n = 2$), and by co-application of the SIRT1 pathway inhibitor EX-527 ($n = 2$). (**J**) Dose-dependent KCC2 protein expression induced by treatment of RTT neurons with piperine ($n = 2$). (**K**) KCC2 protein expression induced by treatment of RTT neurons with piperine, a TRPV1 activator ($n = 2$), and by co-application of the TRPV1 antagonist A784168 ($n = 2$). Data are means ± SEM. *$P < 0.05$ and **$P < 0.01$, Student's *t* test.

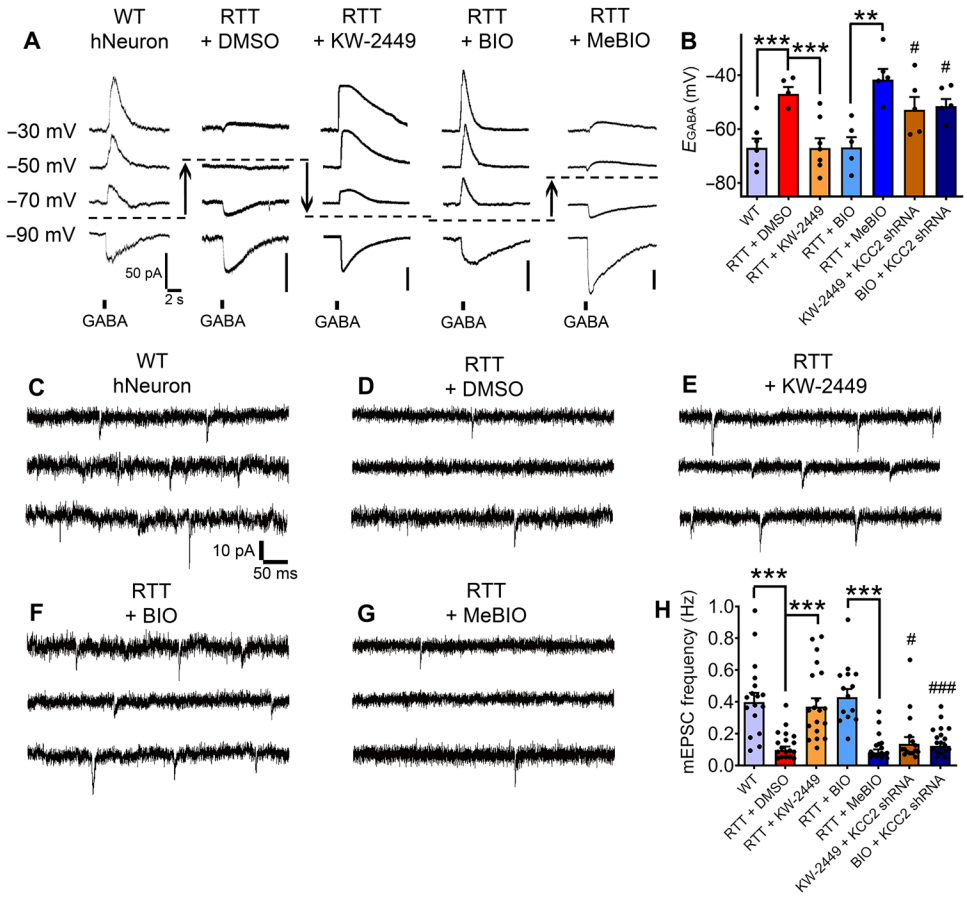

Fig. 4. Restoration of E_{GABA} and in excitatory neurotransmission in cultured human RTT neurons to quantities comparable to isogenic WT control neurons after treatment with KEEC KW-2449 or BIO. (**A**) Representative gramicidin-perforated patch recording results showing the responses to GABA recorded from human neurons (hNeuron) derived from WT or *MECP2* knockout RTT human stem cells and treated with KEECs or controls. Dashed lines indicate E_{GABA} in each condition. (**B**) Quantification of E_{GABA} recorded from WT neurons ($n = 6$) or isogenic RTT neurons treated with KEEC KW-2449 ($n = 7$) or BIO ($n = 5$) or the inactive analog MeBIO ($n = 5$). The effect on E_{GABA} induced by co-administration of KCC2 shRNA with KW-2449 ($n = 5$) or BIO ($n = 5$) was also quantified. $^{\#}P < 0.05$, compared to the KEEC-treated groups without shRNA transfection. (**C** to **G**) Representative mEPSC traces recorded from WT or RTT human neurons treated with KEECs or controls. (**H**) Quantification of mEPSC recorded from WT neurons ($n = 17$) or isogenic RTT neurons treated with KEEC KW-2449 ($n = 19$) or BIO ($n = 14$) or the inactive analog MeBIO ($n = 17$). The effect on E_{GABA} induced by co-administration of KCC2 shRNA with KW-2449 ($n = 15$) or BIO ($n = 19$) was also quantified. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$, compared to the KEEC-treated groups without shRNA transfection. Data are means ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$, one-way ANOVA with Bonferroni correction.

increased the mEPSC amplitude, an indicator of synaptic strength ($P < 0.001$ compared to DMSO control for both KW-2449– and BIO-treated groups; fig. S8, B and C).

We further investigated whether the beneficial effect of KEECs is a direct result of increased KCC2 gene expression. Treatment of RTT neurons transfected with a short hairpin RNA (shRNA) construct that knocks down KCC2 gene expression (*28*) with KEEC KW-2449 or BIO failed to hyperpolarize E_{GABA} ($P = 0.040$ for KW-2449 + KCC2 shRNA and $P = 0.013$ for BIO + KCC2 shRNA; Fig. 4B), suggesting that the hyperpolarizing shift in E_{GABA} in RTT neurons treated with KW-2449 is due to increased KCC2 rather than to reduced NKCC1 expression. Similarly, KCC2 shRNA transfection abolished the effect of KW-2449 or BIO to enhance mEPSC frequency ($P = 0.002$ for KW-2449 + KCC2 shRNA and $P < 0.001$ for BIO + KCC2 shRNA; Fig. 4H). We further showed that, consistent with the KCC2 shRNA transfection results shown in Fig. 4B, the E_{GABA} shift induced by KW-2449 in RTT neurons could be acutely blocked by KCC2 inhibitors furosemide (200 μM) and VU 0240551 (50 μM) ($P < 0.001$; fig. S9) but not by the NKCC1 blocker bumetanide (100 μM). Thus, our results demonstrate that KEECs rescue the deficits in GABAergic and glutamatergic signaling through up-regulation of KCC2 gene expression.

Deficits in a number of morphological measurements, including nuclei size and neurite complexity and branching, are hallmarks of RTT neurons (*54*–*56*). We have quantified these morphological marks in cultured human RTT neurons treated with KEECs including KW-2449 or BIO (1 μM), as well as DMSO or MeBIO as controls (Fig. 5, A to D). Comparing KEEC-treated or control compound–treated groups, a significant increase in the size of neuronal nucleus ($P = 0.011$ for KW-2449 group and $P = 0.034$ for BIO group; Fig. 5F) and an enhancement in the length and complexity of microtubule-associated protein 2 (MAP2) staining–positive neurites ($P < 0.001$ for KW-2449 and BIO groups; Fig. 5, E, G, and H) were observed in KEEC-treated RTT neurons. We also assessed neuronal membrane capacitance (C_m), an indicator of neuronal cell size (*57*) that is reduced in RTT neurons, and found that KW-2449– or BIO-treated RTT neurons displayed a significantly higher C_m, at numbers comparable to isogenic WT human neurons, than RTT human neurons treated with DMSO or MeBIO control, suggesting a recovery of neuronal cell size in KEEC-treated RTT neurons (drugs applied at 1 μM concentration; $P < 0.001$; fig. S8A). Such increase in cell size in RTT neurons treated with KEECs is presumably mediated by enhanced excitatory activities, which induce activity-dependent genes such as brain-derived neurotrophic factor (BDNF) and activate the Akt–mTOR (mammalian target of rapamycin) pathway that regulates cell growth and is deficient in RTT neurons (*55*). As expected, knocking down KCC2 with a shRNA construct abolished the increase in C_m induced by KEECs ($P > 0.50$ compared to the DMSO control group; fig. S8A). In summary, our data demonstrate that KEEC treatment improves both functional and morphological deficits in RTT neurons to the physiological properties observed in WT neurons.

Female patients with RTT are mosaics, with half of the neurons expressing the WT and the other half expressing the mutant *MECP2* allele (*58*). We investigated whether KEEC treatment affects electrophysiological properties of isogenic human *MECP2* WT neurons. Figures S10 and S11 show that E_{GABA} ($P > 0.46$), mEPSC frequency

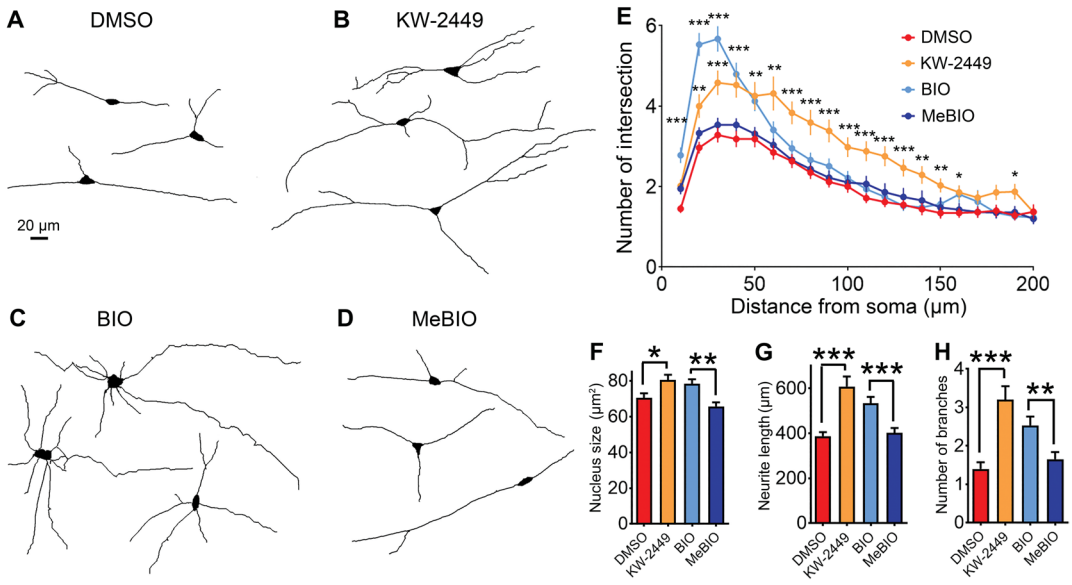

Fig. 5. Rescue of morphological deficits in cultured human RTT neurons with KW-2449 or BIO. (**A** to **D**) Representative MAP2-stained dendritic morphology traces reconstructed from RTT neurons treated with DMSO, KW-2449, BIO, or MeBIO. (**E**) Sholl analysis of RTT neurons treated with KEEC KW-2449 (n = 55) or BIO (n = 62), the inactive analog MeBIO (n = 58), or DMSO control (n = 60). (**F** to **H**) Nucleus size (F), neuron length (G), and total neurite length (H) quantifications in RTT neurons after treatment with KEEC KW-2449 or BIO, the inactive analog MeBIO, or DMSO control. Data are means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, Student's t test.

(P > 0.83), and mEPSC amplitude (P > 0.57) were not changed by KEEC treatment. We further demonstrated that treatment of WT neurons with KEEC did not result in any significant change in the membrane capacitance (C_m; P > 0.17; fig. S12E), an indicator of cell size, or the basic membrane properties including the resting membrane potential (RMP; P > 0.34; fig. S12F) and action potential firing threshold (AP threshold; P > 0.15; fig. S12G). In contrast, treating human RTT neurons with KEECs enhanced the membrane capacitance (P = 0.012; fig. S12L), consistent with increased neuronal cell size (compare Fig. 5). Treatment of RTT neurons with BIO resulted in a small but significant hyperpolarizing shift in resting membrane potential (P = 0.020; fig. S12M) but did not alter the AP threshold (P > 0.47; fig. S12N), whereas treatment of RTT neurons with KW-2449 did not result in any change in resting membrane potential (P > 0.93) or the AP threshold (P > 0.15), further supporting that the observed E_{GABA} change in RTT neurons is caused by enhanced KCC2 expression and reduced intracellular chloride concentrations. Together, our data show that KEEC treatment does not result in morphological or functional change in WT neurons.

KEECs ameliorate behavioral deficits in *Mecp2* mutant mice

Encouraged by the ability of KEECs to enhance KCC2 expression in vitro and to rescue functional deficits in RTT neurons, we assessed the in vivo efficacy of KW-2449 and piperine to affect RTT disease-related symptoms such as breathing pauses and reduced locomotion (*59*) in a *Mecp2* mutant mouse model of RTT (Mecp2 −/y) (*60*). Because previous work had demonstrated that intraperitoneal injection of piperine improved symptoms in mouse models of autism and depression (*61, 62*), we assessed whether KW-2449 or piperine could rescue the breathing and locomotion deficits, which had been shown to reflect impaired GABAergic signaling (*63*).

Plethysmograph and locomotion measurements were made in 4-week-old male *Mecp2* mutant mice as a pretreatment control time point and were then injected daily with KEEC KW-2449 (2 mg/kg) or piperine (6 mg/kg). Injection of the solvent DMSO or ethanol (EtOH) served as vehicle controls (Fig. 6, A and J). The same two behavioral measurements in vehicle or KEEC-injected *Mecp2* mutant mice were performed again 2 and 4 weeks after the start of daily injections. Each mouse's "after injection" data were compared to the "before injection" baseline to assess behavioral changes (Δ). As a result of disease progression, DMSO-injected control *MeCP2* mutant mice showed an increase in breathing pause rate (Fig. 6F, red lines) and a decrease in locomotion activity (Fig. 6O, red lines). In contrast, treatment of *MeCP2* mutant mice with KW-2449 significantly reduced the frequency of breathing pauses [representative plethysmograph traces are shown in Fig. 6 (B and C), breathing pause frequency is shown in Fig. 6F (P = 0.010), and quantified Δbreathing pause frequency is shown in Fig. 6G (P = 0.006)] and induced a remarkable increase in locomotive activities compared to DMSO-injected littermate control mice [Fig. 6P; locomotive activities are shown in Fig. 6O (P = 0.014) and quantified Δlocomotive activities are shown in Fig. 6P (P = 0.040)]. Similarly, treatment of *MeCP2* mutant mice with piperine significantly reduced the frequency of breathing pauses (P = 0.049 for breathing pause frequency and P = 0.018 for Δbreathing pause frequency; Fig. 6, H and I) and increased locomotive activities compared to EtOH-injected littermate control mice (P = 0.0526 for locomotive activities and P = 0.016 for Δlocomotive activities; Fig. 6, Q and R). These in vivo experiment results demonstrate that KEEC treatment ameliorates disease-related behavioral pathologies in *MeCP2* mutant mice, consistent with their therapeutic potential.

DISCUSSION

In this study, we have used CRISPR-Cas9 gene editing technology to develop a compound-screening platform that provides a robust and

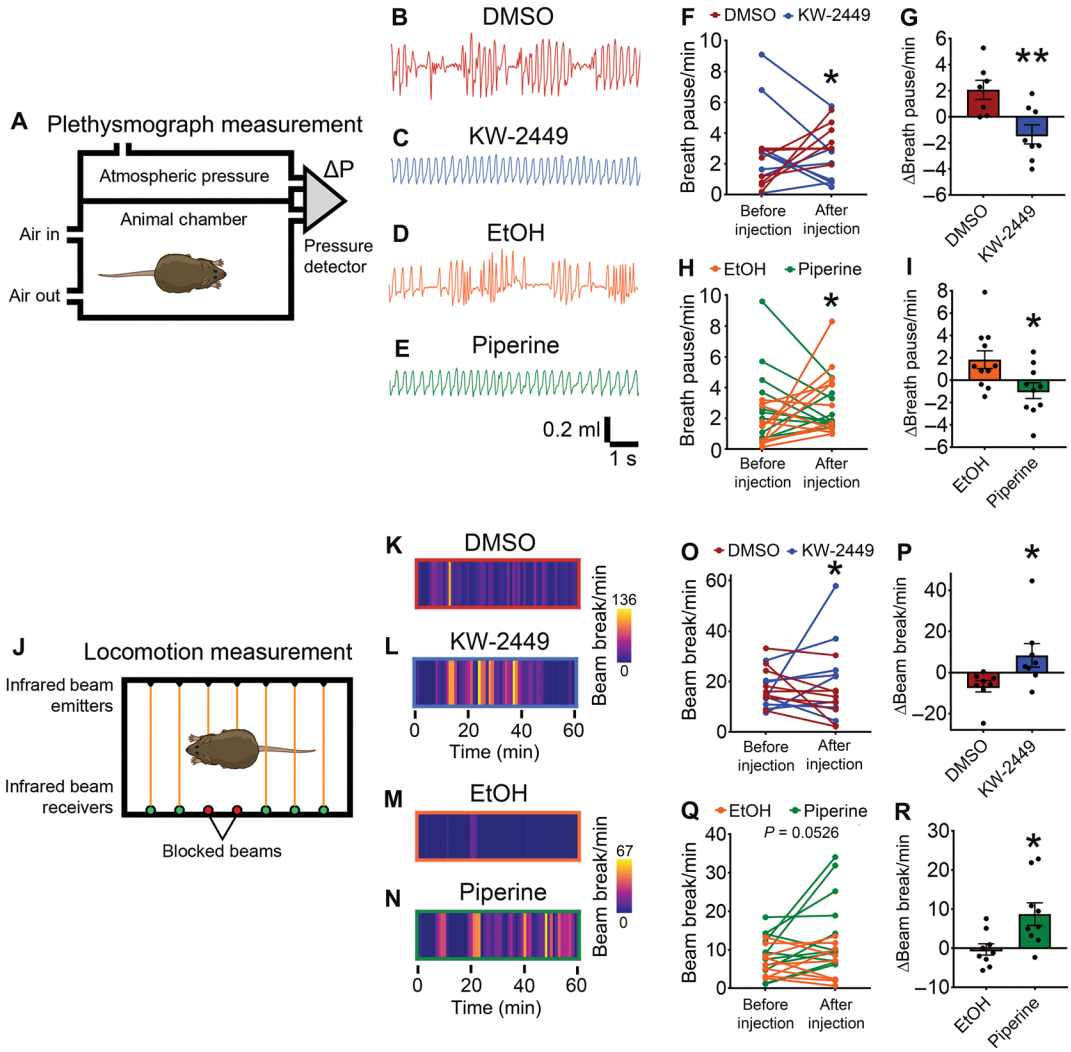

Fig. 6. Amelioration of disease-related breathing pauses and locomotion deficits in *Mecp2* mutant mice by treatment with KW-2449 or piperine. (**A**) Diagram depicting the plethysmograph measurement setup. (**B** to **E**) Representative breathing traces from *Mecp2* mutant mice injected with KEEC KW-2449 (C) (2 mg/kg, $n = 8$) or piperine (E) (6 mg/kg, $n = 11$) or respective vehicle DMSO (B) ($n = 7$) or ethanol (D) ($n = 10$). (**F** to **I**) Quantified breathing pause frequencies and the changes (Δ) between the before and after injection conditions in either KW-2449–injected (F and G) or piperine-injected (H and I) *Mecp2* mutant mice compared to vehicle-injected littermates. (**J**) Diagram depicting the locomotion measurement setup. (**K** to **N**) Representative locomotion activity time series heat map, measured by infrared beam break frequency in home cage, from *Mecp2* mutant mice injected with KEEC KW-2449 (L) ($n = 8$) or piperine (N) ($n = 9$) or respective vehicle DMSO (K) ($n = 8$) or ethanol (M) ($n = 9$). (**O** to **R**) Quantified locomotion activities and the changes (Δ) between the before and after injection conditions in either KW-2449–injected (O and P) or piperine-injected (Q and R) *Mecp2* mutant mice compared to vehicle-injected littermates. Data are means ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$, mixed two-way ANOVA (F, H, O, and Q) and Student's *t* test (G, I, P, and R).

convenient readout of the transcriptional and translational output of the *KCC2* gene from its endogenous locus in human neurons. Unbiased screening approaches led to the identification of a group of small-molecule KEECs that increased the KCC2 reporter signal. The ability for KEECs to increase KCC2 expression was validated by mRNA and protein quantification performed in both cultured human neurons and organotypic mouse brain slices. Previous data showed that neurons in the RTT brain exhibit reduced KCC2 expression, which led to a depolarizing shift in GABA reversal potential and impaired excitatory synapse development (*28*, *54*). We demonstrate here that treatment with KEECs increased KCC2 expression and rescued the deficits in GABA functional switch and excitatory synapse development in RTT neurons equivalent to that which is observed in their isogenic WT control. We also showed that KEEC treatment rescued the immaturity in morphological development in RTT neurons, likely a result of the enhanced excitatory activities that induce activity-dependent genes such as BDNF and activate the Akt-mTOR pathway that promotes growth and is deficient in RTT neurons (*55*). In addition, our results provide evidence that injection of KW-2449 or piperine into *MeCP2* mutant mice ameliorated apnea and locomotor activity reduction, two disease-related phenotypes of *MeCP2* mutant mice associated with impairments in KCC2 expression and GABAergic inhibition (*64*, *65*). The study suggests a novel strategy for brain disease therapy aimed at restoring the impaired E/I balance in the neural circuitry through enhancement of KCC2 expression.

Previous efforts to identify KCC2 modulator chemical compounds have used non-neuronal cells using procedures such as thallium flux

or intracellular [Cl^-] sensor assays as readout (*34, 35, 66*). KCC2 is a neuron-specific gene, and the cellular and molecular context that regulates its expression in neurons is likely absent in non-neuronal cells (*12*) and may have prevented the identification of chemical compounds that augment the expression of KCC2. In contrast to these studies, we have used a target gene–specific reporter approach that robustly detects the transcriptional and translational amounts of a therapeutic target gene, KCC2, in neurons, allowing us to assess the effects of KEECs on relevant neuronal phenotypes. In cultured RTT neurons, treatment with KEECs KW-2449 and BIO restored the impaired KCC2 expression and rescued deficits in both GABAergic and glutamatergic neurotransmissions, as well as abnormal neuronal morphology. Previous data suggested that disrupted Cl^- homeostasis in the brainstem causes abnormalities in breathing pattern (*64*), consistent with breathing abnormalities seen in mice carrying a conditional *Mecp2* deletion in GABAergic neurons (*67*). The reduction in locomotion activity observed in the *Mecp2* mutant mice has also been attributed to abnormalities in the GABAergic system (*65*). Therefore, treatment with the KEEC KW-2449 or piperine may ameliorate disease phenotypes in *MeCP2* mutant mice through restoration of the impaired KCC2 expression and GABAergic inhibition.

The group of KEECs reported here may help to elucidate the molecular mechanisms that regulate KCC2 gene expression in neurons. A previous study conducted with a glioma cell line showed that resveratrol activates the SIRT1 pathway and reduces the expression of NRSF/REST (*50*), a transcription factor that suppresses KCC2 expression (*52*). Our results demonstrate that resveratrol increases KCC2 expression by a similar mechanism, which could contribute to the therapeutic benefit of resveratrol on a number of brain disease conditions (*68, 69*). We also identified a group of GSK3β pathway inhibitors as KEECs. Overactivation of the GSK3β pathway has been reported in a number of brain diseases (*70*). Thus, our results suggest that GSK3β pathway inhibitors could exert beneficial effects on brain function through stimulating KCC2 expression. Another major KEEC target pathway, the FLT3 kinase signaling, has been investigated as a cancer therapy target (*71, 72*). Although FLT3 is expressed in the brain (*73*), drugs that target FLT3 pathway have not been extensively studied as potential treatments for brain diseases. Our results provide the first evidence that FLT3 signaling in the brain is critical for the regulation of key neuronal genes such as KCC2. Therefore, this work lays the foundation for further research to repurpose a number of clinically approved FLT3 inhibitors as novel brain disease therapies.

Our results are valuable for the development of novel therapeutic strategies to treat neurodevelopmental diseases through rectification of dysfunctional neuronal chloride homeostasis. Because of the lack of pharmaceutical reagents that enhance KCC2 expression, bumetanide, a blocker of the inward chloride transporter NKCC1 that counteracts KCC2, has been used as an alternative (*74*). Bumetanide treatment has shown benefits in treating symptoms in mouse models of fragile X syndrome (*75*) and Down's syndrome (*76*) and was shown to confer symptomatic benefit to human patients with autism or fragile X syndrome (*77, 78*). These findings strongly suggest that pharmacological restoration of disrupted chloride homeostasis may provide symptomatic treatment for various neurodevelopmental and neuropsychiatric disorders. However, NKCC1 lacks the neuron-restricted expression pattern of KCC2 and is also expressed in non-brain tissue including kidney and inner ears (*79*), consistent with knockout of *Nkcc1* in mouse model leading to deafness and imbalance (*30*). Therefore, bumetanide treatment may trigger undesirable side effects, thus severely limiting its therapeutic application. In contrast, the expression of KCC2 is restricted to neurons, and a number of the KEECs identified in this study that enhance KCC2 expression in neurons are Food and Drug Administration–approved and have not elicited any severe adverse effects in clinical trials (*80–83*). The promising efficacy of KEECs demonstrated in this study and the known safety of the KCC2 target warrant further preclinical and clinical studies to investigate these drugs and their derivatives as potential therapies for neurodevelopmental diseases.

One potential limitation of this study is that, although we have shown that injection of Mecp2 mutant mice with KEEC KW-2449 or piperine ameliorates disease-relevant physiological phenotypes such as apnea and reduced locomotion, we have not tested the effect of KEEC treatment on social behavior deficits described in mouse model of RTT (*67*). Whether the beneficial effects of KEECs can be generalized to other domains of RTT pathology not investigated in this study is unknown. Given the important role of KCC2 in chloride homeostasis and inhibition efficacy, it would be interesting for future studies to assess whether treatment with KEECs can ameliorate the symptoms of neurodevelopmental disorders other than RTT, such as fragile X syndrome (*75*) or Down's syndrome (*76*), in which disruptions in KCC2 expression or function have been documented. In the current study, we tested the therapeutic efficacy of KEECs in cultured human neurons and in mice and have no information on how these compounds would act in the human. Testing KEECs in larger animal models, such as non-human primates, could help to increase the translational relevance. A further limitation of this study is that the identified KEECs are mostly repurposed drugs that were developed for non-CNS (central nervous system) therapeutic applications. As a result, the detailed pharmacokinetics and blood-brain barrier crossing properties of the KEECs are lacking. Future work that optimizes the chemical structure and delivery methods of lead KEECs to improve drug candidates' access into the brain is necessary before the potential application of KEECs to human clinical trials.

In summary, in this work, we investigated the efficacy of KEECs to rescue a number of well-documented cellular and behavior phenotypes of RTT, including impaired GABA functional switch, reductions in excitatory synapse number and strength, immature neuronal morphology (*53, 54*), as well as an increase in breathing pauses and a decrease in locomotion (*84*). It is possible, however, that KEECs may also be effective in treatment of conditions other than RTT, as impairment in KCC2 expression has been linked to many brain diseases (*17, 85*) including epilepsy (*86–88*), schizophrenia (*19, 20, 89*), brain and spinal cord injury (*21, 90*), stroke and ammonia toxicity conditions (*91–93*), as well as the impairments in learning and memory observed in the senile brain (*23*). Thus, a phenotypically diverse array of brain diseases may benefit from enhancing the expression of KCC2. The newly identified KEECs are potential therapeutic agents for otherwise elusive neurological disorders.

MATERIALS AND METHODS

Study design

The purpose of this study was to develop an unbiased high-throughput drug screening platform to identify small-molecule compounds that enhance the expression of KCC2 in neurons (KEEC) and to investigate the efficacy of KEECs in rescuing the electrophysiological, neuronal morphology, and animal behavior deficits in cellular and rodent

models of RTT. On the basis of our previous publications and power analysis results from pilot experiments, three to six biological replicates were used for each cultured neuron or brain slice biochemical and RNA analysis. A sample size of five to eight neurons was used for E_{GABA} measurements, whereas a sample size of 14 to 19 neurons was used for mEPSC measurements. Electrophysiological data were analyzed blind to the genotype and treatment conditions. Morphological analyses were carried out with about 60 randomly selected neurons per treatment group. Image acquisition and analysis were carried out by different researchers in a double-blind manner, and automated analysis software pipelines were used to reduce human bias. In vivo drug injection and animal behavior experiments were conducted with 7 to 11 *Mecp2* mutant mice per group, which is supported by power analysis results. Animals were randomly assigned to treatment groups with approximately equivalent numbers in each condition. All behavioral analyses were performed by experimenters who were blind to the treatment condition.

Statistical analysis

For all experiments described in the article, the experimentation, quantification, and analysis of data were performed with subjective unbiased methods, and the researchers conducting the experiments were blinded to genotype and treatment conditions except for the screening assay and Western blot analysis in which a large number of drug treatment conditions were tested in parallel. For high-throughput drug screening data analysis, a CellHTS2 analysis script was run using RStudio software to calculate the *B* score. For comparison of two groups such as Western blot protein abundance quantification between vehicle and drug-treated conditions, Student's *t* tests were performed using Microsoft Excel software. For comparison of multiple groups such as electrophysiology data recorded from multiple drug treatment groups, one-way analysis of variance (ANOVA) with Bonferroni post hoc correction was performed using GraphPad Prism software. Permutation test was used to compare mEPSC frequency results recorded from vehicle and drug-treated conditions. Kolmogorov-Smirnov test was used to compare the cumulative distribution between two mEPSC amplitude datasets. Linear mixed modeling statistic test method was used to compare the neuronal morphological metrics measured from vehicle and drug-treated conditions. For the animal behavior experiment data analysis, mixed two-way ANOVA on aligned ranked data was used to assess the drug treatment effect, compared to the effect of DMSO injection, on the number of breathing pauses and beam breaks. Student's *t* test was used to determine the significance of the differences in the changes in breathing pauses or beam crossing numbers between the DMSO-injected and drug-treated groups.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Assay development experiments conducted with neurons derived from ES cell lines with homozygous insertion of luciferase reporter into the *KCC2* locus.

Fig. S2. Western blot experiments demonstrating that treatment of cultured mouse astrocytes with KEECs does not induce ectopic KCC2 expression or alter the amount of NKCC1 gene expression.

Fig. S3. Developmental time course of GABA reversal potential (E_{GABA}) in cultured neonatal mouse cortical neurons.

Fig. S4. Pharmacological validation of GABA responses in the gramicidin-perforated patch clamp recording setup.

Fig. S5. Dose-response properties of KEECs to induce the KCC2 luciferase reporter signal in human RTT KCC2 reporter neurons.

Fig. S6. Enhancement of KCC2 gene expression in immature mouse neurons and human RTT neurons following the knockdown of the FLT3 or GSK3β gene with siRNA.

Fig. S7. Enhancement of the chloride extrusion rate following treatment of human RTT neurons with KW-2449.

Fig. S8. Increase in membrane capacitance and rescue of mEPSC amplitude deficits in human RTT neurons following KEEC treatment.

Fig. S9. Loss of hyperpolarizing shift in E_{GABA} in human RTT neurons treated with KEECs following pharmacological blockade of KCC2.

Fig. S10. Equivalent E_{GABA} in mature (2 to 3 months in vitro) WT human neurons treated with DMSO and those treated with KEECs.

Fig. S11. Equivalent frequency and median amplitude of mEPSCs in WT human neurons treated with DMSO and those treated with KEECs.

Fig. S12. Equivalent basic membrane properties of WT neurons treated with DMSO and those treated with KEECs and enhancement of only cell size in KEEC-treated RTT neurons.

Fig. S13. Representative images of neurons from different genotype and treatment groups recorded in electrophysiological experiments.

Fig. S14. Gramicidin-perforated patch clamp recording data presented as linear regression fits of the average GABA response recorded at different membrane holding potentials (V_{hold}) in different genotype and treatment groups.

Table S1. KEECs identified from screening with WT human KCC2 reporter neurons.

Table S2. KEECs identified from screening with RTT human KCC2 reporter neurons.

Table S3. Statistical results for all figures (provided as separate Excel file).

Table S4. Raw data (provided as separate Excel file).

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Pharmacological enhancement of *KCC2* gene expression exerts therapeutic effects on human Rett syndrome neurons and *Mecp2* mutant mice

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Restoring balance in Rett syndrome

Rett syndrome (RTT) is a neurodevelopmental disorder causing cognitive, motor, and emotional impairments. Excitation/inhibition imbalance has been hypothesized to play a main role in RTT. The neuron-specific K^+/Cl^- cotransporter 2 (KCC2) is critical for maintaining the excitatory balance in the brain, and its expression is reduced in RTT. Now, Tang *et al.* developed a high-throughput screening to identify KCC2 expression–enhancing small molecules. The hit compounds were tested in vitro in RTT neurons and in vivo in a mouse model. The treatments rescued functional and morphological defects in neurons and ameliorated behavioral deficits in mice. Targeting KCC2 might be an effective strategy for treating RTT and possibly other disorders characterized by excitation/inhibition imbalance.