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RESEARCH ARTICLE

Staurosporine and NEM mainly impair WNK-SPAK/OSR1 mediated phosphorylation of KCC2 and NKCC1

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Abstract

The pivotal role of KCC2 and NKCC1 in development and maintenance of fast inhibitory neurotransmission and their implication in severe human diseases arouse interest in post-transcriptional regulatory mechanisms such as (de)phosphorylation. Staurosporine (broad kinase inhibitor) and N-ethylmaleimide (NEM) that modulate kinase and phosphatase activities enhance KCC2 and decrease NKCC1 activity. Here, we investigated the regulatory mechanism for this reciprocal regulation by mass spectrometry and immunoblot analyses using phospho-specific antibodies. Our analyses revealed that application of staurosporine or NEM dephosphorylates Thr¹⁰⁰⁷ of KCC2, and Thr²⁰³, Thr²⁰⁷ and Thr²¹² of NKCC1. Dephosphorylation of Thr¹⁰⁰⁷ of KCC2, and Thr²⁰⁷ and Thr²¹² of NKCC1 were previously demonstrated to activate KCC2 and to inactivate NKCC1. In addition, application of the two agents resulted in dephosphorylation of the T-loop and S-loop phosphorylation sites Thr²³³ and Ser³⁷³ of SPAK, a critical kinase in the WNK-SPAK/OSR1 signaling module mediating phosphorylation of KCC2 and NKCC1. Taken together, these results suggest that reciprocal regulation of KCC2 and NKCC1 via staurosporine and NEM is based on WNK-SPAK/OSR1 signaling. The key regulatory phospho-site Ser⁹⁴⁰ of KCC2 is not critically involved in the enhanced activation of KCC2 upon staurosporine and NEM treatment, as both agents have opposite effects on its phosphorylation status. Finally, NEM acts in a tissue-specific manner on Ser⁹⁴⁰, as shown by comparative analysis in HEK293 cells and immature cultured hippocampal neurons. In summary, our analyses identified phospho-sites that are responsive to staurosporine or NEM application. This provides important information towards a better understanding of the cooperative interactions of different phospho-sites.

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Introduction

The cation chloride cotransporter family (CCCs) consists of electroneutral secondary active cotransporters that mediate the symport of cations (Na^+ and K^+) coupled with chloride ions (Cl^-) across the plasma membrane. CCCs are divided into K^+ - Cl^- outward cotransporters (KCC1-4), Na^+ , K^+ , Cl^- inward cotransporters (NKCC1-2, NCCs), the polyamine transporter CCC9, and the CCC-interacting protein CIP1 [1–3]. These transporters play a crucial role in various physiological processes like regulation of cell volume, directional ion transport across epithelial cells, secretion of K^+ , and regulation of intracellular Cl^- concentration ($[\text{Cl}^-]_i$) in neurons [2, 4–11].

In neurons, KCC2 and NKCC1 are involved in the development and maintenance of inhibitory neurotransmission. For instance, KCC2 generates a low $[\text{Cl}^-]_i$ required for fast inhibitory neurotransmission. Binding of γ -aminobutyric acid (GABA) or glycine to their receptors, which are ligand-gated Cl^- channels, leads to a Cl^- influx and therefore hyperpolarization [8, 12]. By contrast, NKCC1 is more prevalent in immature inhibitory neurons. There, its action results in a high $[\text{Cl}^-]_i$ causing GABA or glycine to elicit a depolarizing action that opens L-type voltage-gated Ca^{2+} channels necessary for proper synapse formation [13–18].

The physiological relevance of the two cotransporters is corroborated by the phenotypes present in knockout mice. Mice with disruption of the gene *Slc12a5* encoding both KCC2 isoforms (KCC2a and KCC2b), die shortly after birth due to motor deficits [19, 20]. Furthermore, dysfunction of KCC2 is associated with neurological and psychiatric disorders including epilepsy, neuropathic pain, spasticity, ischemic insults, brain trauma, schizophrenia, and autism [21–32]. Mice with disruption of *Slc12a2* (NKCC1) are viable, but suffer from peripheral deafness, postnatal hyperexcitability and anti-convulsant, pain perception and male infertility [33–36]. Inhibition of KCC2 promotes formation of pathological conditions whereas inhibition of NKCC1 seems to prevent or at least alleviate pathological phenotypes [37]. This renders these two transporters a prime pharmacotherapeutic target and fosters interest in understanding posttranslational mechanisms of their regulation [12, 38–40]. The main focus is set on phospho-related mechanisms. The broad kinase inhibitor staurosporine was used to enhance KCC2 transport activity in immature auditory brainstem [41] and hippocampal cultured neurons [42], and to decrease NKCC1 activity [43–46]. Furthermore, N-ethylmaleimide (NEM), that likely modulates the activity of kinases and phosphatases, activates KCC2 [47–50] and inhibits NKCC1 [45, 51]. Thus, both compounds reciprocally regulate KCC2 (activation) and NKCC1 (inhibition). Understanding their mode of action provides the basis for further pharmacological studies and important insight into the molecular mechanisms involved in physiological regulation of these two transporters.

Since KCC2 and NKCC1 are present in the same neuronal populations but transport K^+ and Cl^- in opposite direction [52–54], regulatory mechanisms are required to coordinate their activity [4, 38, 55–60]. Indeed, several kinases and phosphatases regulate their transport activity. *In vitro* and *in vivo* analyses revealed that members of the with-no-lysine kinase (WNKs) family in combination with their downstream targets STE20/SPS1-related proline/alanine rich kinase (SPAK) and oxidative stress response kinase (OSR1) are the most prominent kinases that regulate KCC2 and NKCC1 activity in a reciprocal way. SPAK/OSR1, activated by WNK1, phosphorylate Thr⁶ and Thr¹⁰⁰⁷ of KCC2 [48, 59, 61–64]. WNKs also interact with a yet unknown kinase to phosphorylate Thr⁹⁰⁶ of KCC2 [48, 63]. Site-directed mutagenesis studies indicate that simultaneous mutation of Thr⁹⁰⁶ and Thr¹⁰⁰⁷ to alanine (mimicking the dephosphorylated state) activates KCC2 [61, 65–67]. WNK/SPAK/OSR1 also phosphorylates the N-terminal residues Thr²⁰³, Thr²⁰⁷, Thr²¹² and Thr²¹⁷ of NKCC1 [38, 59, 60, 68–76]

thereby activating NKCC1 [72, 75]. Thus dephosphorylation (KCC2) and phosphorylation (NKCC1) of WNK/SPAK/OSR1 specific phospho-sites enhance KCC2 and NKCC1 activity.

Phosphorylation can also enhance KCC2 activity. Phosphorylation of Ser⁹³², Thr⁹³⁴, Ser⁹³⁷, or the protein kinase C (PKC) site Ser⁹⁴⁰, all residing in exon 22, increases its transport activity [47, 68, 77]. The multiplicity of phosphorylation/dephosphorylation sites on KCC2 offers a complex toolbox to gradually fine-regulate its activity and integrate different signaling pathways [4, 40, 65, 68].

Initially, both staurosporine and NEM were thought to act through a similar mechanism [47], but recent findings revealed that they act differentially on specific KCC2 phospho-sites [40]. Furthermore, staurosporine and NEM mediated effects involve both KCC2 phosphorylation and dephosphorylation [40, 48]. To gain insight into their mode of action, we analyzed the impact of these compounds on phosphorylation of specific KCC2 and NKCC1 phospho-sites using large-scale phosphoproteomics studies and phospho-site specific antibodies in stably transfected HEK293 cells and immature primary cultures of hippocampal neurons.

Material and methods

Cell culturing of HEK293 cells

For K⁺-Cl⁻ cotransporter activity measurements, stably transfected rat KCC2b HEK293 cells (HEK^{rmKCC2b}) [55] were plated in a 0.1 mg/ml poly-L-lysine coated black-well 96 well culture dish (Greiner Bio-One, Frickenhausen, Germany) at a concentration of 100,000 cells/well. The remaining cells were plated on a 0.1 mg/ml poly-L-lysine-coated class coverslip and ~18 h later analyzed by immunocytochemistry.

Primary cultures of rat hippocampal neuron

All animal procedures were carried out in accordance with the European Union Directive of 22 September (2010/63/EU). The protocol was approved by the INSERM Local committee (Number 0287.01, delivered by Ministère de l'Éducation et de la Recherche). Hippocampi from 18-day-old rat embryos were dissected and dissociated using trypsin (0.05%) and plated in 60-mm culture dishes at a density of 100,000 cells cm⁻² in minimal essential medium (MEM) supplemented with 10% NU serum (BD Biosciences, Le Pont de Claix, France), 10 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine, and 100 U ml⁻¹ penicillin-streptomycin as previously described [78]. On day 7 of culture incubation, half of the medium was changed to MEM with 2% B27 supplement (Invitrogen). 24 h prior to plating, dishes were coated with poly-ethylenimine (5 µg/ml).

Immunocytochemistry

For immunocytochemistry, all steps were performed at room temperature. HEK293 cells grown on poly-L-lysine-coated coverslips were fixated for 10 min with 4% paraformaldehyde in 0.2 M phosphate buffer (PBS). After three washing steps in PBS, cells were incubated with blocking solution (2% bovine serum albumin, 10% goat serum in PBS) for 30 min. Primary antibody solution (anti-KCC2 N1-12; 1:1,000; Neuromab, Carlsifornia, USA) was added in carrier solution (0.3% Triton X-100, 1% bovine serum albumin, 1% goat serum in PBS) for 1 h. After washing three times in PBS, the secondary antibody, which was conjugated to a fluorescent probe (Alexa Fluor 488 goat anti-mouse; 1:1,000; Thermo Fisher Scientific, Bremen, Germany) was incubated for 1 h. After three washes in PBS, cells were mounted onto glass slides with Mowiol (Roth, Karlsruhe, Germany) and DAPI (Roth; 1:1,000). Photomicrographs were

taken using Biozero BZ-8100E (Keyence, Neu-Isenburg Deutschland) fluorescence microscope (Leica, Wetzlar, Germany).

Determination of K⁺-Cl⁻ cotransport

KCC2 transport activity was determined by Cl⁻ dependent uptake of Tl⁺ in HEK293 cells as previously described [47, 79, 80]. To initiate the flux measurement, the medium in the 96-well culture dish was replaced by 80 μ l preincubation buffer (100 mM N-methyl-D-glucamine-chloride, 5 mM HEPES, 5 mM KCl, 2 mM CaCl₂, 0.8 mM MgSO₄, 5 mM glucose, pH 7.4, 160 mmol / kg \pm 2,04 mmol/kg) supplemented with 2 μ M FlouZin-2 AM dye (Thermo Scientific, Bremen, Germany) plus 0.2% (wt/vol) Pluronic F-127 (Thermo Scientific, Bremen, Germany) and incubated for 48 min at room temperature. Cells were then washed 3 times with 80 μ l preincubation buffer and incubated for 15 min with 80 μ l preincubation buffer plus 0.1 mM ouabain to block Na⁺/K⁺ ATPase activity. Three technical and five biological replicates were performed for each construct. Afterwards, the 96-well plate was placed into a fluorometer (Fluoroskan Accent, Thermo Scientific, Bremen, Germany) and each well was injected with 40 μ l 5 x Tl⁺ stimulation buffer (12 mM Tl₂SO₄, 100 mM NMDG, 5 mM Hepes, 2 mM KCl, 2 mM CaCl₂, 0.8 mM MgSO₄, 5 mM glucose, pH 7.4; 175 mmol/kg \pm 2 mmol/kg). Fluorescence was determined in a kinetic dependent manner (excitation 485 nm, emission 538 nm, 1 frame in 5 s in a 200 s time span) across the entire cell population in a single well. By using linear regression of the initial values of the slope of Tl⁺-stimulated fluorescence increase, transport activity was calculated [79, 80].

To determine the dose-response profile, increasing concentration of staurosporine (8–80 μ M) and NEM (25–3000 μ M) were applied to the preincubation buffer 15 min prior flux measurement. This was done for three technical replicates and at least five independent measurements were performed.

Statistical analyses

The majority of data populations illustrated in Figs 1, 3 and 4 showed non-normal distributions (verified using Shapiro-Wilk normality test at 0.05 significance level). Therefore, a non-parametric test (Wilcoxon-Mann-Whitney rank sum test) was employed for the comparison between different groups of data. The resulting *p*-values were adjusted with the Bonferroni correction for multiple testing. Note that only *p*-values < 0.01 were considered to reduce the chances of false positives (type I errors). The sample size *n* always refers to the number of biological replicates (independent preparations). The activity of each independent preparation was determined as mean over three technical replicates. The average scatter within technical replicates was 3 times smaller than the scatter across biological replicates for a given treatment.

For dose-response analyses, we used the nls function from the stats package in R (version 3.5.1) to model the data points with the Hill-Langmuir equation,

$$y(x; ED_{50}, n, y_{\text{sat}}) = y_0 + y_{\text{sat}} \frac{x^n}{ED_{50}^n + x^n}, \quad (1)$$

where *y* is the Tl⁺ uptake of HEK^{mKCC2b} cells, *y*₀ is the Tl⁺ independent-baseline activity (*y*₀ = 100%), *y*_{sat} is the maximum activity (equivalent to *V*_{max} in reaction rate equation) relative to baseline, *x* is the concentration of the agonist, *n* is the Hill coefficient, and ED₅₀ is the agonist concentration that produces 50% of the saturation response. The 95% confidence intervals for the dose response curves were determined with the function NonlinearModelFit [“MeanPredictionBands”] in Wolfram Mathematica. For the boxplots, the box extends from the upper (Q1) to the lower (Q3) quartile. The line inside the box represents the median. The whiskers

extend to the outermost data point that falls within upper inner and lower inner Quartile fences [$Q1+1.5(IQR)$] and [$Q3-1.5(IQR)$], respectively, where $IQR = Q1-Q3$ is the interquartile range.

Treatment of stably transfected HEK^{rnKCC2b} cells and hippocampal neurons

For mass spectrometry and immunoblot analyses, stably transfected HEK^{rnKCC2b} cells [55] were grown in 75 cm² cell-culture flask. Cells were washed with 5 ml flux hypotonic preincubation buffer (100 mM N-methyl-D-glucamine-chloride, 5 mM HEPES, 5 mM KCl, 2 mM CaCl₂, 0.8 mM MgSO₄, 5 mM glucose, pH 7.4; 160 mmol/kg±2,04 mmol/kg) and then treated with or without 8 μM staurosporine or 1 mM NEM. Cells were centrifuged at 500 rpm for 3 min and the resulting pellets used for immunoblot analyses or mass spectrometry analyses.

For immunoblot analyses of immature (9 days *in vitro*, (DIV)) rat hippocampal neurons, half of the media from culture dishes containing neurons (2.5 ml from each dish) was collected to prepare samples including 16 μM staurosporine (from 10 mM stock solution in DMSO), 1 mM NEM (by direct dissolving of the NEM powder) or DMSO (vehicle, same volume as for staurosporine), respectively. The samples were distributed dropwise into the culture dishes that gave final concentration of 8 μM staurosporine or 0.5 mM NEM and cultures were incubated 15 min at 37 °C (5% CO₂). After incubation, neurons were rinsed twice with Hanks' Balanced Salt solution (HBSS, ThermoFisher Scientific) precooled to 4 °C. The rinsage solution was replaced with ice-cold HBSS containing a cocktail of protease and phosphatase inhibitors (ThermoFisher Scientific) and neurons rapidly scraped and centrifuged at 4 °C (10,000 g, 3 min). Pellets were frozen in liquid nitrogen and kept for future analyses at -80 °C.

Multi-Enzyme Digestion Filter Aided Sample preparation (MED FAS)

For mass spectrometry analyses, cell pellets of untreated, staurosporine or NEM treated HEK^{rnKCC2b} cells were lysed in 2% SDS in 0.1 M Tris-HCl, pH 8.0, containing 0.1 M DTT as described previously [81]. Total protein was determined using WF-assay in micro-titer plate format [82]. Aliquots of the cell lysate containing 1–2 mg were processed in Amicon Ultra 15 Ultracel 30k (Merck Millipore, Darmstadt) devices as described previously [83] with several modifications using the MED FASP method [84]. Briefly, SDS and other low molecular weight material were removed by centrifugation at 4,000 x g in 5 consecutive washes with UA buffer containing 8 M urea (ultrapure, Merck, Darmstadt) in 0.1 M Tris pH 8. Following two washes with 5 ml of 0.1M Tris-HCl, pH 8 (DB buffer), 20 μg of LysC (Wako, Neuss) in 0.5 mL of DB was added to the filter. Samples were digested overnight at 37 °C and peptides were collected by centrifugation. Next, the material retained in the filter was cleaved with 10 μg trypsin in 0.5 ml DB at 37 °C for 4 h and the peptides were eluted as previously. To increase the yield of peptides, filters were washed twice with 0.5 mL DB. Concentration of peptides was determined by WF-assay [82].

TiO₂-based enrichment of phosphopeptides

Phosphopeptides were enriched using TiO₂-beads [85] with several modifications [83]. Briefly, 25 mg of 'Titansphere TiO₂ 10 μm' (GL Sciences, Inc., Japan) were suspended in 50 μl of 3% (m/v) dihydroxybenzoic acid in 80% (v/v) CH₃CN, 0.1% CF₃COOH and diluted 1:4 with water before use. Ten microliters of this slurry (1 mg beads) were added and samples incubated under continuous agitation for 20 min. The mass ratio of the beads and peptides was 3:1. Then, the titanium beads were sedimented by centrifugation at 5,000 x g for 1 min and the supernatants were collected and mixed with another portion of the beads and incubated as

above. The bead-pellets were resuspended in 150 μ l of 30% (v/v) CH₃CN containing 3% (v/v) CF₃COOH and transferred to a 200 μ l pipet tip plugged with one layer of glass microfiber filter GFA (Whatman). The beads were washed three times with 30% (v/v) CH₃CN, 3% CF₃COOH (v/v) solution and three times with 80% CH₃CN (v/v), 0.3% CF₃COOH (v/v) solution. Finally, the peptides were eluted from the beads with 100 μ L of 40% CH₃CN (v/v) containing 15% NH₄OH (m/v) and were vacuum-concentrated to \sim 4 μ l.

Mass-spectrometric analysis

Analysis of peptide mixtures was conducted using a QExactive HF-X mass spectrometer (Thermo-Fisher Scientific, Palo Alto). Aliquots containing <1 μ g of total peptide were chromatographed on a 50 cm column with 75 μ m inner diameter packed C₁₈ material. Peptide separation occurred at 300 nl/min for 95 min using an acetonitrile gradient of 5–30%. The temperature of the column oven was 55°C. The mass spectrometer operated in data-dependent mode with survey scans acquired at a resolution of 60,000. Up to the top 15 most abundant isotope patterns with charge \geq +2 from the survey scan (300–1650 m/z) were selected with an isolation window of 1.4 m/z and fragmented by HCD with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans were 20 and 28 ms, respectively. The ion target value for MS1 and MS2 scan modes was set to 3×10^6 and 10^5 , respectively. The dynamic exclusion was 30 s. The MS spectra were searched using MaxQuant software. A maximum of two missed cleavages was allowed. The maximum false peptide and protein discovery rate was specified as 0.01. The whole mass spectrometry analyses were performed in three technical and two biological replicas per treatment.

Immunoblot and phospho-antibody immunoprecipitation analyses

Lysate protein samples were subjected to immunoblot and immunoprecipitation as previously described [63]. Protein samples (40 μ g) were boiled in sample buffer for 5 min, resolved by 7.5% sodium dodecyl sulfate polyacrylamide-gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane. Membranes were incubated for 30 min with TBST (Tris-buffered saline, 0.05% Tween-20) containing 5% (w/v) skim milk. Blots were then washed six times with TBST and incubated for 1 h at room temperature with secondary HRP-conjugated antibodies diluted 5000-fold in 5% (w/v) skim milk in TBST. After repeating the washing steps, signals were detected with enhanced chemiluminescence reagent. Immunoblots were developed using ChemiDoc™ Imaging Systems (Bio-Rad, Feldkirchen). Figures were generated using Photoshop/Illustrator (Adobe). Band densities of bands were measured with ImageJ. Calculation of intensity ratios is based on (phospho-dimeric KCC2 + phospho-monomeric KCC2) / (total dimeric KCC2 + total monomeric KCC2), (total dimeric KCC2 + total monomeric KCC2)/ β -actin or tubulin, SPAK pThr²³³/SPAK, SPAK pSer³⁷³/SPAK, SPAK/ β -actin or tubulin, NKCC1 pThr^{203,207,212}/NKCC1, NKCC1/ β -actin or tubulin, as reported previously [66]. Mann-Whitney U-test was used for comparison between 2 independent groups and Wilcoxon matched pairs test was employed to compare paired data (GraphPad Prism 7.0, San Diego, CA, USA).

For phospho-antibody immunoprecipitation, KCC isoforms were immunoprecipitated from indicated cell extracts. 2 mg of the indicated clarified cell extract was mixed with 15 μ g of the indicated phospho-specific KCC antibody conjugated to 15 μ l of protein-G-Sepharose, in the added presence of 20 μ g of the dephosphorylated form of the phosphopeptide antigen, and incubated 2 hours at 4 °C with gentle shaking. Immunoprecipitates were washed three times with 1 ml of lysis buffer containing 0.15 M NaCl and twice with 1 ml of buffer A. Bound proteins were eluted with 1x LDS sample buffer.

Antibodies

The following antibodies were purchased from Division of Signal Transduction Therapy Unit at the University of Dundee: KCC2A phospho-Thr⁹⁰⁶ (SAYTYER(T)LMMEQRSRR [residues 975–989 of human KCC3A] corresponding to SAYTYEK(T)LVMEQRSQI [residues 899–915 of human KCC2A], S959C); KCC2A phospho-Thr¹⁰⁰⁷ (CYQEKVHM(T)WTKDKYM [residues 1032–1046 of human KCC3A] corresponding to TDPEKVHL(T)WTKDKSVA [residues 998–1014 of human KCC2A], S961C); SPAK/OSR1 (T-loop) phospho-Thr²³³/Thr¹⁸⁵ antibody (226–238 of human SPAK or residues 178–190 of human OSR1, TRNKVRKpTFVGTP, S204C); SPAK/OSR1 (S-motif) phospho-Ser³⁷³/Ser³²⁵ antibody (367–379 of human SPAK, RRVPGSpsGHLHKT, which is highly similar to residues 319–331 of human OSR1 in which the sequence is RRVPGSpsGRLHKT, S670B); SPAK (427–443 of human SPAK, QSLSVHDSQGPPVGTP, S849C); NKCC1 phospho-Thr203+Thr207+Thr212 (residues 198–217 of human NKCC1, HYYDpTHTNpTYLpTFGHNT, S763B); NKCC1 phospho-Thr²¹²/Thr²¹⁷ (residues 208–223 of human NKCC1, YYLRpTFGHNpTMDAVPR, S063D); NKCC1-total antibody (residues 1–260 of shark NKCC1, S841B). Pan-KCC2 antibody (residues 932–1043 of human KCC2) was from NeuroMab (73–013). KCC2 phospho-Ser940 antibody was from ThermoFisher Scientific (PA5-95678). PKC δ phospho-Thr⁵⁰⁵ antibody (9374) and β -Actin (D6A8) antibody (12620) were from Cell Signaling Technology. Anti (neuronal)- β -Tubulin III antibody was from Sigma-Aldrich (T8578). Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce. IgG used in control immunoprecipitation experiments was affinity-purified from pre-immune serum using Protein G-Sepharose.

Results

Dose-response analyses of staurosporine and NEM in stably transfected HEK^{rnKCC2b} cells

Staurosporine and NEM generally activate KCCs [40, 47, 86, 87]. To closer characterize their mode of action, we used stably transfected HEK^{rnKCC2b} cells, as high amount of the cotransporter is advantageous for subsequent biochemical analyses. Immunoreactivity of HEK^{rnKCC2b} cells against KCC2 was detected in all cells with clear labeling at the cell membrane and cytosol (Fig 1A). This is in agreement with previous cell surface expression analyses that detected $11.8 \pm 1.4\%$ of total KCC2 in stably transfected *rnKCC2b* at the cell surface [79].

To quantify the impact of staurosporine and NEM on *rnKCC2b* transport activity, TI⁺ flux measurements were performed [79, 80, 88]. Transport activity in HEK^{rnKCC2b} cells was 5.6 times higher compared to background (HEK^{rnKCC2b} 100% \pm 5.32%; mock; 17.7% \pm 5.5%; $p = 4.3 \times 10^{-5}$; $n = 5$) (Fig 1B). Here and elsewhere in the text, numbers indicate mean \pm SD, p was determined using Wilcoxon-Mann-Whitney rank sum test. Treatment with 10 μ M staurosporine or 1 mM NEM resulted in an approximately twofold increase of *rnKCC2b* activity compared to untreated HEK^{rnKCC2b} cells (staurosporine: 187.5% \pm 37.1%, $p = 3 \times 10^{-3}$; NEM: 204% \pm 23.2%; $p = 3 \times 10^{-3}$). The loop diuretics furosemide, that specifically inhibits the function of cation chloride cotransporters [89, 90], significantly inhibited the function of *rnKCC2b* (34.7 \pm 12.2%, $p = 4 \times 10^{-5}$).

Next, we determined the dose-response relationships of staurosporine and NEM on *rnKCC2b* transport activity by treating HEK^{rnKCC2b} cells with different concentrations of staurosporine (5–80 μ M) or NEM (25–3,000 μ M). Fig 2 represents the dose-response curve for both agents. The dose-response curve for staurosporine (Fig 2A) was approximately a rectangular hyperbola ($n \approx 1$, not significantly different from unity). This reflects Michaelis-Menten

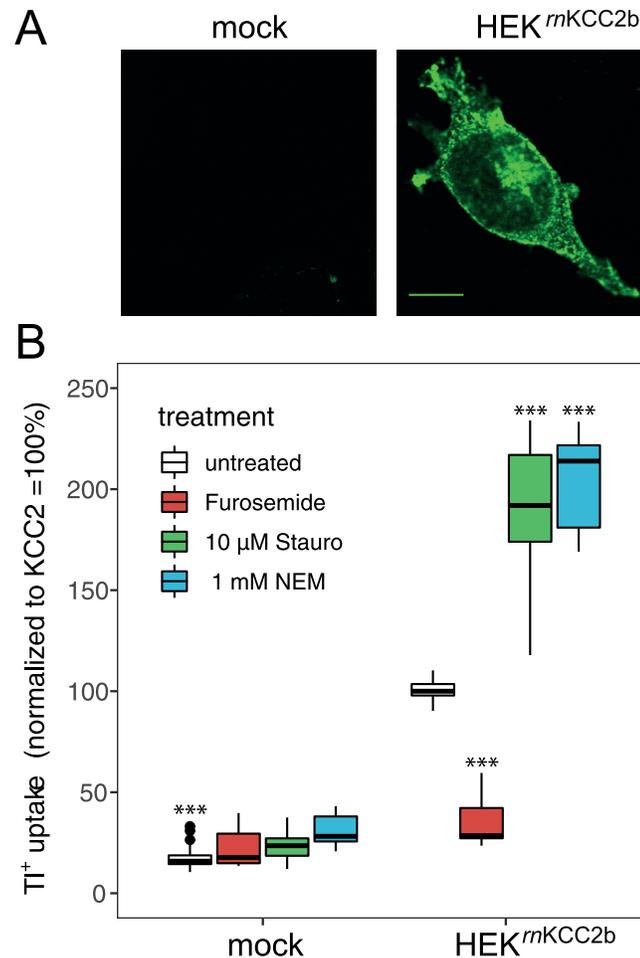


Fig 1. Staurosporine and NEM increase KCC2 activity. A: Immunoreactivity of stably transfected HEK^{*mKCC2b*} cells was detected with labeling at the cell membrane and cytosol. Photomicrographs were taken with a confocal laser-scanning microscope. Scale bar, 10 μm. B: Transport activity of *mKCC2b* was determined by TI⁺ flux measurements. Treatment with 10 μM staurosporine or 1 mM NEM resulted in an approximately twofold increase of *mKCC2b* activity. Application of the loop diuretics furosemide (2 mM) significantly inhibited *mKCC2b* function. The graph represents the data of at least five independent measurements (each consisting of three technical replicates) normalized to *mKCC2b*. Statistical analyses: ***, $p < 0.001$ versus HEK^{*mKCC2b*}; Error bars represent SD.

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kinetics and suggests absence of cooperative effects. In contrast, the dose response curve for NEM (Fig 2B) had a pronounced sigmoidal shape ($n \approx 5$), which reflects cooperative binding kinetics. The effective dose ED₅₀ (representing the potency) for staurosporine of stably transfected HEK^{*mKCC2b*} was 12.8 ± 4.9 μM and the maximal efficacy (E_{\max}) was $205 \pm 40\%$. The ED₅₀ value for NEM was 0.5 ± 1.3 mM and E_{\max} was $105 \pm 6\%$. For further analyses, we used a concentration of 8 μM staurosporine and 1 mM NEM, if not indicated otherwise as these concentrations significantly increase KCC2b transport activity.

Identification of CCC phosphorylation sites in stably transfected HEK^{*mKCC2b*} cells by mass spectrometry analyses upon treatment with staurosporine or NEM

Phosphoproteomics by mass spectrometry has the advantage of providing an unbiased survey of phospho-sites. Therefore, we here used for the first time this technique to gain insight on

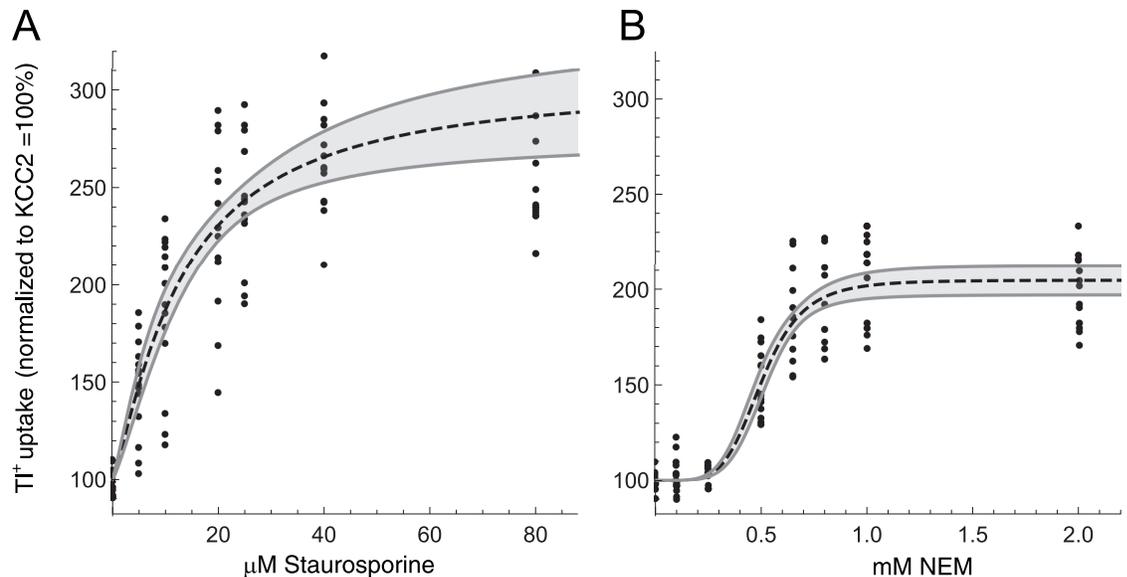


Fig 2. Dose-response relationship of staurosporine and NEM on KCC2 transport activity. HEK^{*rmKCC2b*} cells were treated with different concentrations of staurosporine (5–80 μ M; A) or NEM (25–3,000 μ M; B) for 15 min. Transport activity of *rmKCC2b* was determined by TI^+ flux measurements. Treatment with staurosporine resulted in a classical Michaelis-Menden curve, whereas NEM treatment showed a sigmoidal curve with a Hill coefficient of 5.2 ± 1.3 indicating a positive cooperative effect on *rmKCC2b*. The graph represents the data of at least five independent measurements (each consisting of three technical replicates) normalized to untreated HEK^{*rmKCC2b*}.

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the impact of staurosporine and NEM on *rmKCC2b* phospho-sites in HEK^{*rmKCC2b*}. For each condition, two biological and three technical replicas were performed. Phospho-sites detected in at least one technical experiment are listed in Table 1. We first mapped KCC2b phospho-sites in untreated HEK^{*rmKCC2b*} cells. Twelve phospho-sites were identified: Ser²⁵, Ser²⁶, Ser³¹, Thr³², Thr³⁴ in the cytoplasmic N-terminus and Thr⁹⁰⁶, Ser⁹³⁷, Ser⁹⁴⁰, Thr¹⁰⁰⁷, Ser¹⁰²², Ser¹⁰²⁵, and Ser¹⁰²⁶ in the C-terminus (Table 1, untreated). These sites include all phospho-sites already present in PhosphositePlus (Table 2) [91]. Recently phosphoproteomic data deposited in Phosida and Phosphosite plus revealed that rat KCC2b tissue only harbors seven phospho-sites (Table 2). Thus, we here report five phospho-sites (Ser²⁵, Thr³², Thr⁹⁰⁶, Ser⁹³⁷ and Thr¹⁰⁰⁷) that were so far only reported for mouse but not rat KCC2 tissue (Table 2). These sites most likely reflect different expression of kinases and phosphatases in different tissues (HEK293 vs. rat brain tissue) [69] or increased detection rate in stably transfected HEK^{*rmKCC2b*} cells.

As KCC1, KCC4, and NKCC1 are endogenously expressed in HEK293 cells and as mass spectrometric analysis provides data on most proteins in a given sample, we also investigated phosphorylation sites of the following proteins: *hsKCC1* (Thr⁸⁹³, analogous to *rmKCC2b* Thr¹⁰⁰⁷), *hsKCC4* (Thr⁹²⁶ and Thr⁹⁸⁰, analogous to *rmKCC2b* Thr⁹⁰⁶ and Thr¹⁰⁰⁷) and *hsNKCC1* (Thr²¹², Thr²¹⁷, Ser²⁴², Thr²⁶⁶, Thr²⁶⁸, Ser⁹⁴⁰, Tyr⁹⁵⁶, and Ser⁹⁵⁷). These phospho-sites were already previously deposited in PhosphositePlus and Phosida (S1–S3 Tables). Overall, we detected only a low proportion of all so far deposited phospho-sites for these three cotransporters. This might reflect low expression levels in HEK293 cells.

Next, we investigated the phosphorylation pattern of *rmKCC2b* upon staurosporine and NEM treatment. Phosphorylation at Ser²⁶, Thr³², Thr³⁴, Thr⁹⁰⁶, Ser⁹⁴⁰, Thr¹⁰⁰⁷, Ser¹⁰²², Ser¹⁰²⁵, Ser¹⁰²⁶ of *rmKCC2b* were still present upon treatment with staurosporine or NEM, whereas

Table 1. Phospho-sites of stably transfected HEK^{rnKCC2b} cells. Stably transfected HEK^{rnKCC2b} were treated with or without 1 mM NEM or 8 μ M staurosporine before they were analyzed by mass spectrometry. The protein accession numbers are: *hsNKCC1* (P55011), *hsKCC1* (Q9UP95), *rnKCC2b* (Q9H2X9-2), *hsKCC4* (Q9Y666).

Sample	Phosphorylation sites	Phosphopeptides	untreated	NEM treated	staurosporine treated
<i>hsNKCC1</i>	T212; T217	TFGHNTMDAVPR	(2)		
	S242	LLRPSLAELHDELEK	(1)	(1)	
	T266	EPFEDGFANGEESTPTR	(3)	(2)	(2)
	S940	EGLDISHLQGQEELLSSQEK	(2) (2)	(2) (1)	(3) (1)
	Y956; S957	DVVVSVEYSKK	(3) (2)	(3) (1)	(3) (3)
<i>hsKCC1</i>	T983	IQMTWTRDK	(2)		
<i>rnKCC2b</i>	S25; S26	ESSPFINSTDTEK	(1)		
	S26	ESSPFINSTDTEK	(2) (3)	(3) (3)	(3) (3)
	S31	ESSPFINSTDTEK	(2)	(1)(1)	
	T32	ESSPFINSTDTEK	(1)	(1)	(1)
	T34	ESSPFINSTDTEK	(3) (2)	(2)	(1)
	T906	TLVMEQRSQILK	(3)	(3)	(3)
	S937	EIQSITDESRGSIR	(2)		
	S940	EIQSITDESRGSIR	(2) (3)	(2)	(1)
	T1007	VHLTWTK	(3)	(3)	(3)
	S1022; S1025	GPSPVSEGIK	(3) (1)	(3)	(3) (1)
	S1022; S1025; S1026	GPSPVSEGIK	(1)	(1)	
	S1022; S1026	GPSPVSEGIK	(3)	(3)	(3)
	S1026	GPSPVSEGIK	(3)	(3)	(3)
<i>hsKCC4</i>	T926	TLMMEQR	(2)		
	T980	VQMTWTREK	(3)	(1)	

The number in brackets indicates in how many technical replica a given phospho-site was detected (max. 3). Each bracket provides the results of one biological experiment.

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phosphorylation of Ser²⁵ and Ser⁹³⁷ was not detected anymore in either of the two conditions (Table 1). Additionally, no phosphorylation was detected for Ser³¹ after treatment with staurosporine.

Regarding endogenously expressed CCCs, the phospho-site Thr⁹⁸³ of *hsKCC1* (analogous to *rnKCC2b* Thr¹⁰⁰⁷), Thr⁹²⁶ of *hsKCC4* (analogous to *rnKCC2b* Thr⁹⁰⁶) and Thr^{212/217} and Thr^{266/268} of *hsNKCC1* could not be detected upon application of either of the two reagents. Additionally, the phospho-sites Thr⁹⁸⁰ of *hsKCC4* (analogous to *rnKCC2b* Thr⁹⁰⁶) and Ser²⁴² of *hsNKCC1* were not detected anymore after staurosporine treatment. The phospho-sites Thr²⁶⁶, Ser⁹⁴⁰ and Tyr⁹⁵⁶/Ser⁹⁵⁷ of *hsNKCC1* were still present upon treatment with staurosporine and NEM.

Several kinases were described to directly phosphorylate KCC2 and NKCC1. This includes kinases of the WNK-SPAK/OSR1 and PKC mediated phosphorylation pathways. To gain further insight into the regulatory phosphorylation mechanism, we explored their phosphorylation pattern as well. We detected several phosphorylation sites in *hsWNK1*, *hsWNK2*, and *hsSPAK* [69, 93, 94] (Table 3). Upon all, we observed phosphorylation of the activating T-loop residue Ser³⁸² of *hsWNK1* and the S-loop phosphorylation site of Ser^{372/373} of *hsSPAK* that is phosphorylated by WNK1 [63, 69, 93]. Upon treatment with staurosporine or NEM, Ser³⁸² of *hsWNK1* and Ser^{372/373} of *hsSPAK* were not detected anymore (Table 4). We were not able to detect phosphorylation of Thr²³³ that is located in the T-loop kinase domain of *hsSPAK*. Normally, this site is directly phosphorylated by WNK1 and WNK4 to activate SPAK [60, 63, 69,

Table 2. Phospho-sites in PhosphoSitePlus and PHOSIDA detected by mass spectrometry analyses.

Phosphosite Plus <i>rnKCC2b</i>	Phosphosite Plus <i>hsKCC2b</i>	Phosphosite Plus <i>mmKCC2b</i>	Phosida <i>mmKCC2b</i>	Transport activity measured by:
-	-	S²⁵	S²⁵	[47, 65]
S²⁶	-	S²⁶	S²⁶	[47, 65]
S³¹	S³¹	-	S³¹	(40)
-	-	-	(T³²)	-
T³⁴	T³⁴	T³⁴	T³⁴	[40, 92]
-	Y⁵⁹⁴	-	-	-
-	S⁵⁹⁹	-	-	-
-	-	-	S⁶⁴⁹	-
-	T⁷⁹⁸	-	-	-
-	T⁸⁰⁸	-	-	-
-	-	T⁹⁰⁶	T⁹⁰⁶	[47, 61, 66]
-	-	S⁹¹³	-	-
-	-	S⁹³²	(S⁹³²)	[40]
-	-	-	(T⁹³⁴)	[47]
-	-	S⁹³⁷	S⁹³⁷	[47]
S⁹⁴⁰	-	S⁹⁴⁰	S⁹⁴⁰	[48, 77]
-	-	S⁹⁸⁸	-	-
-	-	T⁹⁹⁹	-	[40]
-	-	T¹⁰⁰⁶	T¹⁰⁰⁶	[47, 48, 61, 66]
-	-	-	T¹⁰⁰⁸	[40]
S¹⁰²²	-	S¹⁰²¹	S¹⁰²¹	[47]
S¹⁰²⁵	-	S¹⁰²⁴	(S¹⁰²⁴)	[47]
S¹⁰²⁶	-	S¹⁰²⁵	S¹⁰²⁵	[47]
-	-	S¹⁰⁴⁴	-	-

Abbreviations used are as follows: *rn*, *Rattus norvegicus*; *hs*, *Homo sapiens*; *mm*, *Mus musculus*. Phospho-sites detected in the present mass spectrometry study are marked in bold.

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95]. Furthermore, we did not detect any phospho-sites of the ubiquitously expressed WNK3 and WNK4. Again, this probably results from low expression rates in HEK293 cells.

PKC mediates the phosphorylation of KCC2 and NKCC1 [48, 77, 96, 97]. The PKC family consists of 10–12 isoforms grouped into three classes [98–101]. We detected several phospho-sites in seven *hsPKC* family members (alpha, beta, delta, epsilon, theta, eta and iota; Table 4).

To summarize, according to mass spectrometry based phosphoproteome analyses, staurosporine and NEM reduce the number of detected phospho-sites of stably expressed *rnKCC2b* and endogenously expressed *hsKCC1*, *hsKCC4*, *hsNKCC1*, *hsWNK1* and *hsSPAK*. Phosphorylation of some sites (*rnKCC2b*: Ser³¹, *hsNKCC1*:Ser²⁴², *hsKCC4*: Thr⁹⁸⁰) was absent only after staurosporine treatment. Yet, these results can only be used as an indication since the absence of phosphorylation sites can reflect detection problems caused by low phosphorylation rates.

Quantitative analyses of phospho-sites of *rnKCC2b* and *hsNKCC1* upon staurosporine and NEM treatment in HEK^{*rnKCC2b*} cells

The experimental setup of our mass spectrometry-based analysis precluded quantification of changes at individual phospho-sites. We therefore applied in a next step phospho-site-specific antibody, as they were previously shown to quantitatively monitor changes in KCC2, NKCC1 and SPAK phosphorylation [23, 48, 59, 66, 70, 102]. Currently, a limited number of this class

Table 3. Phospho-sites of *hs*SPAK, *hs*WNK1 and *hs*WNK2 endogenously expressed in stably transfected HEK^{*rnKCC2b*} cells. Stably transfected HEK^{*rnKCC2b*} were treated with or without 1 mM NEM or 8 μ M staurosporine before they were analyzed by mass spectrometry. The protein accession numbers are: *hs*SPAK (AAC7238.1), *hs*WNK1 (Q9H4A3.2), *hs*WNK2 (Q9Y3S1.4).

sample	Phosphorylation site	Phosphopeptides	untreated	staurosporine treated	NEM treated
<i>hs</i> SPAK	308	EMMKY ^S GK	(2) (1)	(1)	(2)
	354	LLTRTPDIAQRAK	(1)	(2)	(1)
	356	LLTRTPDIAQRAK	(2)	(2)	(1)
	403	AAFS ^S QEK	(3)	(1)	(1)
	518; 520	ALKTLTFK	(3) (2)	(2) (3)	(3) (3)
	372; 373	VRRVPGSSGHLHK	(1)		
	<i>hs</i> WNK1	11	QSSTPGSLFLSPAPAPK	(2)	(1)
167; 170		DRPVSQPSLVGSK	(3)	(2)	(3)
167; 174		DRPVSQPSLVGSK	(3)	(3)	(2)
170		DRPVSQPSLVGSK	(3)		(2)
174		DRPVSQPSLVGSK	(3) (3)	(3) (3)	(2) (3)
183		EEPPPARSGGGGSAK		(1)	(1)
185		EEPPPARSGGGGSAK		(1)	
183; 185		EEPPPARSGGGGSAK	(3)	(3)	(3)
382		SVIGTPEFMAPEMYEEK	(1)		
599		QQVEQSSASQTGIK	(2) (3)	(3) (3)	(2)
1220		DDYGFSGSQK	(2)	(1)	
1849		EGPVLATSSGAGVFK	(3)	(1)	
1978		EGPVASPPFMDLEQAVLPAVIPK	(3)	(3)	(3)
2121		VPPAVIIPPAAPLSGR	(2)		(1)
2245		GTFTDDLHK	(1)		
2372	SISNPPGSNLRTT	(3) (3)	(3) (3)	(3)	
<i>hs</i> WNK2	560	EQQDVGSPDK	(2)	(3)	(3)
	1889	AGSLGPETPSR	(3)	(3)	(3)
	1862	QASLPVSGSVAGDFVK	(1) (3)		(2) (1)

The number in brackets indicates in how many technical replica a given phospho-site was detected (max. 3). Each bracket provides the results of one biological experiment.

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of antibodies is available for CCCs. They are directed against the well-known phospho-sites Ser⁹⁴⁰, Thr⁹⁰⁶ and Thr¹⁰⁰⁷ in *rnKCC2b* and Thr^{203, 207, 212} in *hsNKCC1*. So far, no data are available for the staurosporine and NEM effect on Thr⁹⁰⁶ in *rnKCC2b* and Thr^{203, 207, 212} in *hsNKCC1* and the staurosporine effect on Ser⁹⁴⁰, and Thr¹⁰⁰⁷ in *rnKCC2b*. To examine the impact of staurosporine and NEM on the phosphorylation level of these sites, we treated HEK^{*rnKCC2*} cells with 8 μ M staurosporine, 1 mM NEM or DMSO as a vehicle control for 15 min. Lysates were probed for KCC2 or NKCC1 and phosphorylation levels of each phospho-site were quantified (Fig 3). As previously described, positions Ser⁹⁴⁰, Thr⁹⁰⁶ and Thr¹⁰⁰⁷ are phosphorylated in KCC2b, and Thr^{203, 207, 212} in NKCC1 of untreated HEK^{*rnKCC2*} cells [48, 70, 102] (Fig 3). These data corroborate the phosphoproteome analyses which revealed phosphorylation of Ser⁹⁴⁰, Thr⁹⁰⁶ and Thr¹⁰⁰⁷ in KCC2b and Thr²¹²/Thr²¹⁷ in NKCC1 as well (Table 1).

Next, we observed the impact of staurosporine and NEM on these phospho-sites (Fig 3). Both agents decreased the phosphorylation status of the WNK/SPAK sites Thr⁹⁰⁶ (p -value for NEM or staurosporine: $p = 0.0026$) and Thr¹⁰⁰⁷ (p -value for NEM or staurosporine: $p = 0.0026$) of *rnKCC2* and Thr^{203/207/212} of *hsNKCC1* (p -value for NEM and staurosporine:

Table 4. Phospho-sites of *hsPKC* endogenously expressed in stably transfected HEK^{*rmKCC2b*} cells. Stably transfected HEK^{*rmKCC2b*} were treated with or without 1 mM NEM or 8 μ M staurosporine before they were analyzed by mass spectrometry. The protein accession numbers are: *hsPKC* Alpha (P17252), *hsPKC* Beta (P05771-2), *hsPKC* Delta (Q05655), *hsPKC* Epsilon (Q02156), *hsPKC* Theta (Q04759), *hsPKC* Eta (P24723), and *hsPKC* Iota (P41743).

classes	sample	Phosphorylation site	Phosphopeptides	untreated	staurosporine treated	NEM treated
conventional	PKC Alpha	10;11	ADVFPGNDSTASQDVANRFARK	(3)		(1)
		10;13	ADVFPGNDSTASQDVANRFARK	(3) (3)	(1) (1)	(1) (1)
		226	TIRSTLNPQWNESTFTFK		(2)	(2)
	319	VISPSEDRK	(3) (3)	(3) (2)	(2) (3)	
	PKC Beta	11	ADPAAGPPPSEGEESTVRFARK	(2)	(1)	(2)
641		HPPVLTTPDQEVIR	(3)	(3)	(3)	
novel	PKC Delta	645	ARLSYSDK	(3)	(3)	(3)
		647	ARLSYSDK	(2)	(1)	(1)
		664	NLIDSMQSAFAGFSFVNPK	(1)		(3)
	PKC Epsilon	309	VLADLGVTPDK	(2)	(2)	(2)
	PKC Theta	685	ALINSMDQNMFR	(2)	(1)	(2)
	PKC Eta	317	TLAGMGLQPGNISPTSK	(3)	(2)	(2)
atypical	PKC Iota	564	PNISGEFGLDNFDSQFTNEPVQLTPDDDDIVRK	(3)	(1)	(3)

The number in brackets indicates in how many technical replica a given phospho-site was detected (max. 3). Each bracket provides the results of one biological experiment.

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$p = 0.0026$) [72–75, 103]. The reduced phosphorylation of Thr²¹² in *hsNKCC1* agrees with our phosphoproteome analyses as no phosphorylation of Thr²¹²/Thr²¹⁷ in NKCC1 was observed (Table 1).

Previous analyses showed that SPAK directly phosphorylates Thr¹⁰⁰⁷ of *rmKCC2* [48] and Thr^{203/207/212} of *hsNKCC1* [70]. Treatment of HEK293 cells with NEM resulted in a decrease of phosphorylation of Thr²³³ ($p = 0.0026$), that is located in the T-loop kinase domain, and the S-loop phosphorylation site Ser³⁷³ of *hsSPAK* ($p = 0.0026$) (Fig 3). Both are targets of WNKs [48, 69, 93]. As no data were available for the staurosporine mediated effect, we additionally analyzed its impact on these phospho-sites in HEK293 cells. Staurosporine also reduced the phosphorylation of Thr²³³ ($p = 0.0026$) and Ser³⁷³ ($p = 0.0026$) in *hsSPAK* (Fig 3). Thus, both agents reduced phosphorylation levels of these SPAK phospho-sites. These data conform well to our phosphoproteomic analyses, as no phosphorylated Ser^{372/373} of *hsSPAK* was detected after treatment with either of the two agents.

Furthermore, staurosporine reduced phosphorylation of Ser⁹⁴⁰ ($p = 0.0026$) in HEK^{*rmKCC2b*}, whereas NEM increased phosphorylation of Ser⁹⁴⁰ ($p = 0.046$) significantly (Fig 3). Since, Ser⁹⁴⁰ is directly phosphorylated by PKC [77, 104], we here analyzed the impact of both agents on the T-loop phosphorylation site Thr⁵⁰⁵ of PKC- δ . Autophosphorylation of this site is most probably essential for kinase activity [105–107]. Staurosporine significantly decreases Thr⁵⁰⁵ phosphorylation ($p = 0.0026$, Fig 3), whereas NEM slightly, but not significantly, increases Thr⁵⁰⁵ phosphorylation ($p = 0.064$). The different impact of both agents on the phosphorylation of Thr⁵⁰⁵ of PKC correlates well with their impact on Ser⁹⁴⁰ phosphorylation of KCC2b.

We also determined whether staurosporine or NEM altered the total protein amount of *rmKCC2b*, *hsNKCC1* or *hsSPAK* (Fig 3). Whereas NEM resulted in increased KCC2 amount ($p = 0.0026$), no obvious change was detected upon staurosporine treatment. NKCC1 and SPAK levels were not changed significantly upon treatment with either agent.

To conclude, staurosporine and NEM reduced the phosphorylation status of SPAK (Thr²³³ and Ser³⁷³). This correlated with the reduction of the phosphorylation of Thr¹⁰⁰⁷ in *rmKCC2b*

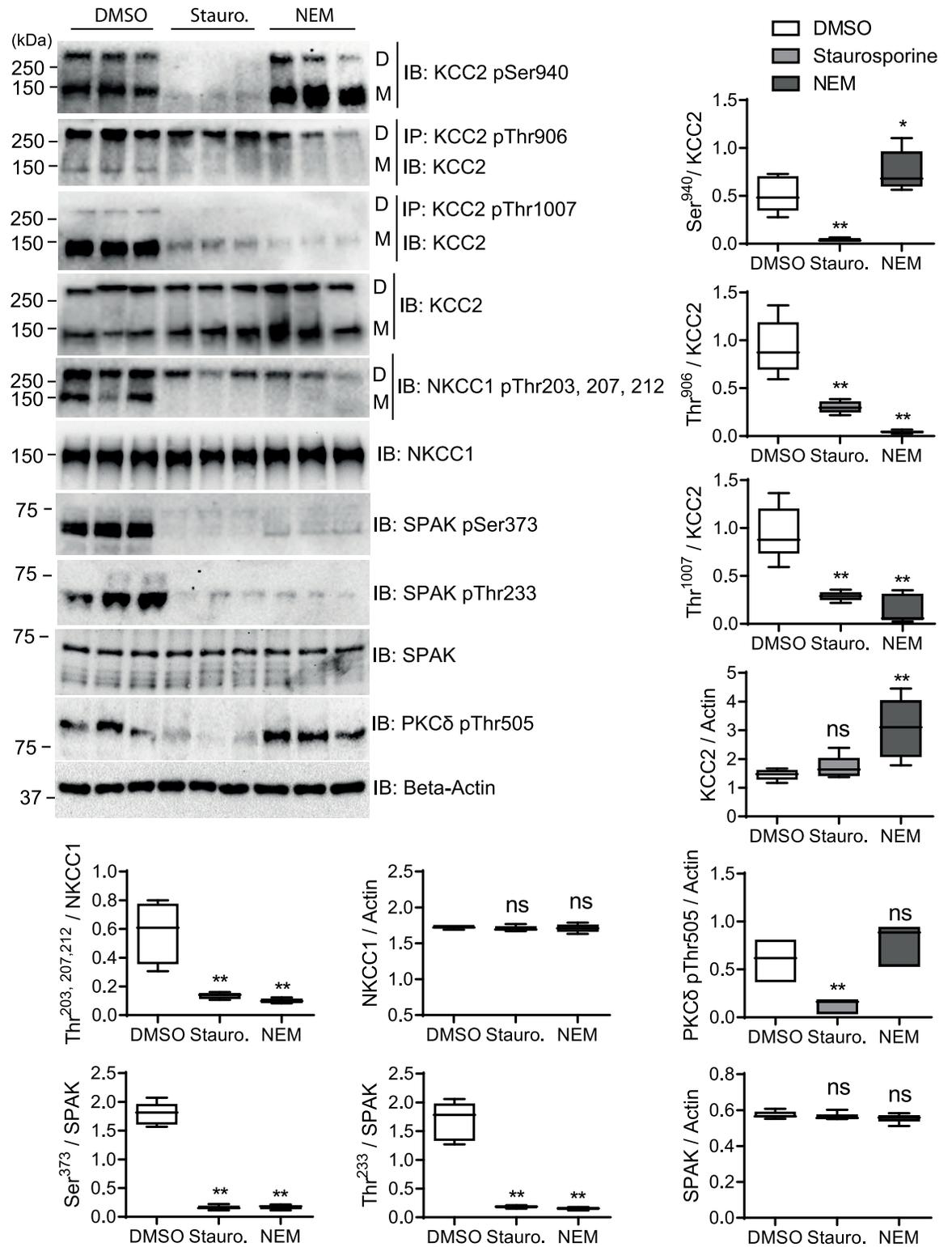


Fig 3. Quantitative analyses of *rnKCC2b* and *hsNKCC1* phospho-sites upon staurosporine and NEM treatment in HEK^{rnKCC2b} cells. Stably transfected HEK^{rnKCC2b} cells were treated with DMSO (control), 8 μM staurosporine or 0.5 mM NEM, respectively, for 15 min. Cell lysates were harvested and subjected to immunoprecipitation (IP) and immunoblot (IB) with indicated antibodies. D, dimeric KCC2; M, monomeric KCC2. Band intensities were quantified with ImageJ software. ***, $p < 0.001$; **, $p < 0.01$; Wilcoxon-Mann-Whitney test ($n = 6$).

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and Thr^{203/207/212} in *hsNKCC1*. Additionally, both agents reduced phosphorylation of Thr⁹⁰⁶ in *mKCC2b*, which is phosphorylated by WNKs and a yet unknown kinase [48]. Staurosporine also reduced phosphorylation of the PKC site Ser⁹⁴⁰ in *mKCC2b*, whereas NEM increased its phosphorylation level. This correlated with the reduction of Thr⁵⁰⁵ phosphorylation of PKC- δ upon staurosporine treatment and the impact of NEM to increase Thr⁵⁰⁵ phosphorylation.

Quantitative analyses of phospho-sites of KCC2 and NKCC1 upon staurosporine or NEM treatment of rat immature hippocampal neurons

As KCC2 is predominantly expressed in neurons [20], we analyzed for the first time the impact of staurosporine and NEM on the phosphorylation of specific phospho-sites of endogenously expressed KCC2 and NKCC1 using immature (9 DIV) primary rat hippocampal neurons (Fig 4). At this age cultured hippocampal neurons exhibit prominent level of Thr⁹⁰⁶ and Thr¹⁰⁰⁷ KCC2 phosphorylation [66] that could be a subject of modulation by staurosporine and NEM. To this end, we treated neurons with 8 μ M staurosporine, 0.5 mM NEM or DMSO as a vehicle control for 15 min. NEM was reduced to 0.5 mM, since higher concentrations induced cell death. As described for the analyses in stably transfected HEK^{*mKCC2b*} cells, we used phospho-specific KCC2 and NKCC1 antibodies to quantify phosphorylation levels of each phospho-site relatively to the DMSO control. In untreated cultured immature hippocampal neurons, Ser⁹⁴⁰, Thr⁹⁰⁶ and Thr¹⁰⁰⁷ in KCC2 and Thr^{203, 207, 212} in NKCC1 were phosphorylated, similar to stably transfected HEK^{*mKCC2b*} cells (Fig 4).

Most actions of staurosporine and NEM, as monitored by immunoblots, were similar between HEK^{*mKCC2b*} cells and immature hippocampal neurons. Both agents resulted in decreased phosphorylation of Thr¹⁰⁰⁷ in KCC2 (p -value for NEM and staurosporine: $p = 0.0026$), Thr^{203/207/212} in NKCC1 (p -value for NEM and staurosporine: $p = 0.0026$), and Ser³⁷³ in SPAK (p -value for NEM: $p = 0.0026$ and staurosporine: $p = 0.0026$). Additionally, both agents reduced phosphorylation of the WNK-dependent phospho-site Thr⁹⁰⁶ in KCC2 (p -value for NEM and staurosporine: $p = 0.0026$). Finally, NEM increased the total protein level of KCC2, as observed in HEK293 cells ($p = 0.015$) (Fig 4). We were not able to detect the phosphorylation of Thr²³³ of SPAK using phospho-specific antibodies.

A marked difference, however, was observed for the PKC dependent phospho-site Ser⁹⁴⁰. Here, treatment with NEM reduced phosphorylation of Ser⁹⁴⁰ ($p = 0.0026$) (Fig 4), contrary to the results obtained in stably transfected HEK^{*mKCC2b*} cells (Fig 3). Treatment with staurosporine also resulted in reduced phosphorylation of Ser⁹⁴⁰ ($p = 0.0026$), which was similar to its action in HEK^{*mKCC2b*} cells. We could not detect Thr⁵⁰⁵ phosphorylation of PKC- δ due to low expression rates.

To sum up, NEM affected the phosphorylation status of Ser⁹⁴⁰ in immature hippocampal neurons (decrease) in the opposite way compared to HEK^{*mKCC2b*} cells (increase). All other effects of staurosporine and NEM were similar between immature hippocampal neurons and HEK293 cells, i.e. both reduced the phosphorylation status of Ser⁹⁴⁰, Thr⁹⁰⁶, and Thr¹⁰⁰⁷ in KCC2 and Thr^{203/207/212} in *hsNKCC1*.

Discussion

KCC2 and NKCC1 are key players for the development and maintenance of fast inhibitory neurotransmission. Their oppositely directed transport of K⁺ and Cl⁻ ions within the same neuronal population necessitates a precisely coordinated regulatory mechanism for efficient setting of the intracellular [Cl⁻] concentration [4, 38, 55–60] (Fig 5).

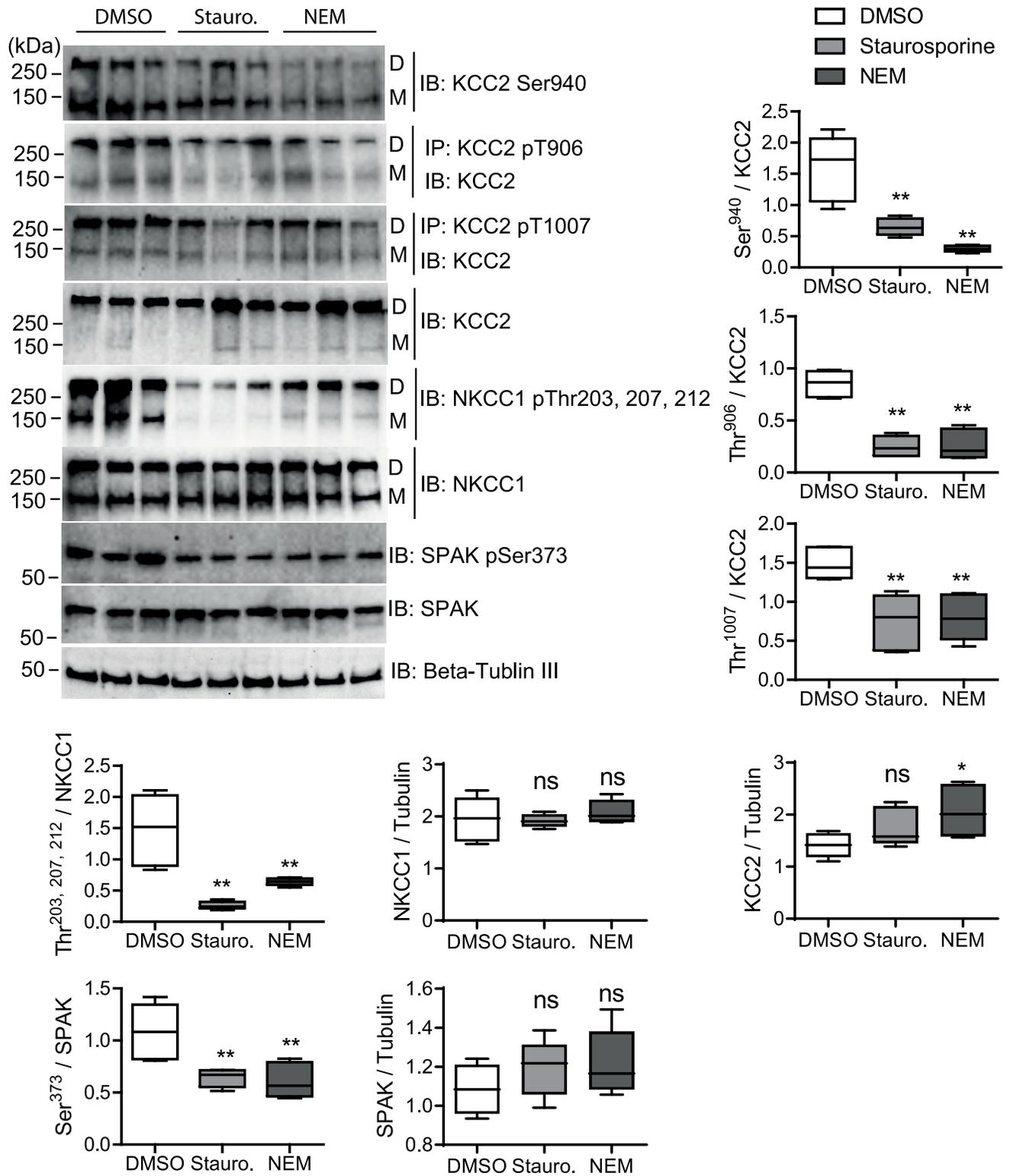


Fig 4. Quantitative analyses of *mKCC2* and *mNKCC1* phospho-sites upon staurosporine or NEM treatment in immature hippocampal neurons. Immature rat hippocampal neurons were treated with DMSO (control), 8 μ M staurosporine or 0.5 mM NEM, respectively, for 15 min. Neuronal lysates were harvested and subjected to immunoprecipitation (IP) and immunoblot (IB) with the indicated antibodies. D, dimeric KCC2; M, monomeric KCC2. Band intensities were quantified using ImageJ software. ***, $p < 0.001$; **, $p < 0.01$; Wilcoxon-Mann-Whitney test ($n = 6$).

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Posttranslational regulation via the WNK-SPAK/OSR1 dependent phosphorylation represents a potent mechanism to regulate transport activity of KCC2 and NKCC1 in a reciprocal way [60, 108–110] (Fig 5). Here, we show that staurosporine and NEM decrease phosphorylation of Thr²³³ and Ser³⁷³ in SPAK, of Thr¹⁰⁰⁷ in *mKCC2* and Thr²⁰³, Thr²⁰⁷ and Thr²¹² in *hsNKCC1* in both HEK293 cells and immature cultured hippocampal neurons. Since SPAK directly impairs phosphorylation of Thr¹⁰⁰⁷ in *mKCC2* and Thr²⁰³, Thr²⁰⁷ and Thr²¹² in *hsNKCC1* [38, 48, 59, 60, 63, 68–76], our data suggest that staurosporine and NEM directly affect the WNK-SPAK/OSR1 mediated phosphorylation of these residues in KCC2 and NKCC1. The data are in line with previous analyses showing that NEM reduces phosphorylation of Ser³⁷³ in SPAK and Thr¹⁰⁰⁷ in KCC2 using HEK293 cells and immature cortical neurons [48]. Furthermore, application of staurosporine and NEM decreases phosphorylation of Thr⁹⁰⁶ in *mKCC2*. This site is directly phosphorylated by WNKs and a yet unknown kinase [48]. However, functional in-depth analyses such as mutagenic approaches are required to prove a causal relation between dephosphorylation of SPAK Thr²³³ and Ser³⁷³ and dephosphorylation of the specific KCC2 and NKCC1 phospho-sites upon staurosporine and NEM treatment.

Recent analyses demonstrated that dephosphorylation of Thr⁹⁰⁶ and Thr¹⁰⁰⁷ increases KCC2 activity [61, 64–66], whereas dephosphorylation of Thr²⁰³, Thr²⁰⁷ and Thr²¹² decreases NKCC1 activity [45, 51]. Furthermore, staurosporine and NEM results in activation of KCC2 (this study and [40, 47, 48, 59, 60, 79, 80], whereas they reduce NKCC1 activity [43–46, 51]. This suggests, that staurosporine and NEM mediated dephosphorylation of these phospho-sites result in a reciprocal regulation of KCC2 (activation) and NKCC1 (inactivation) activity most likely via the WNK/SPAK-dependent phosphorylation pathway. This is also in line with the observation in immature hippocampal neurons that KCC2 can rapidly be activated by staurosporine [42].

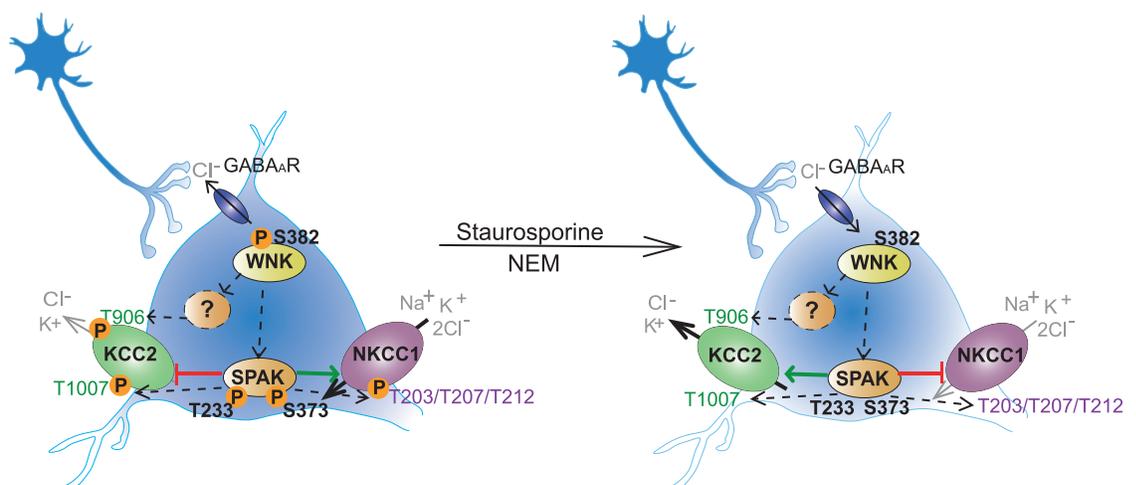


Fig 5. Staurosporine and NEM impair the WNK-SPAK/OSR1 mediated phosphorylation of KCC2 and NKCC1. In immature hippocampal neurons, the WNK mediated phosphorylation of SPAK/OSR1 directly phosphorylates T¹⁰⁰⁷ of KCC2 and T²⁰³, T²⁰⁷ and T²¹² of NKCC1. WNKs also interact with a yet unknown kinase to phosphorylate Thr⁹⁰⁶ of KCC2. Phosphorylation of these residues decreases KCC2 and increases NKCC1 activity. Application of staurosporine or NEM decreases phosphorylation of S³⁸² of WNK1 and T²³³ and S³⁷³ of SPAK. This likely results in reduction of phosphorylation of T⁹⁰⁶ and T¹⁰⁰⁷ in KCC2 and of T²⁰³, T²⁰⁷ and T²¹² in NKCC1. Dephosphorylation of these residues increases KCC2 activity and decreases NKCC1 activity. The figure is modified from Moore et al. [39].

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Another key regulatory KCC2 phospho-site is the PKC-mediated phosphorylation of Ser⁹⁴⁰. Phosphorylation of Ser⁹⁴⁰ enhances KCC2 cell surface expression and increases ion transport activity, whereas mutation of serine to alanine (mimicking the dephosphorylated state) results in transport activity that is equal or decreased compared to wild-type KCC2 (KCC2^{wt}) [65, 77, 111]. Our data demonstrate that staurosporine and NEM can differentially affect Ser⁹⁴⁰ phosphorylation. Treatment of immature hippocampal neurons with either agent results in decreased phosphorylation of Ser⁹⁴⁰. However, treatment of HEK^{rkKCC2b} cells with staurosporine decreases, whereas NEM increases phosphorylation of Ser⁹⁴⁰. This is in line with the different effect of these agents on transport activity of the phosphomutants Ser^{31A/D}, Thr^{34A/D}, Thr^{999A} and Thr^{1008A/D} [40].

Moreover, NEM has a cell-type specific impact on Ser⁹⁴⁰ phosphorylation. In immature cortical neurons [48] and HEK293 cells, NEM increases Ser⁹⁴⁰ phosphorylation, whereas it decreases Ser⁹⁴⁰ phosphorylation in immature cultured hippocampal neurons (Fig 4). The mechanisms that cause this opposite effect of NEM on Ser⁹⁴⁰ phosphorylation in different tissues is unclear. One possibility is that different NEM concentrations (HEK293 cells: 1 mM and 0.1 mM; immature hippocampal neurons: 0.5 mM; immature cortical neurons: 0.1 mM [48]) affect different regulatory pathways. Alternatively, tissues-specific sets of PKC isoforms and phosphatases result in different phosphorylation patterns. Indeed, the PKC family consists of 10–12 isoforms grouped into three classes [98–101] that vary in their expression profile [112] and regulation of their activity through several regulatory proteins, co-factors and second messenger cascades [100, 101, 113]. This offers the opportunity to differentially regulate KCC2 function in distinct neuronal populations through PKC. In HEK^{rkKCC2b} cells, we showed that staurosporine reduced and NEM slightly increased phosphorylation of the T-loop phospho-site Thr⁵⁰⁵ of PKC- δ . This correlated with decreased KCC2 Ser⁹⁴⁰ phosphorylation upon staurosporine treatment and increased phosphorylation of this residue upon NEM treatment. This suggests, that both agents directly act on PKC- δ mediating the phosphorylation of Ser⁹⁴⁰ in HEK^{rkKCC2b} cells. However, more functional in-depth analyses are required to elucidate the causal link between dephosphorylation of PKC- δ Thr⁵⁰⁵ and dephosphorylation of KCC2 Ser⁹⁴⁰ upon staurosporine treatment. Since we were unable to detect phosphorylation of PKC- δ Thr⁵⁰⁵ in immature hippocampal neurons, we suggest that other PKC isoforms are involved in the direct phosphorylation of KCC2 Ser⁹⁴⁰ in immature hippocampal neurons.

Our data furthermore reveal that staurosporine (HEK^{rkKCC2b} cells, immature hippocampal neurons) and NEM treatment (immature hippocampal neurons) decrease Ser⁹⁴⁰ phosphorylation resulting in an equal or diminished transport activity compared to KCC2^{wt} [65, 77, 111]. We therefore conclude that Ser⁹⁴⁰ is not the key regulatory phospho-site mediating the staurosporine and NEM-based stimulation effect on KCC2. This is in line with recent published analyses showing that NEM still enhances the transport activity of Ser^{940A} (mimicking dephosphorylated state), indicating that other phospho-sites are important in NEM-dependent stimulation [40, 48].

NEM but not staurosporine increased total KCC2 amount in HEK293 cells and immature cultured hippocampal neurons. In immature cortical neurons, Deep and coworkers [48] detected the same trend of enhanced total KCC2 abundance (albeit not significant), resulting in increased cell surface expression [48]. This suggests that in contrast to staurosporine, NEM increases KCC2 expression and trafficking that could result in a higher KCC2 activity. The different impact of NEM on total KCC2 abundance in HEK293 in the study of Deep and coworkers (no increase) and our analyses (increase) could result from different NEM concentration used in the experiments (0.1 mM vs. 1 mM).

Via mass spectrometry analysis, we identified several new phosphorylation sites whose function awaits further investigation. These sites are the N-terminal KCC2 phospho-site Thr³², and the C-terminal NKCC1 phospho-sites Ser²⁴², Thr²⁶⁶, Thr²⁶⁸, Ser⁹⁴⁰, Tyr⁹⁵⁶ and

Ser⁹⁵⁷. Future studies should investigate their regulatory impact on KCC2 and NKCC1 activity.

Conclusions

In conclusion, our data identify molecular mechanisms involved in staurosporine and NEM mediated changes in transport activity of KCC2 and NKCC1, which are a defining feature of CCCs [114]. The observation of cell-type specific action of these agents is in line with different reversal potentials in mature neuronal populations [115] and calls for comprehensive neuron sub-type-specific phospho analysis. The recently reported structural data of CCCs [116–119] finally lay the foundation to analyze jointly the physiological role of phosphorylation and underlying structural changes to obtain an integrative and mechanistic view of the action of phosphorylation.

Supporting information

S1 Table. Phospho-sites in PhosphoSitePlus detected by mass spectrometry analyses.
(DOCX)

S2 Table. Phospho-sites in PhosphoSitePlus detected by mass spectrometry analyses.
(DOCX)

S3 Table. Phospho-sites in PhosphoSitePlus detected by mass spectrometry analyses.
(DOCX)

S1 Fig. Quantitative analyses of *rnKCC2* and *hsNKCC1* phospho-sites upon staurosporine and NEM treatment in HEK^{*rnKCC2b*} cells.
(PDF)

S2 Fig. Quantitative analyses of *rnKCC2* and *hsNKCC1* phospho-sites upon staurosporine and NEM treatment in immature hippocampal neurons.
(PDF)

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References

1. Hartmann A-M, Tesch D, Nothwang HG, Bininda-Emonds ORP. Evolution of the Cation Chloride Cotransporter Family: Ancient Origins, Gene Losses, and Subfunctionalization through Duplication. *Molecular biology and evolution*. 2014:mst225.
2. Gamba G. Molecular Physiology and Pathophysiology of electroneutral Cation-Chloride Cotransporter. *Physiological Reviews*. 2005; 85:423–93. <https://doi.org/10.1152/physrev.00011.2004> PMID: 15788703
3. Daigle ND, Carpentier GA, Frenette-Cotton R, Simard CF, Lefoll M-H, Noel M, et al. Molecular characterization of a human cation-Cl⁻ cotransporter (SLC12A8, CCC9A) that promotes polyamine and amino acid transport. *Journal of Cellular Physiology*. 2009; 220:680–9. <https://doi.org/10.1002/jcp.21814> PMID: 19472210
4. Hartmann A, Nothwang HG. Molecular and evolutionary insights into the structural organization of cation chloride cotransporters. *Frontiers in Cellular Neuroscience*. 2015; 8:470. <https://doi.org/10.3389/fncel.2014.00470> PMID: 25653592
5. Payne JA, Rivera C, Voipo J, Kaila K. Cation-Chloride cotransporters in neuronal communication, development and trauma *Trends in Neuroscience*. 2003; 26:199–206.
6. Arroyo JP, Kahle KT, Gamba G. The SLC12 family of electroneutral cation-coupled chloride cotransporters. *Molecular Aspects of Medicine*. 2013; 34:288–98. <https://doi.org/10.1016/j.mam.2012.05.002> PMID: 23506871
7. Kahle KT, Staley KJ, Nahed BV, Gamba G, Hebert SC, Lifton RP, et al. Roles of the cation-chloride cotransporters in neurological disease. *Nature Clinical Practice*. 2008; 4:490–502.
8. Blaesse P, Airaksinen MS, Rivera C, Kaila K. Cation-Chloride Cotransporters and Neuronal Function. *Cell*. 2009; 61:820–38.
9. Adragna NC, Fulvio MD, Lauf PK. Regulation of K-Cl Cotransport: from Function to Genes. *Journal of Membrane Biology*. 2004; 201:109–37. <https://doi.org/10.1007/s00232-004-0695-6> PMID: 15711773
10. Di Fulvio M, Alvarez-Leefmans FJ. The NKCC and NCC genes: an in silico view. *Physiology and Pathology of Chloride Transporters and Channels in the Nervous System*. 2009:169–208.
11. Gagnon KB, Delpire E. Physiology of SLC12 transporters: lessons from inherited human genetic mutations and genetically engineered mouse knockouts. *American Journal of Physiology-Cell Physiology*. 2013; 304:C693–C714. <https://doi.org/10.1152/ajpcell.00350.2012> PMID: 23325410
12. Medina I, Friedel P, Rivera C, Kahle KT, Kourdougli N, Uvarov P, et al. Current view on the functional regulation of the neuronal K⁺-Cl⁻ cotransporter KCC2. *Frontiers in cellular neuroscience*. 2014;8.
13. Achilles K, Okabe A, Ikeda M, Shimizu-Okabe C, Yamada F, Fukuda A, et al. Kinetic properties of Cl⁻ uptake mediated by Na⁺-dependent K⁺-2Cl⁻ cotransport in immature rat neocortical neurons. *Journal of Neuroscience*. 2007; 27:8616–27. <https://doi.org/10.1523/JNEUROSCI.5041-06.2007> PMID: 17687039
14. Sipilä ST, Huttu K, Yamada J, Afzalov R, Voipio J, Blaesse P, et al. Compensatory enhancement of intrinsic spiking upon NKCC1 disruption in neonatal hippocampus. *The Journal of Neuroscience*. 2009; 29:6982–8. <https://doi.org/10.1523/JNEUROSCI.0443-09.2009> PMID: 19474325
15. Yamada J, Okabe M, Toyoda H, Kilb W, Luhmann HJ, Fukuda A. Cl⁻ uptake promoting depolarizing GABA actions in immature rat neocortical neurons is mediated by NKCC1. *Journal of Physiology*. 2004; 557:829–41. <https://doi.org/10.1113/jphysiol.2004.062471> PMID: 15090604
16. Yuste R, Katz LC. Control of postsynaptic Ca²⁺ influx in developing neocortex by excitatory and inhibitory neurotransmitters. *Neuron*. 1991; 6:333–44. [https://doi.org/10.1016/0896-6273\(91\)90243-s](https://doi.org/10.1016/0896-6273(91)90243-s) PMID: 1672071

17. Reichling DB, Kyrozis A, Wang J, MacDermott AB. Mechanism of GABA and glycine depolarization-induced calcium transients in rat dorsal horn neurons. *Journal of Physiology*. 1994; 476:411–21. <https://doi.org/10.1113/jphysiol.1994.sp020142> PMID: 8057250
18. Owens DF, Boyce LH, Davis MBE, Kriegstein AR. Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. *The Journal of Neuroscience*. 1996; 16:6416–23.
19. Hübner CA, Stein V, Hermans-Borgmeyer I, Meyer T, Ballanyi K, Jentsch TJ. Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron*. 2001; 30:515–24. [https://doi.org/10.1016/s0896-6273\(01\)00297-5](https://doi.org/10.1016/s0896-6273(01)00297-5) PMID: 11395011
20. Uvarov P, Ludwig A, Markkanen M, Pruunsild P, Kaila K, Delpire E, et al. A novel N-terminal isoform of the neuron-specific K-Cl Cotransporter KCC2. *Journal of Biological Chemistry*. 2007; 282(42):30570–6. <https://doi.org/10.1074/jbc.M705095200> PMID: 17715129
21. Kim JY, Liu CY, Zhang F, Duan X, Wen Z, Song J, et al. Interplay between DISC1 and GABA signaling regulates neurogenesis in mice and risk for schizophrenia. *Cell*. 2012; 148(5):1051–64. <https://doi.org/10.1016/j.cell.2011.12.037> PMID: 22385968
22. Tyzio R, Nardou R, Ferrari DC, Tsintsadze T, Shahrokhi A, Eftekhari S, et al. Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring. *Science*. 2014; 343(6171):675–9. <https://doi.org/10.1126/science.1247190> PMID: 24503856
23. Kahle KT, Merner ND, Friedel P, Silayeva L, Liang B, Khanna A, et al. Genetically encoded impairment of neuronal KCC2 cotransporter function in human idiopathic generalized epilepsy. *EMBO reports*. 2014; 15(7):766–74. <https://doi.org/10.15252/embr.201438840> PMID: 24928908
24. Merner ND, Chandler MR, Bourassa C, Liang B, Khanna AR, Dion P, et al. Regulatory domain or CpG site variation in SLC12A5, encoding the chloride transporter KCC2, in human autism and schizophrenia. *Frontiers in cellular neuroscience*. 2015;9. <https://doi.org/10.3389/fncel.2015.00009> PMID: 25698924
25. Huberfeld G, Wittner L, Clemenceau S, Baulac M, Kaila K, Miles R, et al. Perturbed chloride homeostasis and GABAergic signaling in human temporal lobe epilepsy. *The Journal of Neuroscience*. 2007; 27(37):9866–73. <https://doi.org/10.1523/JNEUROSCI.2761-07.2007> PMID: 17855601
26. Coull JAM, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, et al. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature*. 2003; 424:938–42. <https://doi.org/10.1038/nature01868> PMID: 12931188
27. Rivera C, Li H, Thomas-Crusells J, Lahtinen H, Viitanen T, Nanobashvili A, et al. BDNF-induced TrkB activation down-regulates the K⁺-Cl⁻ cotransporter KCC2 and impairs neuronal Cl⁻ extrusion. *The Journal of Cell Biology*. 2002; 159(5):747–52. <https://doi.org/10.1083/jcb.200209011> PMID: 12473684
28. Boulenguez P, Liabeuf S, Bos R, Bras H, Jean-Xavier C, Brocard C, et al. Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. *Nature medicine*. 2010; 16(3):302–7. <https://doi.org/10.1038/nm.2107> PMID: 20190766
29. Papp E, Rivera C, Kaila K, Freund TF. Relationship between neuronal vulnerability and potassium-chloride cotransporter 2 immunoreactivity in hippocampus following transient forebrain ischemia. 2008. *Neuroscience*:677–89.
30. Shulga A, Thomas-Crusells J, Sigl T, Blaesse A, Mestres P, Meyer M, et al. Posttraumatic GABA-mediated [Ca²⁺]_i increase is essential for the induction of brain-derived neurotrophic factor-dependent survival of mature central neurons. *The Journal of Neuroscience*. 2008; 28(27):6996–7005. <https://doi.org/10.1523/JNEUROSCI.5268-07.2008> PMID: 18596173
31. Puskarjov M, Ahmad F, Khirug S, Sivakumaran S, Kaila K, Blaesse P. BDNF is required for seizure-induced but not developmental up-regulation of KCC2 in the neonatal hippocampus. *Neuropharmacology*. 2014.
32. Pisella LI, Gaiarsa J-L, Diabira D, Zhang J, Khalilov I, Duan J, et al. Impaired regulation of KCC2 phosphorylation leads to neuronal network dysfunction and neurodevelopmental pathology. 2019; 12(603): eaay0300.
33. Delpire E, Lu J, England R, Dull C, Thorne T. Deafness and imbalance associated with inactivation of the secretory Na-K-2Cl co-transporter. *Nature Genetics*. 1999; 22:192–5. <https://doi.org/10.1038/9713> PMID: 10369265
34. Delpire E, Mount DB. Human and Murine Phenotypes associated with defects in Cation-Chloride-Cotransporter. *Annu Rev Physiol*. 2002; 64:803–43. <https://doi.org/10.1146/annurev.physiol.64.081501.155847> PMID: 11826289
35. Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, et al. NKCC1 transporter facilitates seizures in the developing brain. *Nature Medicine*. 2005; 11:1205–13. <https://doi.org/10.1038/nm1301> PMID: 16227993

36. Zhu L, Polley N, Mathews GC, Delpire E. NKCC1 and KCC2 prevent hyperexcitability in the mouse hippocampus. *Epilepsy research*. 2008; 79(2–3):201–12. <https://doi.org/10.1016/j.eplepsyres.2008.02.005> PMID: 18394864
37. Ben-Ari Y. NKCC1 chloride importer antagonists attenuate many neurological and psychiatric disorders. *Trends in neurosciences*. 2017; 40(9):536–54. <https://doi.org/10.1016/j.tins.2017.07.001> PMID: 28818303
38. Alessi DR, Zhang J, Khanna A, Hochdörfer T, Shang Y, Kahle KT. The WNK-SPAK/OSR1 pathway: Master regulator of cation-chloride cotransporters. *Science signaling*. 2014; 7(334):re3–re. <https://doi.org/10.1126/scisignal.2005365> PMID: 25028718
39. Moore YE, Kelley MR, Brandon NJ, Deeb TZ, Moss SJ. Seizing control of KCC2: a new therapeutic target for epilepsy. 2017; 40(9):555–71.
40. Cordshagen A, Busch W, Winkhofer M, Nothwang HG, Hartmann A-M. Phosphoregulation of the intracellular termini of K⁺-Cl⁻ cotransporter 2 (KCC2) enables flexible control of its activity. *Journal of Biological Chemistry*. 2018; 293(44):16984–93. <https://doi.org/10.1074/jbc.RA118.004349> PMID: 30201606
41. Blaesse P, Guillemain I, Schindler J, Schweizer M, Delpire E, Khiroug L, et al. Oligomerization of KCC2 Correlates with Development of Inhibitory Neurotransmission. *The Journal of Neuroscience*. 2006; 26(41):10407–19. <https://doi.org/10.1523/JNEUROSCI.3257-06.2006> PMID: 17035525
42. Khirug S, Huttu K, Ludwig A, Smirnov S, Voipo J, Rivera C, et al. Distinct properties of functional KCC2 expression in immature mouse hippocampal neurons in culture and in acute slices. *European Journal of Neuroscience*. 2005; 21(4):899–904. <https://doi.org/10.1111/j.1460-9568.2005.03886.x> PMID: 15787696
43. Flatman PW. Regulation of Na-K-2Cl cotransport by phosphorylation and protein-protein interactions. *Biochimica et biophysica Acta*. 2002; 1566:140–51.
44. Lytle C, McManus TJ, Haas M. A model of Na-K-2Cl cotransport based on ordered ion binding and glide symmetry. *American Journal of Physiology-Cell Physiology*. 1998; 274(2):C299–C309.
45. Muzyamba M, Cossins A, Gibson J. Regulation of Na⁺-K⁺-2Cl⁻ cotransport in turkey red cells: the role of oxygen tension and protein phosphorylation. *The Journal of physiology*. 1999; 517(2):421–9.
46. Palfrey HC, Leung S. Inhibition of Na-K-2Cl cotransport and bumetanide binding by ethacrynic acid, its analogues, and adducts. *American Journal of Physiology-Cell Physiology*. 1993; 264(5):C1270–C7.
47. Weber M, Hartmann A-M, Beyer T, Ripperger A, Nothwang HG. A novel regulatory locus of phosphorylation in the C-terminus of the potassium chloride cotransporter KCC2 that interferes with N-ethylmaleimide or staurosporine mediated activation. *Journal of Biological Chemistry*. 2014;jbc-M114.
48. Conway LC, Cardarelli RA, Moore YE, Jones K, McWilliams LJ, Baker DJ, et al. N-Ethylmaleimide increases KCC2 cotransporter activity by modulating transporter phosphorylation. *Journal of Biological Chemistry*. 2017; 292(52):21253–63. <https://doi.org/10.1074/jbc.M117.817841> PMID: 29092909
49. Bize I, Güvenç B, Buchbinder G, Brugnara C. Stimulation of human erythrocyte K-Cl cotransport and protein phosphatase type 2A by n-ethylmaleimide: role of intracellular Mg⁺⁺. *The Journal of membrane biology*. 2000; 177(2):159–68. <https://doi.org/10.1007/s002320001109> PMID: 11003690
50. Jennings ML, Schulz RK. Okadaic acid inhibition of KCl cotransport. Evidence that protein dephosphorylation is necessary for activation of transport by either cell swelling or N-ethylmaleimide. *The Journal of general physiology*. 1991; 97(4):799–817. <https://doi.org/10.1085/jgp.97.4.799> PMID: 1647439
51. Gagnon KBE, England R, Delpire E. Characterization of SPAK and OSR1, regulatory kinases of the Na-K-2Cl cotransporter. *Molecular and cellular biology*. 2006; 26(2):689–98. <https://doi.org/10.1128/MCB.26.2.689-698.2006> PMID: 16382158
52. Vardi N, Zhang L-L, Payne JA, Sterling P. Evidence that different cation chloride cotransporters in retinal neurons allow opposite responses to GABA. *The Journal of Neuroscience*. 2000; 20(20):7657–63. <https://doi.org/10.1523/JNEUROSCI.20-20-07657.2000> PMID: 11027226
53. Balakrishnan V, Becker M, Löhke S, Nothwang HG, Güresir E, Friauf E. Expression and Function of Chloride Transporters during Development of Inhibitory Neurotransmission in the Auditory Brainstem. *The Journal of Neuroscience*. 2003; 23:4134–45. <https://doi.org/10.1523/JNEUROSCI.23-10-04134.2003> PMID: 12764101
54. Price TJ, Trussel LO. Estimate of the chloride concentration in a central glutamatergic terminal: gramicidin perforated-patch study on the calyx of Held. *Journal of Neuroscience*. 2006; 26:11432–6. <https://doi.org/10.1523/JNEUROSCI.1660-06.2006> PMID: 17079672
55. Hartmann A-M, Blaesse P, Kranz T, Wenz M, Schindler J, Kaila K, et al. Opposite effect of membrane raft perturbation on transport activity of KCC2 and NKCC1. *Journal of Neurochemistry*. 2009; 111(2):321–31. <https://doi.org/10.1111/j.1471-4159.2009.06343.x> PMID: 19686239
56. Wenz M, Hartmann A-M, Friauf E, Nothwang HG. CIP1 is an activator of the K⁺-Cl⁻ cotransporter KCC2. *Biochemical and Biophysical Research Communications*. 2009; 381:388–92. <https://doi.org/10.1016/j.bbrc.2009.02.057> PMID: 19232517

57. Caron L, Rousseau F, Gagnon É, Isenring P. Cloning and functional characterization of a cation-chloride cotransporter-interacting protein. *J Biol Chem*. 2000; 275:32027–36. <https://doi.org/10.1074/jbc.M000108200> PMID: 10871601
58. De los Heros P, Kahle KT, Rinehart J, Bobadilla NA, Vázquez N, San-Cristobal P, et al. WNK3 bypasses the tonicity requirement for K-Cl cotransporter activation via a phosphatase-dependent pathway. *PNAS*. 2005; 103(6):1976–81.
59. Heubl M, Zhang J, Pressey JC, Al Awabdh S, Renner M, Gomez-Castro F, et al. GABA A receptor dependent synaptic inhibition rapidly tunes KCC2 activity via the Cl⁻-sensitive WNK1 kinase. *Nature communications*. 2017; 8(1):1776. <https://doi.org/10.1038/s41467-017-01749-0> PMID: 29176664
60. Shekarabi M, Zhang J, Khanna AR, Ellison DH, Delpire E, Kahle KT. WNK kinase signaling in ion homeostasis and human disease. *Cell metabolism*. 2017; 25(2):285–99. <https://doi.org/10.1016/j.cmet.2017.01.007> PMID: 28178566
61. Rinehart J, Maksimova YD, Tanis JE, Stone KL, Hodson CA, Zhang J, et al. Sites of Regulated Phosphorylation that Control K-Cl Cotransporter Activity. *Cell*. 2009; 138:525–36. <https://doi.org/10.1016/j.cell.2009.05.031> PMID: 19665974
62. Markkanen M, Ludwig A, Khirouq S, Pryazhnikov E, Soni S, Khirouq L, et al. Implications of the N-terminal heterogeneity for the neuronal K-Cl cotransporter KCC2 function. *Brain research*. 2017; 1675:87–101. <https://doi.org/10.1016/j.brainres.2017.08.034> PMID: 28888841
63. De los Heros P, Alessi DR, Gourlay R, Campbell DG, Deak M, Macartney TJ, et al. The WNK-regulated SPAK/OSR1 kinases directly phosphorylate and inhibit the K⁺-Cl⁻ co-transporters. *Biochem J*. 2014; 458:559–73. <https://doi.org/10.1042/BJ20131478> PMID: 24393035
64. Moore YE, Deeb TZ, Chadchankar H, Brandon NJ, Moss SJ. Potentiating KCC2 activity is sufficient to limit the onset and severity of seizures. *Proceedings of the National Academy of Sciences*. 2018; 115(40):10166–71.
65. Titz S, Sammler EM, Hormuzdi SG. Could tuning of the inhibitory tone involve graded changes in neuronal chloride transport? *Neuropharmacology*. 2015.
66. Friedel P, Kahle KT, Zhang J, Hertz N, Pisella LI, Buhler E, et al. WNK1-regulated inhibitory phosphorylation of the KCC2 cotransporter maintains the depolarizing action of GABA in immature neurons. *Sci Signal*. 2015; 8(383):ra65–ra. <https://doi.org/10.1126/scisignal.aaa0354> PMID: 26126716
67. Inoue K, Furukawa T, Kumada T, Yamada J, Wang T, Inoue R, et al. Taurine inhibits K⁺-Cl⁻ cotransporter KCC2 to regulate embryonic Cl⁻ homeostasis via With-no-lysine (WNK) protein kinase signaling pathway. *Journal of Biological Chemistry*. 2012; 287(25):20839–50. <https://doi.org/10.1074/jbc.M111.319418> PMID: 22544747
68. Hartmann A-M, Nothwang HG. Molecular and evolutionary insights into the structural organization of cation chloride cotransporters. *Frontiers in cellular neuroscience*. 2014; 8.
69. Richardson C, Alessi DR. The regulation of salt transport and blood pressure by the WNK-SPAK/OSR1 signalling pathway. *Journal of cell science*. 2008; 121(20):3293–304.
70. Thastrup JO, Rafiqi FH, Vitari AC, Pozo-Guisado E, Deak M, Mehellou Y, et al. SPAK/OSR1 regulate NKCC1 and WNK activity: analysis of WNK isoform interactions and activation by T-loop trans-autophosphorylation. *Biochemical Journal*. 2012; 441(1):325–37. <https://doi.org/10.1042/BJ20111879> PMID: 22032326
71. Geng Y, Hoke A, Delpire E. The Ste20 Kinases SPAK and OSR1 regulate NKCC1 function in sensory neurons. *The Journal of Biological Chemistry*. 2009; 284:14020–8.
72. Gagnon KB, England R, Delpire E. A single binding motif is required for SPAK activation of the Na-K-2Cl cotransporter. *Cellular Physiology and Biochemistry*. 2007; 20(1–4):131–42. <https://doi.org/10.1159/000104161> PMID: 17595523
73. Moriguchi T, Urushiyama S, Hisamoto N, Iemura S-I, Uchida S, Natsume T, et al. WNK1 regulates phosphorylation of Cation-Chloride-coupled Cotransporters via the STE20-related kinases, SPAK and OSR1. *Journal of Biological Chemistry*. 2005; 280(52):42685–93. <https://doi.org/10.1074/jbc.M510042200> PMID: 16263722
74. Vitari AC, Thastrup J, Rafiqi FH, Deak M, Morrice Na, Karlsson H, K., et al. Functional interactions of the SPAK/OSR1 kinases with their upstream activator WNK1 and downstream substrate NKCC1. *Biochemical Journal* 2006; 397:223–31. <https://doi.org/10.1042/BJ20060220> PMID: 16669787
75. Darman RB, Forbush B. A regulatory locus of phosphorylation in the N terminus of the Na-K-Cl cotransporter, NKCC1. *Journal of Biological Chemistry*. 2002; 277(40):37542–50. <https://doi.org/10.1074/jbc.M206293200> PMID: 12145304
76. Dowd BFX, Forbush B. PASK (Proline-Alanine-rich-related Kinase), a regulatory kinase of the Na-K-Cl cotransporter (NKCC1). *The Journal of Biological Chemistry*. 2003; 278(30):27347–53. <https://doi.org/10.1074/jbc.M301899200> PMID: 12740379

77. Lee HHC, Walker JA, Jeffrey RW, Goodier RJ, Payne JA, Moss SJ. Direct PKC-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter, KCC2. 2007.
78. Buerli T, Pellegrino C, Baer K, Lardi-Studler B, Chudotvorova I, Fritschy J-M, et al. Efficient transfection of DNA or shRNA vectors into neurons using magnetofection. *Nature protocols*. 2007; 2(12):3090. <https://doi.org/10.1038/nprot.2007.445> PMID: 18079708
79. Hartmann A-M, Pisella LI, Medina I, Nothwang HG. Molecular cloning and biochemical characterization of two cation chloride cotransporter subfamily members of *Hydra vulgaris*. *PloS one*. 2017; 12(6): e0179968. <https://doi.org/10.1371/journal.pone.0179968> PMID: 28662098
80. Hartmann A-M, Wenz M, Mercado A, Störger C, Mount DB, Friauf E, et al. Differences in the large extracellular loop between the K⁺-Cl⁻ cotransporters KCC2 and KCC4. *The Journal of Biological Chemistry*. 2010; 285(31):23994–4002. <https://doi.org/10.1074/jbc.M110.144063> PMID: 20516068
81. Wisniewski JR. Quantitative Evaluation of Filter Aided Sample Preparation (FASP) and Multienzyme Digestion FASP Protocols. *Analytical chemistry*. 2016; 88(10):5438–43. <https://doi.org/10.1021/acs.analchem.6b00859> PMID: 27119963
82. Wisniewski JR, Gaugaz FZ. Fast and sensitive total protein and Peptide assays for proteomic analysis. *Analytical chemistry*. 2015; 87(8):4110–6. <https://doi.org/10.1021/ac504689z> PMID: 25837572
83. Wisniewski JR, Nagaraj N, Zougman A, Gnad F, Mann M. Brain phosphoproteome obtained by a FASP-based method reveals plasma membrane protein topology. *Journal of proteome research*. 2010; 9(6):3280–9. <https://doi.org/10.1021/pr1002214> PMID: 20415495
84. Wisniewski JR, Mann M. Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Analytical chemistry*. 2012; 84(6):2631–7. <https://doi.org/10.1021/ac300006b> PMID: 22324799
85. Pinkse MW, Uitto PM, Hilhorst MJ, Ooms B, Heck AJ. Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns. *Analytical chemistry*. 2004; 76(14):3935–43. <https://doi.org/10.1021/ac0498617> PMID: 15253627
86. Lytle C, McManus T. Coordinate modulation of Na-K-2Cl cotransport and K-Cl cotransport by cell volume and chloride. *American Journal of Physiol Cell Physiology*. 2002; 283:1422–31.
87. Gagnon É, England R, Delpire E. Volume sensitivity of cation-Cl⁻ cotransporter is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. *American Journal of Physiol Cell Physiol*. 2005a; 290:134–42.
88. Delpire E, Days E, Lewis LM, Mi D, Kim K, Lindsley CW, et al. Small-molecule screen identifies inhibitors of the neuronal K-Cl cotransporter KCC2. *PNAS*. 2009; 106(13):5383–8. <https://doi.org/10.1073/pnas.0812756106> PMID: 19279215
89. Gillen CM, Brill S, Payne JA, Forbush B. Molecular Cloning and Functional Expression of the K-Cl Cotransporter from Rabbit, Rat and Human. *The Journal of Biological Chemistry*. 1996; 271:16237–44.
90. Culliford SJ, Ellory JC, Lang H-J, Englert H, Staines HM, Wilkins RJ. Specificity of classical and putative Cl⁻-transport inhibitors on membrane transport pathways in human erythrocytes. *Cellular Physiology and Biochemistry*. 2003; 13(4):181–8. <https://doi.org/10.1159/000072420> PMID: 12876375
91. Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, et al. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic acids research*. 2011; 40(D1):D261–D70.
92. Bergeron MJ, Gagnon KBE, Caron L, Isenring P. Identification of Key functional domains in the C Terminus of the K⁺-Cl⁻ cotransporters. *J Biol Chem*. 2006; 281:15959–69. <https://doi.org/10.1074/jbc.M600015200> PMID: 16595678
93. Vitari AC, Deak M, Morrice NA, Alessi DR. The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. *Biochemical Journal*. 2005; 391(1):17–24.
94. Zagórska A, Pozo-Guisado E, Boudeau J, Vitari AC, Rafiqi FH, Thastrup J, et al. Regulation of activity and localization of the WNK1 protein kinase by hyperosmotic stress. *The Journal of cell biology*. 2007; 176(1):89–100. <https://doi.org/10.1083/jcb.200605093> PMID: 17190791
95. Uchida S, Sohara E, Rai T, Sasaki SJBotC. Regulation of with-no-lysine kinase signaling by Kelch-like proteins. 2014; 106(2):45–56.
96. Smith L, Smallwood N, Altman A, Liedtke CM. PKC δ acts upstream of SPAK in the activation of NKCC1 by hyperosmotic stress in human airway epithelial cells. *Journal of Biological Chemistry*. 2008; 283(32):22147–56. <https://doi.org/10.1074/jbc.M801752200> PMID: 18550547
97. Del Castillo IC, Fedor-Chaikin M, Song JC, Starlinger V, Yoo J, Matlin KS, et al. Dynamic regulation of Na⁺-K⁺-2Cl⁻ cotransporter surface expression by PKC- ϵ in Cl⁻-secretory epithelia. *American Journal of Physiology-Cell Physiology*. 2005; 289(5):C1332–C43. <https://doi.org/10.1152/ajpcell.00580.2004> PMID: 16000638

98. Palmer R, Ridden J, Parker P. Identification of multiple, novel, protein kinase C-related gene products. *FEBS letters*. 1994; 356(1):5–8. [https://doi.org/10.1016/0014-5793\(94\)01202-4](https://doi.org/10.1016/0014-5793(94)01202-4) PMID: 7988719
99. Webb BL, Hirst SJ, Giembycz MA. Protein kinase C isoenzymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis. *British journal of pharmacology*. 2000; 130(7):1433–52. <https://doi.org/10.1038/sj.bjp.0703452> PMID: 10928943
100. Parker PJ, Murray-Rust J. PKC at a glance. *Journal of cell science*. 2004; 117(2):131–2.
101. Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chemical reviews*. 2001; 101(8):2353–64. <https://doi.org/10.1021/cr0002801> PMID: 11749377
102. Flemmer AW, Gimenez I, Dowd BFX, Darman RB, Forbush B. Activation of the Na-K-Cl cotransporter NKCC1 detected with a Phospho-specific antibody. *The Journal of Biological Chemistry*. 2002; 277(40):37551–8. <https://doi.org/10.1074/jbc.M206294200> PMID: 12145305
103. Darman RB, Flemmer A, Forbush B. Modulation of ion transport by direct targeting of protein phosphatase type 1 to the Na-K-Cl cotransporter. *The Journal of Biological Chemistry*. 2001; 276(37):34359–62. <https://doi.org/10.1074/jbc.C100368200> PMID: 11466303
104. Lee HH, Deeb TZ, Walker JA, Davies PA, Moss SJ. NMDA receptor activity downregulates KCC2 resulting in depolarizing GABA A receptor-mediated currents. 2011; 14(6):736.
105. Salzer E, Santos-Valente E, Keller B, Warnatz K, Boztug K, Joci. Protein kinase C δ : a gatekeeper of immune homeostasis. 2016; 36(7):631–40.
106. Cheng N, He R, Tian J, Dinauer MC, Richard DY. A critical role of protein kinase C δ activation loop phosphorylation in formyl-methionyl-leucyl-phenylalanine-induced phosphorylation of p47phox and rapid activation of nicotinamide adenine dinucleotide phosphate oxidase. 2007; 179(11):7720–8.
107. Stempka L, Girod A, Müller H-J, Rincke G, Marks F, Gschwendt M, et al. Phosphorylation of Protein Kinase C δ (PKC δ) at Threonine 505 is not a prerequisite for enzymatic activity expression of rat pkc δ and an alanine 505 mutant in bacteria in a functional form. 1997; 272(10):6805–11. <https://doi.org/10.1074/jbc.272.10.6805> PMID: 9045715
108. Kahle KT, Deeb TZ, Puskarjov M, Silayeva L, Liang B, Kaila K, et al. Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter KCC2. *Trends in neurosciences*. 2013; 36(12):726–37. <https://doi.org/10.1016/j.tins.2013.08.006> PMID: 24139641
109. Kahle KT, Delpire E. Kinase-KCC2 coupling: Cl⁻ rheostasis, disease susceptibility, therapeutic target. *Journal of neurophysiology*. 2015; 115(1):8–18. <https://doi.org/10.1152/jn.00865.2015> PMID: 26510764
110. Kahle KT, Rinehart J, Lifton RP. Phosphoregulation of the Na-K-2Cl and K-Cl cotransporters by the WNK kinases. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*. 2010; 1802(12):1150–8.
111. Silayeva L, Deeb TZ, Hines RM, Kelley MR, Munoz MB, Lee HH, et al. KCC2 activity is critical in limiting the onset and severity of status epilepticus. *Proceedings of the National Academy of Sciences*. 2015; 112(11):3523–8.
112. Naik MU, Benedikz E, Hernandez I, Libien J, Hrabec J, Valsamis M, et al. Distribution of protein kinase M ζ and the complete protein kinase C isoform family in rat brain. *Journal of Comparative Neurology*. 2000; 426(2):243–58. [https://doi.org/10.1002/1096-9861\(20001016\)426:2<243::aid-cne6>3.0.co;2-8](https://doi.org/10.1002/1096-9861(20001016)426:2<243::aid-cne6>3.0.co;2-8) PMID: 10982466
113. Liu W, Heckman C. The sevenfold way of PKC regulation. *Cellular signalling*. 1998; 10(8):529–42. [https://doi.org/10.1016/s0898-6568\(98\)00012-6](https://doi.org/10.1016/s0898-6568(98)00012-6) PMID: 9794251
114. Lauf PK, Adragna NC. K-Cl cotransport; properties and molecular mechanism. *Cellular Physiology and Biochemistry*. 2000; 10:341–54. <https://doi.org/10.1159/000016357> PMID: 11125215
115. Yassin L, Radtke-Schuller S, Asraf H, Grothe B, Hershinkel M, Forsythe ID, et al. Nitric oxide signaling modulates synaptic inhibition in the superior paraolivary nucleus (SPN) via cGMP-dependent suppression of KCC2. 2014; 8:65.
116. Chew TA, Orlando BJ, Zhang J, Latorraca NR, Wang A, Hollingsworth SA, et al. Structure and mechanism of the cation-chloride cotransporter NKCC1. 2019; 572(7770):488–92.
117. Liu S, Chang S, Han B, Xu L, Zhang M, Zhao C, et al. Cryo-EM structures of the human cation-chloride cotransporter KCC1. 2019; 366(6464):505–8.
118. Delpire E, Guo JJAJoP-CP. Cryo-EM structures of Dr NKCC1 and hKCC1: a new milestone in the physiology of cation-chloride cotransporters. 2020; 318(2):C225–C37.
119. Yang X, Wang Q, Cao EJNC. Structure of the human cation-chloride cotransporter NKCC1 determined by single-particle electron cryo-microscopy. 2020; 11(1):1–11.