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The small molecule CLP257 does not modify activity of the K⁺–Cl[–] co-transporter KCC2 but does potentiate GABA_A receptor activity

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To the Editor:

The neuronal K⁺–Cl[–] co-transporter KCC2 (also known as solute carrier family 12 member 5, or SLC12A5) critically maintains the neuronal Cl[–] gradient to establish hyperpolarizing signaling by GABA_A receptors, the primary mediators of fast synaptic inhibition in the brain. These receptors are also the principal targets of benzo-diazepines, neurosteroids, and intravenous general anesthetics¹. The downregulation of KCC2 in neuropathic pain models

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AUTHOR CONTRIBUTIONS

R.A.C., T.Z.D., I.M., P.A.D., S.Z., J.D., R.J.M., N.J.B., and S.J.M. designed experiments. R.A.C., K.J., L.I.P., H.J.W., L.J.M., D.J.B., I.C., J.G., L.S., and T.Z.D. performed all the experiments and subsequent analysis. P.M.S., M.P.B., D.H.M., N.D., J.H., M.E.D., R.J.M., and Q.W. produced all reagents or oversaw production of reagents. R.A.C., T.Z.D., I.M., N.J.B., and S.J.M. wrote the manuscript.

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and seizure-related disorders identifies it as a putative therapeutic target. Through a small-molecule screen in neuroblastoma/glioma-derivative NG108–15 cells, Gagnon *et al.*² identified CLP257 as a ‘KCC2 activator’. We therefore sought to confirm and further determine its mechanism of action.

We began our investigation by using gramicidin perforated-patch recordings to measure the levels of intracellular Cl[−] ([Cl[−]]_i) in NG108–15 cells that were cultured as detailed in the original report. We observed no change in intracellular Cl[−] values after a 5-h exposure to CLP257, which according to the original paper would produce an easily detectable ~23 mM reduction in Cl[−] (Fig. 1a). Furthermore, acute administration of the selective KCC2 inhibitor VU0463271 did not affect Cl[−] levels in either vehicle- or CLP257-treated cells (Supplementary Fig. 1)³.

In parallel experiments, we analyzed the actions of CLP257 on KCC2 function using fluorescence resonance energy transfer (FRET)-based Cl[−] imaging that reproduced the conditions of the original publication². Importantly, upon dissolution, CLP257 formed a bright yellow solution, confounding interpretation of CFP-YFP conjugate-based Cl[−] imaging, which was not explicitly described in the original report. In contrast to the original findings, after stimulation of NG108–15 cells with medium containing increased extracellular Cl[−], CLP257 increased intracellular Cl[−]. This effect was further potentiated by VU0463271 and therefore is not mediated by KCC2 (Fig. 1b and Supplementary Fig. 2a,b). In addition, we confirmed a previously reported resting Cl[−] current in NG108–15 cells (Supplementary Fig. 2c,d)⁴, which further complicates data interpretation in these cells⁵.

To further investigate the expression of KCC2 in NG108–15 cells, we used standard biochemical analyses. To match the original report², we performed immunoprecipitations with a KCC2-specific antibody and control IgG in NG108–15 cell lysates and rat cortical neuronal extracts. KCC2 protein was specifically detected in lysates from rat cortical neurons. In contrast, no KCC2 protein was found in NG108–15 cells obtained from a commercial source or in NG108-cl cells (stably expressing the Cl[−]-sensitive indicator Clomeleon) from the cell stock obtained from Y. De Koninck at Laval University and used in the original CLP257 report (Fig. 1c and Supplementary Fig. 3a,b). This result is in agreement with a previous report indicating a lack of KCC2 protein in NG108–15 cells⁶. We also did not detect KCC2 protein after a 5-h exposure to CLP257 (Supplementary Fig. 3c,d). Collectively, our electrophysiological, imaging, and biochemical experiments suggest that NG108–15 cells do not express detectable levels of KCC2 protein or activity, calling into question the use of these cells in a screen to identify KCC2 activators.

To further examine whether CLP257 functions through KCC2, we exogenously expressed human KCC2 in HEK293 cells. We used fluorescence-based TI⁺ influx assays that have repeatedly been used to determine KCC2 activity⁷. Five-hour exposure of HEK-KCC2 cells to CLP257 did not accelerate TI⁺ transport. The well-established KCC2 potentiator *N*-ethylmaleimide (NEM) substantially increased TI⁺ uptake, and VU0463271 reduced TI⁺ uptake (Fig. 1d and Supplementary Fig. 4a)⁸. Additionally, CLP257 did not modify the activity of NKCC1 (also known as solute carrier family 12 member 2, or SLC12A2), whereas exposure to bumetanide, a known NKCC1 inhibitor, resulted in a clear reduction in

activity (Supplementary Fig. 4b). We then used perforated-patch electrophysiological assays to assess Cl⁻ homeostasis. Baseline Cl⁻ levels were unaffected by CLP257 even though the calculated flux reversal point of KCC2 under these recording conditions was lower than the values we observed⁸, indicating that there is a window to potentiate KCC2 function (Supplementary Fig. 4c). After a 2-min exposure to bathing solution containing high K⁺, the rate of Cl⁻ uptake was not affected by CLP257 (Fig. 1e). Likewise, after switching back to solutions with normal K⁺ levels for a 2-min period, the rate of Cl⁻ extrusion was not affected by CLP257 (Fig. 1e). Isolation of the KCC2 component of the Cl⁻ flux with furosemide again revealed no effect of CLP257 on Cl⁻ transport (Supplementary Fig. 4d)^{8,9}. Thus, CLP257 does not modulate KCC2 activity.

CLP257 was stated to activate KCC2 by increasing cell-surface levels in the original report². To examine this claim, we exogenously expressed tagged KCC2 constructs together with previously published KCC2 mutants that exhibit either increased (KCC2 A/A) or decreased (KCC2 E/E) cell-surface expression in N2a cells (Fig. 1f and Supplementary Fig. 5)¹⁰. At low concentrations, CLP257 did not modify cell-surface levels of KCC2, whereas at higher concentrations of CLP257 these levels were reduced. Thus, CLP257 does not increase the cell-surface expression of KCC2 as originally stated.

We then employed backscattering interferometry technology to determine whether CLP257 binds to KCC2 (ref. 11). Whereas VU0463271 robustly bound to human KCC2 in this assay with an affinity close to its half-maximal inhibitory concentration (IC₅₀)³, no CLP257 binding was observed (Supplementary Table 1). Given this observation, we sought to identify other targets of CLP257 that could explain the observed benefits in animal models. CLP257 demonstrated potent inhibition of MAO-B with nanomolar efficacy and binding to other targets in the low micromolar concentration range including PPAR γ , the 5-HT_{1A} receptor, the adenosine transporter, and GABA_A receptors. Further functional examination of GABA_A receptors in cultured rat hippocampal neurons showed that CLP257 potentiated activity of these receptors (Fig. 1g,h). This potentiation was independent of KCC2 activity, as it was not modified by exposure to VU0463271 or short hairpin RNA (shRNA)-mediated knockdown of *KCC2* (Fig. 1i).

Collectively, our experiments suggest that the physiological and behavioral effects of CLP257 are likely to be independent of KCC2; rather, these effects are mediated by other mechanisms, including potentiation of GABA_A receptors. Thus, conclusions drawn from experiments using CLP257 in the context of KCC2 activity should be reinterpreted^{12–15}.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

ONLINE METHODS

Cell culture.

The HEK293 and NG108–15 cell lines were selected on the basis of the lines used in the original report, and mouse neuroblastoma cells (N2a) were chosen on the basis of previous use in the relevant assays^{2,16}. HEK293 cells (ATCC, CRL-1573) and N2a cells (ATCC, CCL-131) were grown in DMEM containing 10% FBS and 1% penicillin-streptomycin. NG108–15 cells (ATCC, HB-12317) were grown in DMEM containing 10% FBS, 1% penicillin-streptomycin, and 2% HAT (hypoxanthine–aminopterin–thymidine) medium. The NG108-cl cell line that stably expresses Chlomeleon was obtained from the laboratory of Y. De Koninck and was grown in the standard NG108–15 cell medium supplemented with G418. HEK293 cells were transfected with cDNA encoding human KCC2b (NM_001134771.1), the rat glycine $\alpha 1$ subunit (NM_013133.1), and eGFP using Lipofectamine and were grown for 48 h before experimentation. NG108–15 cells were transfected using similar methods but without *KCC2* cDNA. Hippocampal and cortical neurons were cultured from embryonic day (E) 18 rat embryos as previously described¹⁷. Cultures were maintained in Neurobasal medium supplemented with B-27 neural supplement, GlutaMAX, and penicillin-streptomycin for 18–21 d before experiments. For the *KCC2* knockdown experiments, DIV10 neurons were cotransfected (using Lipofectamine) with GFP and the shRNA targeting *KCC2* (*KCC2_K11* construct) and maintained for 5 d before experiments according to previously described methods¹⁸. Cell culture materials were purchased from Invitrogen.

Compounds.

CLP257 ((5*Z*)-5-[(4-fluoro-2-hydroxyphenyl)methylene]-2-(tetrahydro-1-(2*H*)-pyridazinyl)-4(5*H*)-thiazolone) was purchased from Tocris Bioscience or synthesized by AstraZeneca using protocols detailed in a patent application submitted by CHLORION PHARMA (WO2009–97695). In both cases, CLP257 was solubilized in bath saline with 0.1% DMSO. Data were pooled between the compound sources. VU0463271 was also synthesized by AstraZeneca as previously detailed¹⁹. Muscimol, bicuculline, and NPPB were purchased from Tocris Bioscience, and NEM was purchased from Sigma-Aldrich.

Immunoprecipitation and immunoblotting.

Expression of KCC2 in commercially available NG108–15 cells, NG108-cl cells (Laval University), and rat cortical neurons (DIV18) was assessed by western blot analysis of immunoprecipitated samples. To investigate the effects of CLP257 on KCC2 expression, NG108–15 cells were treated with 30 μ M of the compound or DMSO for 5 h. Cells were lysed in RIPA buffer (Boston Bio Products) supplemented with protease inhibitors (Complete, Roche), 2 mM EDTA, and 1 mM EGTA. Equal amounts of protein lysates were added to an immunoprecipitation matrix (ImmunoCruz) conjugated to monoclonal mouse antibody against KCC2 (75–013, Antibodies Inc.) or IgG control. Beads were resuspended in 2 $\times$ denaturing Laemmli buffer and incubated for 40 min at room temperature to avoid excessive KCC2 dimerization. Rabbit polyclonal antibody to KCC2 (07–432, Millipore) was used for detection. Immunoblots were visualized using a Chemidoc MP imaging system (Bio-Rad). Results were verified in three independent replicates.

Electrophysiology.

NG108–15 or HEK293 cells were preincubated at room temperature for 5 h in vehicle (saline + 0.1% DMSO) with or without CLP257 (10–30 μ M). NG108–15 and HEK293 cell recordings were conducted at room temperature, and neuron recordings were performed at 34 °C. Bath saline contained 140 mM NaCl, 2.5 mM KCl, 2.5 mM MgCl₂, 2.5 mM CaCl₂, 10 mM HEPES, and 11 mM glucose, pH 7.4. Single NG108–15 or HEK293 cells were selected for recording, which was perforated using gramicidin (50 μ g/ml) in the recording pipette. The patch pipette solution for perforated-patch recordings contained 140 mM KCl, 10 mM HEPES, and 10 mM KOH, pH 7.4. Positive-going voltage-ramp protocols were used to measure the reversal potential of glycine-activated currents (20 mV over 1 s) and of NPPB-sensitive currents (120 mV over 2 s, from –60 to +60 mV). Acute application of glycine, muscimol, bicuculline, VU0463271, furosemide, NPPB, and CLP257 was performed using a fast-exchange pipette (three barrel, 700 μ m, Warner Instruments) positioned just above the cells. For ion substitution experiments in HEK293 cells, NaCl was replaced by an equimolar amount of 70 mM KCl. Data were acquired at 10 kHz with an Axopatch 200B amplifier and Clampex 10 software (Molecular Devices).

Cl[–] imaging.

Analysis of changes in Cl[–]-dependent fluorescence in NG108–15 cells was performed according to the protocol described in the original report². We used SuperClomeleon, kindly provided by G.J. Augustine (Korea Institute of Science and Technology)²⁰, to detect Cl[–]-sensitive fluorescence. For fluorescence recording, cells were transfected in suspension with a vector encoding SuperClomeleon using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After transfection, cells were plated in black 96-well plates with transparent bottoms (F-bottom, Greiner) at $\sim 1.5 \times 10^5$ cells/well and incubated in DMEM supplemented with 10% FBS for 40 h. Green epifluorescence signal was detected in >90% of cells using an inverted microscope.

Immediately before the experiment, cells were rinsed once with DMEM without phenol red and twice with a low-Cl[–] assay medium containing 30 mM HEPES, 2 mM CaCl₂, 2 mM MgSO₄, 10 mM K-gluconate, 2 mM NaH₂PO₄, and 20 μ M bumetanide (pH 7.4), adjusted to 310 mOsm with Na-gluconate. The volume of low-Cl[–] assay medium was 70 μ l/well, and the external Cl[–] concentration was 4 mM. Cells were then placed into the microplate fluorescence reader (FluostarOptima, BMG Labtech). Because we observed a strong decrease in intracellular Cl[–] after transfer to low-Cl[–] assay medium, the timing of the manipulations was precisely monitored. Fluorescence recording began 20 min after the change in external medium. After 30 min of recording, an equal volume of assay medium with 104 mM [Cl[–]]_o and 60 μ M CLP257 or DMSO vehicle (pH 7.4) was applied using an automated injection system. VU0463271 (30 μ M) was applied manually with low-Cl[–] assay medium before fluorescence recording. The composition of the high-Cl[–] assay medium was 100 mM NaCl, 30 mM HEPES, 2 mM CaCl₂, 2 mM MgSO₄, 10 mM K-gluconate, and 2 mM NaH₂PO₄, adjusted to 310 mOsm with Na-gluconate. Thus, the resulting medium had a final [Cl[–]]_o of 54 mM, and the final concentrations of the applied drugs were 10 μ M bumetanide, 15 μ M VU0463271, and 30 μ M CLP257. Ratiometric fluorescence measurements of SuperClomeleon, a fusion of Cl[–]-insensitive CFP and mutated YFP, were

performed with two filter sets for visualization of CFP (excitation = 440 nm, emission = 480 nm) and YFP (excitation = 500 nm, emission = 540 nm). The net fluorescent signal for each channel was obtained as the difference between the fluorescence of transfected cells in a given well and the mean background fluorescence of wells containing non-transfected cells. Notably, CLP257 is yellow in color, such that wells containing the compound had a different level of background fluorescence. Raw data were expressed as the mean of the ratio of net fluorescence of CFP to YFP ($F_{\text{CFP}}/F_{\text{YFP}}$) measured in three wells. Increased $F_{\text{CFP}}/F_{\text{YFP}}$ corresponds to increased $[\text{Cl}^-]_i$ (ref. 16). To keep the experimental paradigm consistent with the original CLP257 report², data were normalized to the values obtained in wells exposed to a final Cl^- concentration of 54 mM (set as 0%) and a low Cl^- concentration of 4 mM (set as -100%). Results are expressed as percentages.

Thallium FLIPR assays.

Primary pharmacology with human KCC2 was tested in a cellular thallium assay. Briefly, cDNA encoding human KCC2b (NM_001134771.1) was transiently transfected into HEK293 cells. Cells were plated in 384-well black-walled and clear-bottom plates. Ti^+ FLIPR assays were performed using the FluxOR Potassium Ion Channel Assay (F10017, Life Technologies). First, cells were washed with HBSS buffer using the Biotek Elx 405. FluxOR dye was added in assay buffer (HBSS supplemented with 20 mM HEPES (pH 7.3), BackDrop Background Suppressor (Life Technologies, B10512), 20 μM ouabain, and 10 μM bumetanide) using a Multidrop Combi (Thermo Fisher). Cells were incubated at room temperature for 60 min. Compound plates were prepared with DMSO (0.1%, 5 h), NEM (100 μM , 5 h), CLP257 (50 μM , 5 h), and twofold serial dilutions of VU0463271 (12.5–50 μM , 90 min) in HBSS assay buffer. After 10 s of baseline reading in the presence of compound, we added stimulus buffer containing Ti^+ while preserving pH and osmolarity. KCC2 activity was measured using the maximal response over 2 min. The initial value of the slope of Ti^+ -stimulated fluorescence was also monitored; however, no difference was observed using this approach. Each well's fluorescence trace was normalized by dividing each data point after compound addition by the initial fluorescence values obtained on the FLIPR before compound addition. NKCC1 assays were run using the same protocol as the KCC2 assay but were performed on untransfected HEK293 cells.

Secondary pharmacology screen.

Selectivity of CLP257 was tested in binding and functional assay panels (CEREP). The assay methodology can be found online at <http://www.cerep.fr/>.

Transfection and cell-surface labeling of N2a cells.

N2a cells were transfected with previously described pcDNA vectors encoding rat KCC2- pH_{ext} , A/A-KCC2- pH_{ext} , or E/E-KCC2- pH_{ext} (ref. 10) using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol and used 48 h after transfection. For labeling of cell-surface-expressed KCC2- pH_{ext} , rabbit antibodies against GFP (recognizing the extracellular pHluorin tag) were diluted in culture medium and applied to cells for 2 h at 37 °C, 5% CO_2 . Cells were then washed for 10 min at room temperature in HEPES-buffered saline containing 150 mM NaCl, 2.5 mM KCl, 2.0 mM MgCl_2 , 2.0 mM CaCl_2 , 20 mM HEPES, and 10 mM D-glucose, pH 7.4, labeled with Cy3-

conjugated anti-rabbit antibody (dissolved in HEPES-buffered saline) for 20 min at 13 °C, and fixed in Antigenfix (Diapath). To visualize labeled internalized proteins, cells were subsequently permeabilized with 0.3% Triton X-100, blocked with 5% goat serum, and labeled at room temperature for 1 h with Alexa Fluor 647–conjugated anti-rabbit antibody. For visualization of total overexpressed KCC2-pH_{ext}, cells were labeled overnight (4 °C) with mouse antibody against GFP and for 1 h at room temperature with Alexa Fluor 488–conjugated anti-mouse antibody. For quantitative analysis, images were acquired with an Olympus Fluorview-500 confocal microscope (40× (NA 1.0) or 60× (NA 1.4) oil-immersion objective; zoom 1–5). We randomly selected and focused on a transfected neuron by only visualizing eGFP or pHluorin fluorescence and then acquired images of membrane clusters. The cluster properties of each cell were analyzed with Metamorph software (Roper Scientific). First, we created a binary mask of total eGFP- or total pHluorin-labeled cells and then analyzed KCC2 membrane fluorescence in regions overlapping the binary mask. Analysis parameters were the same for all experimental conditions, and analysis was performed in a blinded manner. After analysis, data were normalized to the mean value of cells transfected to express KCC2-pH_{ext} and treated with DMSO.

Backscattering interferometry.

Examination of CLP257 and VU0463271 binding to KCC2 was performed by Molecular Sensing (<http://www.molsense.com/>). For sample preparation, a construct encoding human KCC2 (SLC12A5–11, residues 1–1116) with a FLAG tag at the N terminus and a decaHis tag at the C terminus was generated in pTT5 for transient expression in Expi293F HEK293 cells following the supplier's protocol (Invitrogen). Cells from a 0.5-liter culture were collected 72 h after transfection and resuspended in 50 ml of lysis buffer containing 150 mM NaCl and 50 mM HEPES, pH 7.4, supplemented with EDTA-free protease and phosphatase inhibitor tablets. The suspension was homogenized with a Turrax (IKA), and cells were lysed by a single 20-kpsi pass on a cell disruption system (Constant Systems). Cell debris was removed by centrifugation for 10 min at 7,400g, and membranes were collected by ultracentrifugation for 45 min at 200,000g. Membranes were resuspended in 10 ml of lysis buffer using a Dow homogenizer to an approximate total membrane protein concentration of 10 mg/ml. Total protein concentration was determined using BCA in the presence of 1% SDS with BSA as a reference. Membranes were aliquotted and stored at –80 °C for further use. Membranes were also isolated from non-transfected HEK293 cells for control experiments. Membranes (0.07 mg/ml) were incubated for 3 h with threefold serial dilutions of compound (0–40 μM) in a final volume of 100 μl each. Samples were analyzed on a glass microfluidic chip on a TruBind 100 system. Control signal (non-transfected cell membranes) was subtracted from assay signal for each compound dilution point. The dissociation constant (K_d) was derived from a nonlinear least-squares fit of the data using the one-sited saturation binding model.

Statistics.

All experimental data derived from experiments in this manuscript were analyzed as previously described^{10,17}. Statistical analysis was performed using GraphPad Prism software. Current–voltage (I–V) relationships were fit by linear regression analysis using Clampex. Two-tailed Student's *t* tests, indicated as paired or unpaired as appropriate, or one-

way ANOVA with Dunnett's or Holm–Sidak's multiple-comparisons test as indicated was used with $\alpha = 0.05$. All data are reported as the mean $\pm$ s.e.m.

Life Sciences Reporting Summary.

Further information on experimental design is available in the **Life Sciences Reporting Summary**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Moore YE, Kelley MR, Brandon NJ, Deeb TZ & Moss SJ Trends Neurosci. 40, 555–571 (2017). [PubMed: 28803659]
2. Gagnon M et al. Nat. Med. 19, 1524–1528 (2013). [PubMed: 24097188]
3. Delpire E et al. Bioorg. Med. Chem. Lett. 22, 4532–4535 (2012). [PubMed: 22727639]
4. Rouzair-Dubois B, Bostel S & Dubois JM Neuroscience 88, 307–317 (1999). [PubMed: 10051209]
5. Rowe SM & Verkman AS Cold Spring Harb. Perspect. Med. 3, a009761 (2013).
6. Williams JR, Sharp JW, Kumari VG, Wilson M & Payne JA J. Biol. Chem. 274, 12656–12664 (1999). [PubMed: 10212246]
7. Zhang D, Gopalakrishnan SM, Freiberg G & Surowy CS J. Biomol. Screen. 15, 177–184 (2010). [PubMed: 20086212]
8. Payne JA Am. J. Physiol. 273, C1516–C1525 (1997). [PubMed: 9374636]
9. Delpire E et al. Proc. Natl. Acad. Sci. USA 106, 5383–5388 (2009). [PubMed: 19279215]
10. Friedel P et al. Sci. Signal. 8, ra65 (2015).
11. Bornhop DJ et al. Science 317, 1732–1736 (2007). [PubMed: 17885132]
12. Hamidi S & Avoli M Neurobiol. Dis. 79, 51–58 (2015). [PubMed: 25926348]
13. Ostroumov A et al. Neuron 92, 493–504 (2016). [PubMed: 27720487]
14. Ferando I, Faas GC & Mody I Nat. Neurosci. 19, 1197–1200 (2016). [PubMed: 27500406]
15. Ferrini F, Lorenzo LE, Godin AG, Quang ML & De Koninck Y Sci. Rep. 7, 3870 (2017). [PubMed: 28634406]
16. Friedel P, Bregestovski P & Medina I Front. Mol. Neurosci. 6, 7 (2013). [PubMed: 23596389]
17. Deeb TZ, Nakamura Y, Frost GD, Davies PA & Moss SJ Eur. J. Neurosci. 38, 2453–2467 (2013). [PubMed: 23627375]
18. Pellegrino C et al. J. Physiol. (Lond.) 589, 2475–2496 (2011). [PubMed: 21486764]
19. Sivakumaran S et al. J. Neurosci. 35, 8291–8296 (2015). [PubMed: 26019342]
20. Grimley JS et al. J. Neurosci. 33, 16297–16309 (2013). [PubMed: 24107961]

Figure 1.

CLP257 does not affect KCC2 function. **(a)** Values of intracellular Cl^- in NG108-15 cells were unchanged after 5-h exposure to CLP257 (30 μ M) ($n = 22$ cells for DMSO and 18 cells for CLP257; $t = 0.57$, $P = 0.57$, unpaired). **(b)** Cl^- imaging demonstrated that CLP257 (30 μ M; orange trace) increased intracellular Cl^- values relative to those in the control condition (high $[Cl^-]_o$; red trace), while addition of VU0463271 (VU, 15 μ M; purple trace) further increased values. VU0463271 applied by itself (blue trace) also elevated intracellular Cl^- . Values were normalized to the difference between the high $[Cl^-]_o$ and low $[Cl^-]_o$ (black trace) conditions. Each trace represents the mean $\pm$ s.e.m. of fluorescence recordings from five independent cultures (Online Methods). The green trace shows expected values based on the original CLP257 report². **(c)** Immunoprecipitation (IP) and immunoblot (IB) analysis of KCC2 in commercially available NG108-15 cells and NG108-cl cells obtained from Laval University revealed no KCC2 protein expression, in contrast to high expression in rat cortical neurons (Rat ctx). The blot shown is representative of three independent experiments. **(d)** TI^+ influx assays conducted in HEK293 cells exogenously expressing KCC2 showed that CLP257 (50 μ M; green traces) did not increase KCC2-mediated TI^+ influx as compared to DMSO (black traces), whereas NEM (100 μ M; blue traces) increased KCC2 activity and VU0463271 (10 μ M; pink traces) decreased it. The red arrow indicates the time of TI^+ addition. **(e)** Values indicating the rate of change in glycine responses

measured during $[K^+]_o$ elevation (Cl^- uptake over 2 min) or $[K^+]_o$ reduction (Cl^- extrusion over 2 min). CLP257 did not increase the rate of uptake ($n = 7$ cells for DMSO and 6 cells for CLP257; $t = 0.28$, $P = 0.79$, unpaired) or the extrusion rate ($n = 7$ cells for DMSO and 6 cells for CLP257; $t = 0.37$, $P = 0.71$, unpaired). (f) CLP257 did not increase KCC2 surface expression. Representative images of total KCC2 signal (F_t), membrane-localized KCC2 signal (F_m) and internalized KCC2 signal (F_i) in N2a cells expressing KCC2-pHext. Cell shape is shown in dark green in each image; scale bar, 15 μm . Images are representative of four independent experiments. (g) CLP257 rapidly and reversibly potentiated whole-cell $GABA_A$ currents activated by a subsaturating concentration of muscimol in cultured hippocampal neurons. (h) Concentration–response relationship in CLP257 potentiation of muscimol- activated currents in cultured neurons ($n = 7$ –14 neurons for each concentration; half-maximal effective concentration (EC50) of CLP257 = 4.9 μM). (i) VU0463271 (10 μM ; $n = 9$ neurons) and shRNA-mediated knockdown of *KCC2* ($n = 12$ neurons) did not prevent potentiation of $GABA_A$ receptor currents by CLP257 (control, untreated), $n = 13$ neurons; one-way ANOVA: $F(2, 31) = 1.11$, $P = 0.34$; Holm–Sidak’s multiple-comparisons test). All data are reported as the mean $\pm$ s.e.m.