Title: WNK1-regulated inhibitory phosphorylation of KCC2 maintains depolarizing GABA activity in immature neurons

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ABSTRACT

Activation of Cl^--permeable $GABA_A$ receptors elicits synaptic inhibition in mature neurons but excitation in immature neurons, which is essential for brain maturation. This developmental "switch" in GABA function is dependent on a post-natal KCC2 cotransporter-mediated decrease in intraneuronal Cl^- $[Cl^-]_i$, but the mechanisms regulating KCC2 in immature neurons are poorly understood. Here, we showed the serine-threonine kinase WNK1 forms a physical complex with KCC2 in the developing mouse brain, and dominant-negative mutation, genetic depletion, or chemical inhibition of WNK1 in immature neurons is sufficient to trigger a hyperpolarizing shift in GABA activity by facilitating KCC2-mediated Cl^- extrusion. These effects resulted from removal of KCC2 phosphorylation at Thr^{906} and Thr^{1007}, a critical inhibitory motif of KCC2 activity we showed to be significantly up-regulated in immature neurons. Together, these data provide insights into the mechanism regulating Cl^- homeostasis in immature neurons, and suggest changes in the WNK1-regulated inhibitory phosphorylation of KCC2 might play a role in the GABA excitatory/inhibitory developmental sequence.

One Sentence Summary: We elucidate a novel WNK1 kinase-dependent mechanism that regulates KCC2-mediated Cl^- homeostasis and GABA activity in immature neurons.

INTRODUCTION

Intracellular Cl^- concentration $[Cl^-]_i$ is precisely regulated to maintain cell volume (*1*), drive transepithelial transport (*2*), and modulate neuronal excitability (*3*). Mechanisms that sense alterations in $[Cl^-]_i$ and transduce these signals to plasmalemmal Cl^- transporting molecules are critical to maintain Cl^- homeostasis, and are required for cell and organismal survival (*4*). While the proteins mediating Cl^- transport including channels, transporters, and exchangers are now largely known (*5*), the molecules and pathways that regulate them to establish context-specific activity are incompletely characterized.

Human and mouse genetics have unequivocally demonstrated that the *SLC12A* family of cation-Cl^- cotransporters (CCCs), including the Cl^--intruding Na^+-K^+-$2Cl^-$ cotransporters (N[K]CCs) and Cl^--extruding K^+-Cl^- cotransporters (KCCs), are primary determinants of $[Cl^-]_i$ in multiple cell types (*2*, *3*, *6*). The WNK (*w*ith *n*o lysine [*K*])-SPAK (SPS1-related proline/alanine-rich kinase) serine-threonine kinases are the master regulators of these Cl^- transporters across evolution (*7*). While the WNK-SPAK signaling pathway has been extensively characterized in epithelial tissues active in water and solute transport, such as the kidney's distal nephron (*8–11*), its role in the central nervous system (CNS) is not well understood.

The emergence of inhibitory GABAergic signaling in the developing CNS demonstrates how changes in $[Cl^-]_i$ can modulate GABA activity and consequently, the function of neurons and circuits. $GABA_A$ receptors ($GABA_ARs$) are ligand-gated, Cl^--permeable ion channels that allow the bidirectional flux of Cl^- ions, the direction of which is dictated by $[Cl^-]_i$ and the membrane potential (*12*). In the adult brain, $GABA_AR$ activation triggers membrane hyperpolarization and synaptic inhibition. Conversely, in the developing brain, $GABA_AR$ activation triggers depolarization and in some instances excitation, which is critical for neuronal proliferation and migration, and synaptogenesis (*13*). This developmental

"switch" in GABA function from excitatory to inhibitory has been attributed to a difference in the $[Cl^-]_i$ of immature (15 - 20 mM) versus mature neurons (~4 mM), which results from a KCC2-dependent increase in neuronal Cl^- extrusion beginning in the first week after birth in mice and rats (*14*).

KCC2 protein levels increase during development in some neuronal populations (*15*), but it is unclear if protein level alone, versus changes in the functional regulation of the transporter, explain the overall increase in KCC2 activity (*16*). In hippocampi and cortices of both rats and mice, KCC2 protein expression begins in late embryonic stages and increases progressively during the first post-natal week (*14*, *15*, *17*, *18*). However, the first signs of KCC2 function are not detectable until post-natal days 5-6 in hippocampi and days 6-8 in cortex (*15*, *18–20*), and GABA remains depolarizing until post-natal days 8-13 (*21*, *22*). Discordance between the level of KCC2 protein expression and KCC2 activity have also been reported in cultured immature hippocampal neurons (*19*). Together, these data suggest other regulatory factors may contribute to maintaining low KCC2 activity in immature neurons in the developing brain. Recent work has demonstrated that phosphorylation can significantly alter KCC2 activity, neuronal $[Cl^-]_i$, and GABA reversal potential (E_{GABA}) (*16*, *23–26*). It has been found also that the level of KCC2 phosphorylation (residue Thr^{906}) is relatively high in immature brain and decreases progressively during neuronal development (*16*).

The Cl^--sensitive (*27*) WNK kinases regulate the phosphorylation state and the associated activity of the CCCs either directly or via SPAK or the related oxidative-stress responsive-1 protein (OSR1) kinases (*7*, *28*). Mutations in genes that regulate the proteolytic degradation of the WNKs (CUL3 and KLCH3) (*29*), the WNKs themselves (WNK1 and WNK4) (*30*), or WNK targets (NCC and NKCC2, which are kidney-specific CCCs) (*31*) are all mutated in Mendelian forms of renal electrolyte imbalance and blood pressure

dysregulation due to dysregulated Cl^- reabsorption (along with Na^+ and/or K^+) in the kidney's nephron (*7*). However, WNKs (including WNK2 and WNK3) are highly-expressed outside the kidney, including the developing and mature CNS (*32–34*), and each gene encodes multiple isoforms, some of which exhibit remarkable specificity in the brain or spinal cord (*35*, *36*). The function of the WNK kinase pathway in the CNS, however, is largely unknown. Interestingly, mutations in WNK1 (WNK1/HSN2), one WNK1 isoform with particularly robust CNS expression, causes a severe autosomal recessive disease in humans characterized by congenital insensitivity to pain (OMIM# 201300 (*35*)), suggesting an essential but yet undefined role for the WNK kinases in the human CNS.

Given the expression of the WNK kinases in the CNS, their role in regulating the CCCs in other tissues (including the CNS of lower organisms (*37*)), and the conservation of phospho-regulatory mechanisms of all CCC family members (*7*), WNKs are compelling candidate regulators of neuronal Cl^- homeostasis via KCC2. One family member, WNK1, is ubiquitously-expressed with multiple tissue-specific isoforms (*36*), including a CNS isoform that causes a Mendelian syndrome of congenital pain insensitivity (*35*). *WNK1* transcripts are expressed in the developing CNS, including the CA1, CA2 and CA3 areas of the hippocampus (*36*). WNK kinases potently inhibit KCCs in oocytes, but activate the KCCs when inhibited (*33*); WNK1 is required for KCC3 phosphorylation in HEK-293 cells (*16*); and WNK1 kinase activity is regulated by changes in $[Cl^-]_i$ (*27*).

Here, we elucidate a mechanism mediated by WNK1 that modulates GABA activity in immature neurons via the regulated inhibitory phosphorylation of the Cl^--extruding KCC2 cotransporter. Antagonism of WNK1 expression or activity significantly enhances KCC2-dependent Cl^--extrusion, lowers $[Cl^-]_i$, and is sufficient to cause a ~15 mV hyperpolarizing shift of the E_{GABA}. Our data suggest WNK1, complementing other mechanisms that regulate

123 gene expression (*14*), contributes to the depolarizing action of GABA in immature neurons
124 by promoting the inhibitory phosphorylation of KCC2 at Thr^{906} and Thr^{1007}.
125

RESULTS

WNK1 kinase inhibition facilitates KCC2-dependent Cl^- extrusion and causes a hyperpolarizing shift in E_{GABA} in immature neurons

We tested whether WNK1 regulates KCC2-dependent neuronal Cl^- homeostasis by expressing constitutively-active (CA, S382E) or a kinase-dead dominant-negative (DN, D368A) WNK1 mutants (*24*), (herein termed "WNK1-CA" and "WNK1-DN", respectively) in cultured hippocampal neurons of different days in vitro (div) and measured the E_{GABA} using gramicidin-perforated patch clamp recordings (see Materials and Methods), which can be used to calculate $[Cl^-]_i$ (**Figure 1A**). Since WNK1 can potentially regulate the activity of Cl^--intruder NKCC1 (*38*), which is also expressed in neurons (*3*), all measurements of E_{GABA} were performed in presence of bumetanide, a relatively specific inhibitor of NKCC1 at low concentrations (10 μM). In our preparations of cultured hippocampal neurons, 10 μM bumetanide produced a 5 mV negative shift of E_{GABA} in immature neurons (6-7 div), and an 8 mV negative shift in more mature cells (13-15 div) **(Figure 1B)**.

In immature neurons, genetic silencing of WNK1 using specific shRNAs (**Supplementary Figure 1**) or dominant negative WNK1-DN produced a highly reproducible and significant ~15 mV hyperpolarizing shift of E_{GABA} from -57.9+/-1.5 mV (n=14) to -73.1+/-2.7 mV (n=11) and -76.4+/-2.3 mV (n=17), respectively **(Figure 1C,** columns 1, 2 and 3**)**. The effect of WNK1 shRNA was specific, as it was rescued by the expression of a shRNA-resistant WNK1-CA mutant (**Figure 1C,** column 4). Critically, the hyperpolarizing shift of E_{GABA} associated with shRNA-mediated WNK1 knockdown was dependent on KCC2 expression, since neurons co-expressing WNK1 shRNA and KCC2 shRNA failed to elicit a E_{GABA} depolarization (**Figure 1C,** column 5).

Conversely, constitutive activation of WNK1 in 6-7 div neurons achieved via expression of WNK1-CA did not produce a detectable change of E_{GABA} as compared to

control neurons transfected with scrambled shRNA (**Figure 1C,** columns 1 and 6**)**. These values were reminiscent of those recorded from immature neurons with knocked-down KCC2 (-53.7±1.9 mV, **Figure 1C,** column 7**)**. These results show antagonism of WNK1 in immature neurons elicits a KCC2-dependent hyperpolarizing shift of E_{GABA}, whereas constitutive WNK1 activation has no detectable effect.

In more mature neurons (13-15 div) that were characterized by significantly more hyperpolarized E_{GABA} values (-83.1±1.7 mV, n=9, **Figure 1C,** column 1**),** the expression of WNK1 shRNA or WNK1-DN did not elicit a further hyperpolarization of E_{GABA}; the mean±SEM values of WNK1 shRNA or WNK1-DN cells did not differ statistically from those transfected with scrambled shRNA (**Figure 1C,** columns 1, 2 and 3**)**. Conversely, mature neurons (13-15 div) expressing WNK1-CA produced a depolarizing shift of E_{GABA} to -62.4±3.7 mV (**Figure 1C,** column 6**)**; these values were, however, more negative that those measured in neurons with knocked-down KCC2 (-54.5±1.9 mV, p=0.02, n=12, **Figure 1C,** column 7**)**.

Consistent with a potentiating effect of WNK1 knockdown on Cl^- extrusion, analysis of the amplitudes of $GABA_AR$ responses in immature neurons, recorded at a fixed membrane potential of -80 mV after isoguvacine application, an agonist of $GABA_A$ receptor, revealed a shortened time of recovery after Cl^- loading in neurons expressing WNK1 shRNA, an effect rescued by expression of shRNA-resistant WNK1-CA **(Figures 1D and 1E).**

Taken together, the results illustrated in **Figure 1** suggested that endogenous WNK1 contributes to the KCC2-dependent control of Cl^- homeostasis in immature (6-7 div), but not more mature (13-15 div) cultured hippocampal neurons.

In utero inhibition of WNK1 lowers neuronal $[Cl^-]_i$ and causes a hyperpolarizing shift in GABA activity

Are the electrophysiological findings above relevant *in vivo*? We next studied whether WNK1 knockdown in immature neurons *in utero* affects $[Cl^-]_i$ and GABA activity. Constructs encoding Cl-Sensor plus a scrambled shRNA (control) or Cl-Sensor plus WNK1 shRNA were electroporated *in utero* in rats at E15 (see Materials and Methods for details). Transverse slices of cortex were prepared on postnatal days P3-P5 and P30. Electroporated slices harbored hundreds of neurons expressing Cl-Sensor in cortical layers III-V (**Figure 2A**), which exhibited variable absolute values of $R_{430/500}$ (**Figure 2B and 2C)**. Cl-Sensor does not allow measurement of the exact concentration of $[Cl^-]_i$ due to its sensitivity to $[H^+]$ and some organic ions; however, it does allow the detection of even weak (2-4 mM) oscillations of resting $[Cl^-]_i$ (e.g., in response to $GABA_AR$ or glycine receptor-channel activation), and is suitable for measurements of neuronal Cl^- extrusion capacity *(39)*. We therefore avoided using ratiometric Cl-Sensor recordings to measure $[Cl^-]_i$ or the magnitude of $[Cl^-]_i$ changes, and designed all experiments to determine the *directionality* of Cl^--dependent fluorescence changes in response to $GABA_AR$ activation, thereby analyzing the *kinetics* of fluorescence recovery after neuronal Cl^- loading.

Consistent with previously-described age-dependent depolarizing and hyperpolarizing actions of GABA in, respectively, immature and mature neurons (*39*), brief exposure of brain slices from control mice (animals electroporated with Cl-Sensor and scrambled shRNA) to isoguvacine (30 μM, 3 minutes, min) produced bi-directional fluorescence responses dependent on the age of the animal: in slices from immature P3-P5 mice, most of neurons (80%) exhibited an uniform decrease of $R_{430/500}$ (indicated with arrow in **Figure 2B**), reflecting an outward direction of Cl^- flux characteristic of GABA depolarizing activity. In slices from more mature (P30) animals, similar isoguvacine applications elicited no change (~60% of neurons) or an increase of $R_{430/500}$ (indicated with arrow in **Figure 2F**), reflecting either Cl^- equilibrium or Cl^- influx, typical for GABA hyperpolarizing activity.

To test whether WNK1 knockdown affects Cl^- extrusion efficacy in this model, we applied artificial cerebrospinal fluid (ACSF) solution containing 25 mM KCl (to depolarize neurons) and 30 μM isoguvacine (to load neurons with Cl^- via $GABA_AR$ activation) in P3-P5 and P30 slice preparations as above. This protocol produced a robust increase of $R_{430/500}$, reflecting a rise in neuronal $[Cl^-]_i$ that recovered progressively to the control values after isoguvacine washout (**Figure 2B and 2F**). In immature P3-P5 slices expressing WNK1 shRNA, the half-recovery time was significantly shorter than those measured in slices expressing scrambled control shRNA (**Figure 2B-2E**). In contrast, WNK1 knockdown did not affect the half-recovery time of the fluorescence after imposed Cl^- overload in P30 slices (**Figure 2F-2I**). These results suggest that *in utero* WNK1 knockdown facilitates KCC2-dependent neuronal Cl^- extrusion and causes a hyperpolarizing shift in GABA activity in immature but not mature neurons.

The absolute mean values of $R_{430/500}$ were higher in immature than mature slices (compare **Figures 2B-2C versus 2F-2G),** which is in agreement with the developmental profile of the resting neuronal $[Cl^-]_i$ and the $R_{430/500}$ half-recovery times after Cl-overload measured in immature slices that were unexpectedly faster than those measured in more mature slices (3.1±0.1 min versus 4.6±0.2 min, $p<0.01$, Figures 2E and I). The reason such slower fluorescence recovery times were documented in mature slices is unclear; we hypothesize the tissue in mature slices is denser and, therefore, the time of KCl wash-out from the extracellular space is longer. Consequently, residual KCl in extracellular space could maintain KCC2 in reverse or close to zero transport mode (*40*), and, thus, slow Cl-extrusion. Since the main purpose of our study was to compare Cl- extrusion between two sets of slices from the same littermate animals, the different age-dependent kinetics of Cl-recovery are not critical for the given study, but certainly will be a subject of future detailed investigations.

KCC2 harbors significantly more inhibitory phosphorylation at Thr^{906} and Thr^{1007} in immature versus mature neurons

How does WNK1 regulate KCC2 activity in immature neurons? KCC2 exhibits significantly more inhibitory phosphorylation of Thr^{906} in the immature whole mouse brain versus the adult brain in a temporal sequence that parallels the developmental increase in KCC2 activity (*16*, *26*). Given the stimulatory effect of WNK1 antagonism on KCC2-mediated Cl^-extrusion and keeping with previous suggestion of Inoue et al., (*24*) on the potential involvement of WNK1 in control of KCC2, we speculated WNK1 inhibition activates KCC2 by relieving KCC2 Thr^{906} and Thr^{1007} inhibitory phosphorylation.

We assayed KCC2 Thr^{906} and Thr^{1007} phosphorylation in dissociated rat hippocampal and cortical neurons that grow *in vitro* 6-7 and 14-15 days in the same conditions used for physiological experiments above. Briefly, we purified KCC2 from neurons using anti-KCC2 antibody, and probed the resulting immunoprecipitates using phosphor-specific antibodies that recognize Thr^{906} or Thr^{1007} (*26*) **(Figure 3A)**. Phosphorylation at both Thr^{906} and Thr^{1007} is significantly elevated in 6-7 div neurons compared to 14-15 div neurons. We calculated the phospho-KCC2: total KCC2 ratio at each time point, and this revealed a 2-6 fold increase in P-Thr^{906}, and 5-12 fold increase in P-Thr^{1007}, in 6-7 div versus 14-15 div neurons (**Figure 3B**). We also measured the total levels, and phosphorylation status of WNK1 and SPAK at residues required for the activation of these enzymes (*26*), in the same cultures **(Figure 3A and 3B)**. Phosphorylation of WNK1 Ser^{382} and SPAK/OSR1 Ser^{373}/Ser^{325} were also significantly elevated in 6-7 div neurons compared to 14-15 div neurons. These results show that KCC2 Thr^{906} and Thr^{1007} inhibitory phosphorylation, and WNK1 pathway activating phosphorylation, is elevated in immature neurons relative to mature neurons.

Alteration of KCC2 Thr^{906}/Thr^{1007} phosphorylation modulates KCC2 function and GABA activity

Does modulation of KCC2 Thr^{906} and Thr^{1007} phosphorylation affect KCC2 activity in immature neurons? Mutations mimicking KCC2 phosphorylation at Thr^{906} and Thr^{1007} (T906E/T1007E) partially inhibit KCC2 activity in HEK-293 cells (*16*, *26*) and neuronal cells electroporated *in utero* (*24*), whereas mutations mimicking de-phosphorylation (T906A/T1007A) activate KCC2 (*16*, *24*, *26*). We validated that genetic mutation preventing or mimicking KCC2 phosphorylation at these sites alters KCC2 activity in cultured hippocampal neurons. We engineered non-phosphorylatable T906A/T1007A (KCC2$^{A/A}$) and phospho-mimetic T906E/T1007E (KCC2$^{E/E}$) KCC2 mutants in an shRNA-resistant KCC2 expression plasmid enabling KCC2 mutant protein expression in the context of endogenous *KCC2* depletion, achieved via a previously characterized rat anti-KCC2 shRNA (*41*). Using this experimental scheme, expression of KCC2$^{A/A}$ in 10 div cultured rat hippocampal neurons elicited a strong -14 mV hyperpolarizing shift of E_{GABA} compared to neurons expressing KCC2$^{E/E}$ (**Figure 4A**). Although KCC2$^{E/E}$ was less active than KCC2$^{A/A}$, KCC2$^{E/E}$ still triggered a significant hyperpolarizing shift of E_{GABA} relative to control cells with depleted KCC2, suggesting T906E/T1007E phosphorylation decreases, but does not eliminate transporter activity, consistent with previous reports (*16*, *24*). The E_{GABA} of neurons expressing WT KCC2 had intermediate values between KCC2$^{E/E}$ and KCC2$^{A/A}$ (**Figure 4A**).

KCC2 Thr^{906} and Thr^{1007} phosphorylation regulates cotransporter activity by altering its surface expression

How does KCC2 Thr^{906} and Thr^{1007} phosphorylation alter transporter activity? Phosphorylation could affect the intrinsic transport activity of molecules, or alternatively, could affect the transport of functional molecules to or from the cell surface. We assessed the

surface expression of WT KCC2, KCC2$^{E/E}$ and KCC2$^{A/A}$ using a previously described KCC2 construct that harbors a tag (pHluorin) in an external loop of KCC2 (KCC2-pH$_{ext}$, (*42*)). The multi-step immunolabelling protocol of living 10 div cultured hippocampal neurons expressing KCC2-pH$_{ext}$ mutants allowed visualization of the total KCC2-pH$_{ext}$ protein (F$_t$, total fluorescence), transporter expressed at the cell surface (F$_m$, membrane fluorescence), and transporter internalized during 2-hour period of incubation with primary anti-GFP antibody (F$_i$, internalized fluorescence, see **Figure 4B** for scheme and Materials and Methods for details). Analysis revealed that while the levels of the intracellular expression of WT KCC2-pH$_{ext}$ and its KCC2$^{E/E}$-pH$_{ext}$ and KCC2$^{A/A}$-pH$_{ext}$ mutants were similar **(Figure 4C and 4D)**, the intensity of the surface-expressed pool of KCC2 Thr^{906} and Thr1007 mutants strongly differed from that of WT KCC2. The phospho-mimetic KCC2$^{E/E}$-pH$_{ext}$ mutant showed an almost three fold lower level of the surface expression than the non-phosphorylatable KCC2$^{A/A}$-pH$_{ext}$ transporter mutant. Similar to electrophysiology studies shown in **Figure 4A**, WT KCC2-pH$_{ext}$ exhibited intermediate values. Analysis of the fluorescence intensities emitted by internalized clusters in neurons expressing phospho-mimetic KCC2$^{E/E}$-pH$_{ext}$ revealed a prominent accumulation of latter in the region of soma, whereas the non-phosphorylatable KCC2$^{A/A}$-pH$_{ext}$ mutant showed much less internalized clusters distributed along the length of dendrites **(Figure 4C).** Neurons expressing WT KCC2-pH$_{ext}$ exhibited an intermediate pattern of staining. Overall, the relative F$_i$ signal from KCC2$^{E/E}$-pH$_{ext}$ expressing neurons was 4-fold stronger than KCC2$^{A/A}$-pH$_{ext}$ mutant. The mean values of F$_i$ in WT KCC2-pH$_{ext}$–expressing neurons were similar to those of KCC2$^{A/A}$-pH$_{ext}$ mutant, although they showed higher degree of variability **(Figure 4D)**. Related to cultured hippocampal neurons, in mouse Neuro2A (N2a) cells, immortalized neuronal-like cells without significant endogenous KCC2, expression of KCC2$^{E/E}$ exhibited a significantly lower Cl^--extrusion capacity, as measured using non-invasive Cl-Sensor (*43*), than cells expressing

KCC2$^{A/A}$ (**Figure 5A-5D**). The Cl^--extrusion ability of WT KCC2 in these cells was similar to that of KCC2$^{E/E}$ mutant (**Figure 5D**). Consistent with this finding, the phosphorylation of KCC2 at Thr^{906} and Thr^{1007}, assessed using specific phospho-antibodies that recognize these sites (*26*), revealed a robust level of baseline KCC2 Thr^{906} and Thr^{1007} phosphorylation (e.g., see **Figure 6F**). These data suggest KCC2 Thr^{906} and Thr^{1007} phosphorylation reduces transporter activity by enhancing its internalization rate and decreasing its surface expression

Catalytic inhibition of WNK1 activates KCC2 and decreases KCC2 inhibitory phosphorylation at Thr^{906} and Thr^{1007}

Does WNK1 modulate KCC2 activity by regulating Thr^{906} and Thr^{1007} phosphorylation? To test this hypothesis, we measured the rate of Cl-Sensor fluorescence recovery in N2a cells co-expressing WT KCC2 and WNK1-CA or WNK1-DN. Consistent with the robust phosphorylation of KCC2 in these cells (**Figure 6F**), WNK1-CA did not further affect the kinetics of fluorescence recovery, whereas WNK1-DN significantly reduced the half-recovery time of the ratiometric fluorescence from 5.5±1.0 min to 2.9±0.3 min (**Figure 5E)**. The effect of WNK1-DN was dependent on KCC2, because it was absent in cells expressing only WNK1-DN **(Figure 5F)**. Importantly, the effect of WNK1-DN was rescued by substituting expression of phospho-mimetic KCC2$^{E/E}$ instead of WT KCC2. These functional data suggest that WNK1-DN triggers KCC2-mediated Cl^--extrusion by preventing the inhibitory phosphorylation of KCC2 at Thr^{906} and Thr^{1007}.

We next utilized a chemical genetic approach (*44*, *45*) to test whether specific inhibition of WNK1 kinase activity alters the activity and phosphorylation of KCC2 (**Figure 6 and Supplementary Figure 2**). Many active site kinase inhibitors are relatively promiscuous due to residue conservation in the ATP binding site. The essential feature of the chemical genetic approach is that it combines a genetic change, in which a kinase-of-interest

can be mutated to generate a unique binding pocket, and a chemical one, in which the modified kinase is sensitized to inhibitor analogs that do not affect wild-type (WT) kinases. We created an analog-specific mutant of WNK1 (WNK1-AS) in which the "gatekeeper" amino acid residue (a large, conserved hydrophobic residue that lines the ATP-binding site, T^{301} in WNK1) was replaced by a smaller amino acid (e.g., alanine). This enlarges the ATP-binding pocket and allows the binding and utilization of ATP analogs modified with bulky substitutions (e.g., N^6-benzyl-ATP-γ-S), and also renders the kinase susceptible to inhibition by cell-permeable derivatives of the Src inhibitor PP1, like 1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-PP1 ("1-NA-PP1"), with high affinity and selectivity; see Materials and Methods for details (*44–46*).

WNK1-AS utilized N^6-benzyl-ATP-γ-S with much greater efficiency than WNK1-CA in autophosphorylation reactions (**Supplementary Figure 2A)** and catalyzed the transfer of benzyl-ATP-γ-S to a known WNK1 substrate, OSR1, (**Supplementary Figure 2B),** but failed to transfer benzyl-ATP-γ-S to KCC2 directly (**Supplementary Figure 2B)**, suggesting WNK1 does not directly phosphorylate KCC2. WNK1-AS was also inhibited by 1-NA-PP1 in a dose-dependent fashion (**Supplementary Figure 2C**). We exploited the sensitivity of WNK1-AS to inhibition with 1-NA-PP1 to test the effect of inhibiting WNK1 kinase activity on KCC2 function and Thr^{906} and Thr^{1007} phosphorylation (**Figures 6**). We transfected N2a cells with WNK1-AS or WNK1-CA and measured KCC2-mediated Cl^- extrusion in the presence or absence of 1-NA-PP1. The application of 1-NA-PP1 (10 μM for 10 min) to cells co-expressing KCC2 and WNK1-CA had no effect on the KCC2-dependent fluorescence recovery after loading with Cl^- **(Figure 6B and 6E)**. However, in cells co-expressing KCC2 and WNK-AS, 1-NA-PP1 produced a robust acceleration of KCC2-dependent fluorescence recovery by 3.4±0.9 min (**Figure 6C and 6E)**. Similar to experiments with WNK1-DN (see **Figure 5F**), KCC2 activation was reversed by substituting expression of phospho-mimetic

KCC2$^{E/E}$ for WT KCC2 (**Figure 6D and 6E)**. These experiments demonstrate that chemical inhibition of WNK1 kinase activity is sufficient to rapidly (within 10 min) stimulate KCC2 activity, and this effect is dependent on the dephosphorylation of KCC2 at Thr^{906} and Thr^{1007}.

To directly test whether WNK1 inhibition alters KCC2 phosphorylation at Thr^{906} and Thr^{1007}, we chemically inhibited WNK1-AS with 1-NA-PP1 and individually assessed the phosphorylation status of KCC2 at Thr^{906} and Thr^{1007} using phospho-specific antibodies that recognize these sites (*26*) (**Figure 6F**). Lysates from N2a cells co-expressing HA-tagged KCC2 and WNK1-CA or WNK1-AS, with or without exposure to 1-NA-PP1 (10 μM for 2 hours), were harvested, subjected to SDS-PAGE, and the phosphorylation status of Thr^{906} and Thr^{1007} was assessed by Western blotting with phospho-specific antibodies. Chemical inhibition of WNK1 activity resulted in a significant decrease in the phosphorylation of both Thr^{906} and Thr^{1007}, with a mildly stronger effect on the Thr^{906} residue (**Figure 6F**). This effect was not seen in cells expressing WNK1-AS without 1-NA-PP1 exposure, or in cells expressing WNK1-CA with 1-NA-PP1 exposure, suggesting a specific effect of inhibition of WNK1 catalytic activity. Together, these results show WNK1 regulates KCC2 Thr^{906} and Thr^{1007} inhibitory phosphorylation, and WNK1 inhibition activates KCC2 by decreasing this phosphorylation.

WNK1 and SPAK kinase forms a physical complex with KCC2 in immature neurons and the developing mouse brain

WNK1 regulates KCC2 activity by modulating its phosphorylation at Thr^{906} and Thr^{1007}, but evidence suggests this is achieved via direct phosphorylation by other kinases. SPAK kinase is a known downstream kinase of WNK1, and its phosphorylation by WNK1 is required for its activation and phosphorylation (*28*). The KCC2a isoform processes an RFxV motif in its N terminus, which is an SPAK/OSR1 binding site; this RFxV motif is also

present in WNK1 (*47*). KCC2b isoform does not have this putative SPAK-binding site (RFxV), and there are 40 unique N-terminal amino acid residues difference between KCC2a and KCC2b (*47*). SPAK phosphorylates all KCC isoforms, including KCC2, at KCC2 Thr^{1007}, but the direct kinase phosphorylating Thr^{906} is unknown (*26*). Since the WNK kinases often physically interact with the CCCs they regulate (*48*), we investigated whether WNK1 associated with KCC2. Controlled, reciprocal co-immunoprecitation experiments with specific antibodies (*26*) revealed that WNK1, along with SPAK, form a physical complex with KCC2 in both immature cultured hippocampal and cortical neurons (**Figure 7A**) and the developing mouse brain (**Figure 7B**). These results suggest WNK1 could regulate KCC2 Thr^{906} and Thr^{1007} phosphorylation by serving as scaffold that bridges SPAK to KCC2 for phosphorylation of Thr^{1007} and potentially another yet undefined kinase that directly phosphorylates Thr^{906}.

DISCUSSION

A paradoxical depolarizing action of GABA due to an elevated $[Cl^-]_i$ is an evolutionary-conserved hallmark of immature neurons (*39*) and is related to the delayed post-natal induction of the Cl^--extruding KCC2 cotransporter activity (*14*, *15*, *20*, *49*, *50*). The mechanisms underlying the developmental switch in KCC2 activity are not well understood, and it is unclear if protein level alone, versus alterations in transporter regulation, account for the net increase in KCC2 activity. Indeed, previous studies have documented a discrepancy between the level of KCC2 protein expression and the KCC2-dependent Cl^- extrusion capacity in immature neurons (*15*, *19*, *20*).

We have shown here that WNK1 kinase inhibits KCC2 to decrease neuronal Cl^- extrusion capacity in immature neurons, thereby contributing to the maintenance of the depolarizing action of GABA (**Figure 8**). The likely mechanism of this event is the WNK1-dependent inhibitory phosphorylation of KCC2 at Thr^{906} and Thr^{1007}, a potent switch of KCC2 activity. Our results corroborate and extend previous work by Rinehart et al. (*16*), who showed KCC2 phosphorylation at Thr^{906} inversely correlates with KCC2 activity in the developing mouse brain, and Inoue et al. (*24*), who demonstrated a phosphorylation-dependent inhibitory effect of taurine on KCC2 activity in immature neurons that was recapitulated by WNK1 over-expression in the absence of taurine.

In our model of immature and mature neuronal cultures, we found the inhibitory phosphorylation of KCC2 at Thr^{906} and Thr^{1007} to be significantly up-regulated in immature versus mature neurons. We therefore propose a inhibitory Thr^{906} and Thr^{1007} phosphorylation regulated by WNK1 is a novel factor contributing to the low activity of KCC2 in the developing brain (**Figure 8**), complementing other known regulatory mechanisms such as changes in protein level (e.g., (*14*)).

Our conclusions are supported by several corroborating lines of evidence utilizing multiple non-overlapping approaches in both *in vitro* and *ex vivo* systems. Using two complementary methods of WNK1 silencing (dominant-negative over-expression of a kinase-dead WNK1 mutant and shRNA-mediated depletion of endogenous WNK1 expression) and physiological $[Cl^-]_i$ recording (gramicidin perforated-patch), we showed that inhibition of WNK1 kinase activity decreases $[Cl^-]_i$ in immature but not mature cultured hippocampal neurons, affecting GABA function. These effects are specific, as they are rescued by over-expression of constitutively active WNK1 in the context of endogenous WNK1 depletion, and are dependent on KCC2 expression, as they are not present when KCC2 is silenced. *Ex vivo* experiments in immature rat cortical slices (P3-P5) support these *in vitro* results, as *in utero* WNK1 inhibition is sufficient to prematurely shift the E_{GABA} of immature neurons to less depolarizing values by lowering neuronal $[Cl^-]_i$. Chemical genetic inhibition of WNK1 kinase activity, mimicking drug targeting of the WNK1 kinase domain, demonstrates that WNK1 catalytic activity is required for the inhibitory phosphorylation of KCC2 at Thr^{906} and Thr^{1007}, and antagonism of WNK1 is sufficient to activate KCC2 activity by promoting the dephosphorylation at these sites.

As ubiquitously expressed kinase, WNK1 can contribute to control of many other molecules including KCC3 (*26*), NKCC1 (*38*) and, potentially, other transporters and channels. In the cartoon shown in **Figure 8,** we included only NKCC1, as another potent WNK1-dependent contributor to neuronal Cl^- homeostasis. In the present work, in keeping with potential implication of NKCC1 to WNK1-dependent change of the Cl^- we performed part of the physiology experiments in presence of NKCC1 inhibitor bumetanide to highlight the contribution of WNK1-KCC2 pathways. An important subject for future projects will be to determine the contribution of WNK1-dependent NKCC1 pathway and its interplay with KCC2-dependent Cl^- extrusion in neuronal development and pathology conditions.

Previous studies suggested the existence of at least two distinct mechanisms of KCC2 regulation during development, including the Neurotropic Factor (NF)–dependent up-regulation of *KCC2* transcription in immature neurons (reviewed by (*51*, *52*), and the post-translational regulation of KCC2 activity via (de)phosphorylation (reviewed by (*25*, *52*)). In immature neurons, KCC2 is phosphorylated and almost fully inactive despite clear neuronal expression of the KCC2 protein (*15*, *19*, *20*, *24*); indeed, phosphorylation at Thr^{906} is reduced 33% by P3 and >90% by P21; in the adult, phosphorylation at Thr^{906} is negligible (*16*) when the ion-transport activity of KCC2 is maximal (*14*, *19*). Despite this inverse correlation between the level of KCC2 phosphorylation at these sites and transporter activity, little experimental data to date has linked these two phenomena. So far, only two studies showed that staurosporine, a broad kinase inhibitor, produces a rapid and potent stimulation of KCC2 activity in immature but not mature cultured hippocampal neurons (*19*) and immature (E18.5), but not more mature (P7) cortical slices (*24*). Here, we identify a specific kinase (WNK1) that contributes to the developmental control of the KCC2 activity, reveal its likely mechanism of action (promoting Thr^{906} and Thr^{1007} phosphorylation), and report a novel way of facilitating Cl^- extrusion via KCC2 by developing a chemical genetic method of WNK1 kinase inhibition.

What are the drivers contributing to maintenance of a high level of KCC2 inhibitory phosphorylation and low level of its activity? One could be ongoing neuronal activity. Immature neuronal networks in organisms from worms to primates are characterized by the existence of ongoing neuronal activity and synchronous oscillations of intracellular Ca^{2+} that are result of synergistic depolarizing actions of both GABA and glutamate (*39*). During development, the strength of the depolarizing action of GABA decreases due to progressive KCC2-mediated Cl^- extrusion, and synchronous oscillations of Ca^{2+} and neuronal activity disappear. In developing neurons, prolonged inhibition of spontaneous network activity

463 triggers a slow-down of the progressive activation of KCC2 (*53–55*). Whether this
464 phenomenon is related to KCC2 phosphorylation at Thr^{906} and Thr^{1007} remains unknown. A
465 second putative regulatory mechanism might involve taurine, an abundant free amino acid in
466 the brain, that was found to contribute into KCC2 inactivation in immature, but not mature
467 neurons (*24*).

468 The WNK serine-threonine kinases are master regulators of the cation-Cl^-
469 cotransporters (CCCs) (*7*), and are sensors of intracellular Cl^- concentration, extracellular
470 osmolarity, and cell volume, transducing signals about changes in these parameters to the
471 CCCs to regulate transporter activity. The *PRKWNK1* gene encodes multiple alternatively-
472 spliced WNK1 isoforms. One isoform is mutated in Mendelian disease featuring impaired Cl^-
473 transport in the distal nephron causing hypertension (*30*). Multiple full-length WNK1
474 isoforms, including the *HSN2* splice variant mutated in congenital pain insensitivity, are
475 prominently expressed in embryonic and the early post-natal brain, and particularly in the
476 *cornu ammonis 1* (CA1), CA2 and CA3 areas of the hippocampus (*34*, *36*) and in cortex (*24*),
477 suggesting a role in brain development. WNK1 expression, in contrast to WNK2 and WNK3,
478 declines into adulthood in these brain regions (*34*, *36*).

479 WNK-mediated regulation of the CCCs is triggered by an interaction between RFXV/I
480 motifs within the WNKs and CCCs and a conserved carboxyl-terminal docking domain in the
481 SPAK and OSR1 kinases. WNKs directly phosphorylate SPAK/OSR1, which in turn
482 phosphorylate KCC2 at Thr^{1007} but not Thr^{906} (*26*). The kinase regulating KCC2 Thr^{906}
483 phosphorylation is currently unknown. However, WNK1 knockdown in HEK-293 cells
484 decreases KCC3 phosphorylation at Thr^{991}, a site homologous to Thr^{906} in KCC2 (*16*). We
485 speculate, given the similar effects of WNK1 shRNA, WNK1-DN, and chemical inhibition of
486 WNK on KCC2 activity, that WNK1 is required for and regulates the phosphorylation of
487 KCC2 at both Thr^{906} and Thr^{1007} in a kinase-dependent manner. However, WNK1 is likely

not the direct phosphorylating kinase at either Thr^{906} (see **Figure 3**) or Thr^{1007} (which is mediated by WNK1-regulation of SPAK/OSR1 kinase) (*26*). Further phosphoproteomic experiments will be required to identify the direct kinase involved in KCC2 phosphorylation at Thr^{906}, as well as the stimuli that might account for changes in WNK1-mediated KCC2 phosphoregulation during development.

Importantly, our chemical genetic work has established a new tool for studying WNK signaling, allowing for the specific and dynamic modulation of WNK1 kinase activity in a cellular context. This technique exploits a functionally silent mutation in the catalytic active site to sensitize a target kinase to small molecule inhibition that does not inhibit wild-type kinases (*56*). This is of particular relevance for the WNK family, which contains multiple family members and splice variants, and for which no current pharmacological inhibitors exist, thus allowing the differentiation between WNK1 and other family members, like WNK3/4 (which are also amenable to chemical genetic inhibition, data not shown). Chemical genetic experiments with WNK1 might also allow for the specific study of WNK1 protein function *in vivo*, for example, in knock-in mice created with the WNK1-AS mutation, since WNK1 KO mice are embryonic lethal (*57*).

Our study revealed also a high level of both Thr^{906} and Thr^{1007} KCC2 phosphorylation when expressed in N2a cells. Although it remains unclear whether these residues were phosphorylated by one of the ubiquitously expressed WNKs or other threonine kinases, the N2a cells expressing KCC2 and its mutants could serve as useful model to delineate signaling pathways involved in control of KCC2 phosphorylation.

Lastly, our results in neurons suggest inhibition of WNK signaling in the CNS might be a novel means of enhancing neuronal Cl^- extrusion to restore GABAergic inhibition by stimulating KCC2. This might be of value neuropsychiatic conditions in which KCC2 activity is suppressed and GABAergic disinhibition fosters the hyperexcitability of neurons

and circuits. This strategy may be particularly relevant in the immature brain where KCC2 phosphorylation is highest, and therefore of interest for neurodevelopmental disorders like autism (*58*) or neonatal seizures (*59*), which reveal pathologic excitatory GABA responses due to elevations in neuronal Cl^- levels at time points when KCC2 is likely present but functionally inhibited. Moreover, it is tempting to speculate that inhibitory KCC2 Thr^{906} and Thr^{1007} phosphorylation might also be pathologically up-regulated in mature neurons, accounting for the documented decrease in KCC2-mediated Cl^- extrusion capacity and GABAergic disinhibition in diseases like temporal lobe epilepsy (*60*) and neuropathic pain (*61*). Interestingly, we and others have recently demonstrated that the first mutations in KCC2 associated with a human disease, severe idiopathic generalized epilepsy in a large French Canadian patient cohort (*42*) and febrile seizures in an Australian family (*62*), are clustered in the C-terminus and reside in residues close to the Thr^{906} and Thr^{1007} motif, altering KCC2 activity. These subjects will be rich topics of future investigation with potential clinical relevance.

MATERIALS AND METHODS

Animals

The animal care and handling was performed in accordance with the guidelines of the European Union Council and the INSERM regulations on the use of laboratory animals.

Primary cultures and transfection of rat hippocampal neurons

For immunocytochemistry, electrophysiology and non-invasive Cl-Sensor analysis, neuronal cultures were plated on coverslips placed in 35 mm culture dishes. 24 hour prior to plating, dishes with coverslips were coated with poly-ethylenimine (5 µg/ml).

Hippocampi and cortices from 18-day-old rat embryos were dissected and then dissociated using trypsin and plated at a density of 70,000 cells cm^{-2} in minimal essential medium (MEM) supplemented with 10% NU serum (BD Biosciences, Le Pont de Claix, France), 0.45% glucose, 1 mM sodium pyruvate, 2 mM glutamine and 10 IU ml−1 penicillin–streptomycin as previously described (Buerli et al. 2007). On days 7, 10 and 13 of culture incubation, half of the medium was changed to MEM with 2% B27 supplement (Invitrogen). For physiology and immunocytochemistry experiments neurons were plated in 35 mm culture dishes containing 14 mm coverslips. For Western Blot experiments neurons were plated in 60 mm dishes (4 dishes per culture and *in vitro* age).

Transfections of neuronal cultures were performed at 4 div (for recordings on 6-7 div) and 10 div (for recordings on 13-15 div) as described previously (Buerli et al. 2007). For transfection of cultures growing in 35 mm dishes, 300 µL of Opti-MEM media was mixed with 7 µL of Lipofectamine reagent 2000 (Invitrogen), 1 µL of Magnetofection CombiMag (OZ Biosciences, France) and 1–1.5 µg of different pcDNAs pre-mixed in desired proportions. The mixture was incubated for 20 min at room temperature (RT) and thereafter distributed dropwise above the neuronal culture. Culture dishes were placed on a magnetic

554 plate (OZ Biosciences, France) and incubated for 30–35 min at 37°C. Transfection was
555 terminated by the substitution of 90% of the incubation solution with fresh culture media.

556 The majority of experiments were based on co-transfection into the same cell of two or
557 three different pcDNAs encoding a fluorescent marker of transfection (enhanced green
558 fluorescent protein (eGFP) or Cl-Sensor), shRNAs, WNK1-related constructs and/or mutants
559 of KCC2. Prior to electrophysiology or imaging experiments, we specifically studied the
560 efficacy of neuronal co-transfection with mixtures of pcDNAs in different proportions as
561 described in (*41*). We found that use of proportion 0.15 + 0.6 + 0.6 for co-transfection of
562 three constructs (marker + shRNA + KCC2 or marker + WNK1 + KCC2) insures expression
563 of both constructs of interest into the eGFP or Cl-Sensor-positive neurons and N2a cells. For
564 transfection of two constructs, we used routinely mixtures of 0.2 μg of marker + 1.0 μg of the
565 construct of interest.

566

567 **Buffers for Western Blots**

568 Buffer A contained 50 mM Tris/HCl, pH7.5 and 0.1mM EGTA. Lysis buffer was 50
569 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium
570 pyrophosphate, 1 mM sodium orthovanadate, 1% (w/v) Triton-100, 0.27 M sucrose, 0.1%
571 (v/v) 2-mercaptoethanol and protease inhibitors (complete protease inhibitor cocktail tablets,
572 Roche, 1 tablet per 50 mL). TBS-Tween buffer (TTBS) was Tris/HCl, pH 7.5, 0.15 M NaCl
573 and 0.2% (v/v) Tween-20. SDS sample buffer was 1X NuPAGE LDS sample buffer
574 (Invitrogen), containing 1% (v/v) 2-mercaptoethanol. Isotonic high K^+ buffer was 95 mM
575 NaCl, 50 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 1 mM Na_2SO_4 and 20 mM
576 HEPES (pH 7.4). Hypotonic high K^+ buffer was 80 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$,
577 1mM Na_2HPO_4, 1 mM Na_2SO_4 and 20 mM HEPES (pH 7.4). Isotonic buffer was 135 mM
578 NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 0.5mM Na_2SO_4 and 15

mM HEPES (pH 7.5). Hypotonic low chloride buffer was 67.5 mM Na-gluconate, 2.5 mM K-gluconate, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.5 mM Na_2HPO, 0.5 mM Na_2SO_4 and 7.5 mM HEPES (pH 7.5).

Phospho-antibody immunoprecipitations

KCCs phosphorylated at the KCC2 Thr^{906} and Thr^{1007} equivalent residue were immunoprecipitated from clarified hippocampal and cortical culture lysates. The phospho-antibody was coupled with protein-G–Sepharose at a ratio of 1 mg of antibody per 1 mL of beads. A total of 2 mg of clarified cell lysate were incubated with 15 µg of antibody conjugated to 15 µL of protein-G–Sepharose in the presence of 20 µg/mL of lysate of the corresponding dephosphopeptide. Incubation was for 2 hours at 4°C with gentle agitation, and the 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.

Immunoblotting

Cell lysates (15 µg) in SDS sample buffer were subjected to electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated for 30 min with TTBS containing 5% (w/v) skimmed milk. The membranes were then immunoblotted in 5% (w/v) skimmed milk in TTBS with the indicated primary antibodies overnight at 4°C. Sheep antibodies were used at a concentration of 1-2 µg/ml. The incubation with phosphospecific sheep antibodies was performed with the addition of 10 µg/mL of the dephosphopeptide antigen used to raise the antibody. The blots were then washed six times with TTBS and incubated for 1 hour at RT with secondary HRP-conjugated antibodies diluted 5000-fold in 5% (w/v) skimmed milk in TTBS. After repeating the

washing steps, the signal was detected with the enhanced chemiluminescence reagent. Immunoblots were developed using a film automatic processor (SRX-101; Konica Minolta Medical) and films were scanned with a 600-dpi resolution on a scanner (PowerLook 1000; UMAX). Figures were generated using Photoshop/Illustrator (Adobe).

Electrophysiology recordings

The gramicidin-perforated whole cell patch-clamp recordings from transfected neurons (eGFP-positive) were performed according to protocols described previously (*41*, *63*). The external solution contained (in mM): 140 NaCl, 2.5 KCl, 20 HEPES, 20 D-glucose, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 0.001 tetrodotoxin and 0.0003 strychnine, pH 7.4. Coverslips with transfected neuronal cells were placed onto an inverted microscope and perfused via a fast perfusion system placed in front of the recording neuron to insure removal of trace amounts of gramicidin that could diffuse from the patch pipette. Patch pipettes (5 MΩ) were filled with a solution containing (in mM): KCl 150, HEPES 10, 20 $\mu g \cdot mL^{-1}$ gramicidin A, pH 7.2. The gigaseals were formed by rapid 5-10 s approaching of the patch pipette to neuronal surface without applying positive pressure (to diminish leak of gramicidin). After sealing, series resistance (Rs), membrane resistance (Rm) and neuron capacitance (C) were monitored routinely at holding potential (V_h) -80 with 5 mV hyperpolarizing pulses, typically taking 10-15 min for the series resistance to stabilize at 15–60 MΩ. Membrane potential values were corrected off-line for junction potential (VJ) between the pipette and bath solutions (-4.0 mV) and series resistance as described (*64*, *65*).

Isoguvacine (30 μM) was focally applied to the neuron soma and proximal dendrites via a micropipette connected to a Picospritzer (General Valve Corporation). The pipette position, pulse duration (50-150 ms) and pressure 10000-30000 Pa were adjusted for each neuron by applying test pulses at Vh -80 and -60 mV with the final aim being to produce

currents with the slope of voltage–current relationship (I-V) below 4.0 $pA \cdot mV^{-1}$. This procedure allowed minimizing changes in $[Cl^-]_i$ during I-V recording. Depending on the direction of the above test currents, four isoguvacine responses were then recorded at voltages -120, -100, -80, -60 mV (for neurons showing outwardly directed [positive] responses at both -80 and -60 mV), -100, -80, -60, -40 mV (for neurons showing outward responses at -80 and inward [negative] responses at -60 mV) or -80, -60, -40 and -20 mV (for inwardly-directed isoguvacine-induced responses at -60 mV) as shown in **Figure 1A**.

All experiments were performed at 23–24°C. Recordings were made using an Axopatch-200A amplifier and pCLAMP acquisition software (Axon Instruments). Data were low-pass filtered at 2 kHz and acquired at 10 kHz.

In utero electroporation

In utero injections and electroporations were performed as previously described (*66*) in embryos from timed pregnant rats (embryonic day 15) that were anaesthetized with ketamine (100 mg/kg, IMALGENE 1000; Merial, Lyon, France) / xylazine (10 mg/kg, Rompun 2%; Bayer Healthcare, Leverkusen, Germany). Wistar rats (Janvier, Le Genest-Saint-Isle, France) were raised and mated at INMED Post Genomic Platform (PPGI) animal facility in agreement with the European Union and French legislations. The uterine horns were exposed, and a lateral ventricle of each embryo was injected using pulled glass capillaries and a microinjector (PV 820 Pneumatic PicoPump; World Precision Instruments, Sarasota, FL) with Fast Green (2 mg/mL; Sigma, St Louis, MO, USA) combined with the constructs encoding Cl-Sensor plus scrambled shRNA, or WNK1 shRNA (ratio 1:3). Plasmids were further electroporated by discharging a 4000 µF capacitor charged to 40 V with a BTX ECM 830 electroporator (BTX Harvard Apparatus, Holliston, MA, USA). The voltage was discharged in five electrical pulses at 950 ms intervals via tweezer-type

electrodes placed on the head of the embryo across the uterine wall. We performed *in utero* electroporation in embryonic rats at E15, corresponding to an active period of both radial and tangential migration of newborn neurons in the cortex.

Cl-Sensor fluorescence recordings from brain slices

Experiments were performed on acute transverse cortical slices (350 µm) that were cut into ice-cold (2–4ºC) artificial cerebrospinal fluid (ACSF) composed of (mM): NaCl, 126; KCl, 3.5; $CaCl_2$, 2; $MgCl_2$, 1.3; NaH2PO4, 1.2; NaHCO3, 25; glucose, 11; pH 7.4, when equilibrated with 95% O_2 and 5% CO_2 using a Vibratome (VT1000E; Leica, Nussloch, Germany). After cut slices were maintained in oxygenated (95% O_2 and 5% CO_2, pH 7.3) ACSF at RT for at least 1 hour before use. Individual slices were transferred to a specially designed recording chamber where they were fully submerged and superfused with oxygenated ACSF (complemented with 1 µM tetrodotoxin, 1 µM strychnine and 10 µM NBQX to prevent spontaneous neuronal activity and non-controlled $[Cl^-]_i$ changes) at 30–32°C at a rate of 2–3 mL/min. The acquisition of fluorescence images was performed using a customized imaging set-up and consecutive cells excitation at 430 and 500 nm as described previously (*43*). The frequency of acquisition was 0.05 Hz. The duration of excitation was selected to avoid use-dependent bleaching of the signal as described and was kept identical for all experiments (*43*). The applications of the ACSF solution containing isoguvacine (30 µM) or KCl (25 mM) + isoguvacine (30 µM) were performed via a perfusion system. The recovery of fluorescence after Cl^- overload produced by KCl + isoguvacine was recorded in ACSF containing 10 µM Bicuculline in addition to mentioned above blockers to avoid Cl^- efflux through $GABA_AR$.

Immunocytochemistry and quantitative immunofluorescence analysis of primary neuronal cultures

For immunocytochemistry on living neurons, rabbit anti-GFP antibodies were diluted in culture media and applied above neurons for 2 hours at 37°C, 5% CO_2. Neurons were then washed for 10 min (RT) in HEPES-buffered saline solution containing (in mM): NaCl 150, KCl 2.5, $MgCl_2$ 2.0, $CaCl_2$ 2.0, HEPES 20 and D-glucose 10, pH 7.4, labelled with anti-rabbit Cy3 antibody (dissolved in the HEPES-buffered saline) for 20 min at 13°C and fixed in Antigenfix (Diapath, Martingo, Italy). To reveal intracellular proteins, cells were subsequently permeabilized with 0.3% Triton X-100, blocked by 5% goat serum, labeled overnight (4°C) with mouse anti-GFP antibody and for 1 hour (RT) with anti-mouse Alexa-488 antibody. Cell nuclei were stained using 5-min staining with Hoechst 33258 (1µg/mL, Sigma-Aldrich).

For quantitative analysis, images were acquired with an Olympus Fluorview-500 confocal microscope (oil-immersion objectives 40x, (NA1.0) or 60x (NA1.4); zoom 1-5). We randomly selected and focused on a transfected neuron by only visualizing eGFP/pHluorin fluorescence and then acquired images of membrane clusters. The cluster properties of each cell were analyzed with Metamorph software (Roper Scientific sas, Evry, France). First, we created a binary mask of eGFP/pHluorin-fluorescent cells and then analyzed KCC2 membrane fluorescence in regions overlapping with the binary mask. Analysis parameters were the same for all experimental conditions. All experiments were performed in a blinded manner. After analysis, data were normalized to the mean value of cells transfected with KCC2-pH_{ext}.

Constructs and materials

Human *WNK1* in the *pCS2* vector with a CMV promoter, containing exons 1 to 28, including the *HSN2* exon but excluding exons 11 and 12, and engineered to express c6 N-terminal MYC tags, was previously described (*67*). This construct was used for biochemistry. For electrophysiology, the full-length insert minus the Myc tag was cloned into a vector containing an N-terminal mCherry tag. Other constructs included HA-tagged KCC2 (Gift from C. Rivera), HA-tagged OSR1 (*38*); eGFP and mCherry (Clontech); rat mCherry-KCC2 (*43*); rat KCC2-pHluorin (pHluorin was introduced in the second extracellular loop of KCC2, Kahle et al., 2014) and Cl-Sensor in gw1 vector (Waseem et al. 2010). All mutations were generated using the QuikChange (Stratagene) site-directed method and verified by DNA sequencing.

shRNA KCC2 in mU6-pro vector was described previously (*41*) and targeted the following sequence in rat KCC2 mRNA GACATTGGTAATGGAACAACG (NP_599190). A control construct with a scrambled sequence (GATGAACCTGATGACGTTC) lacked homology to any known mammalian mRNAs. shRNA WNK1 (OriGene Technologies, Inc, Rockville, MD USA in pRFP-C-RS retroviral vector) targeted the CATTCAGATGTTGCTTCTGGTATGAGTGA sequence of rat WNK1 mRNA (NM_001002823), and is predicted to knockdown known isoforms of *WNK1* in the brain, including *HSN2*.

For characterization of the WNK1 shRNA, rat PC-12 cells were transfected with control firefly (FF) luciferase (luc) shRNA or rat WNK1 shRNA in a puromycin-resistant vector (pRFP-C-RS, Origene) with FuGENE 6 (Roche Applied Science) at a 3:1 ratio (DNA: transfection reagent) according to manufacturer's directions. Transfected cells were incubated for 48 hours prior to addition of puromycin at 8 μg/mL. After selection, lysates were harvested and subjected to SDS-PAGE, and assessed by Western blotting with the indicated antibodies (see below in Materials and Methods).

Antibodies

Primary antibodies used for immunocytochemistry included: polyclonal rabbit anti-GFP (Molecular Probes, Life Technologies, France) and monoclonal mouse anti-GFP (Novus Biologicals, Interchim, France). The secondary antibodies included anti-mouse Alexa-488 (dilution 1:1000; FluoProbes, Interchim, France); anti-rabbit Cy3 (dilution 1:1000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

Antibodies used for Western Blots included antibodies were raised in sheep and affinity-purified on the appropriate antigen by the Division of Signal Transduction Therapy Unit at the University of Dundee:]; KCC2a total antibody [residues 1-119 of human KCC2a]; KCC3a phospho-Thr^{991} [residues 975-989 of human KCC3a phosphorylated at Thr^{991}, SAYTYER(T)LMMEQRSRR, corresponding to residues of rat KCC2 phosphorylated at Thr^{906}, SAYTYEK(T)LMMEQRSRR]; KCC2a phospho-Thr^{906} [residues 975-989 of human KCC3a phosphorylated at Thr^{991}, SAYTYER(T)LMMEQRSRR]; KCC3a phospho-Thr$^{1039/1048}$ [residues 1032-1046 or 1041-1055 of human KCC3a phosphorylated at Thr $^{1039/1048}$, CYQEKVHM(T)WTKDKYM, corresponding to residues of rat KCC2 phosphorylated at Thr^{1006}, TDPEKVHLTW(T)KDKSV]. NKCC1 total antibody [residues 1-288 of human NKCC1]; NKCC1 phospho-Thr^{203}/Thr^{207}/Thr^{212} antibody [residues 198-217 of human NKCC1 phosphorylated at Thr^{203}, Thr^{207} and Thr^{212}, HYYYD(T)HTN(T)YYLR(T)FGHNT]; WNK1-total antibody [residues 2360-2382 of human WNK1]; WNK1phospho-Ser^{382} antibody [residues 377-387 of human WNK1 phosphorylated at Ser^{382}, ASFAK(S)VIGTP]; SPAK-total antibody [full-length GST-tagged human SPAK protein]; SPAK/OSR1 (S-motif) phospho-Ser^{373}/Ser^{325} antibody [367–379 of human SPAK, RRVPGS(S)GHLHKT, which is highly similar to residues 319–331 of human OSR1 in which the sequence is RRVPGS(S)GRLHKT,); ERK1 total antibody [full-length

752 human ERK1 protein]. KCC2 total antibody [residues 932-1043 of rat KCC2] was purchased
753 from NeuroMab. The anti-β-Tubulin III (neuronal) antibody (T8578) was purchased from
754 Sigma-Aldrich. Secondary antibodies coupled to horseradish peroxidase used for
755 immunoblotting were obtained from Pierce. IgG used in control immunoprecipitation
756 experiments was affinity-purified from pre-immune serum using Protein G-Sepharose.
757
758 **Generation of an analog-sensitive WNK1 kinase**
759 To make an analog-sensitive (AS) WNK1, which can utilize bulky ATP analogs
760 instead of ATP and be inhibited by kinase inhibitors such as 1-NA-PP1 (*44*), we mutated the
761 WNK1-CA ATP binding pocket gatekeeper threonine to alanine (T301A) based on extensive
762 homology searches with other kinases in which this method has been successfully employed
763 (*46*). Many protein kinases tolerate replacement of the gate-keeper mutation to alanine, which
764 enlarges the catalytic pocket enough to accommodate bulky purine analogs; however, 20% of
765 all kinases exhibit decreased activity when this residue is mutated unless a suppressor
766 mutation in the amino-terminal lobe of the ATP-binding pocket is also present (*68*). We
767 therefore also engineered the I357L and G367A mutations in the WNK1-CA T301A kinase
768 domain, as previously described (*68*). This combination of mutations yielded an analog-
769 sensitive WNK1 kinase, herein termed "WNK1-AS", capable of phospho-transfer of N6-
770 substituted ATPγS (N^6 Benzyl-ATP-γ-S) to substrates and inhibition in the micromolar range
771 by 1-NA-PP1.
772
773 **Thiophosphorylation of kinase substrates in cellular lysates**
774 The following method was used to determine if a candidate protein (e.g., OSR1 kinase
775 or KCC2) was a substrate of WNK1-AS in a complex protein mixture, as described in detail
776 (*44*). Briefly, Myc-tagged WNK1-AS, WNK1-DN, or WNK1-CA was transiently co-

expressed with HA-OSR1 or HA-KCC2 in N2A cells in 10-cm dishes. After 48 hours, cells were treated with 1-NA-PP1 (1 μM or 10 μM) or vehicle control (DMSO) for 2 hours before media was removed. Cells were then rinsed once with 5 mL cold PBS, harvested with a scraper, and lysed on ice in 500 μL of 1X RIPA buffer + 1X protease inhibitor + 1X phosphatase inhibitor. Cells were then centrifuged at 10,000 x g for 10 min at 4°C to remove cell debris. Supernatant was saved, and to this was added 100 μM N^6 Benzyl-ATP-γ-S, 100 μM ATP, and 3 mM GTP to each sample to initiate the substrate labeling reaction. Thiophosphorylation of kinase substrates was allowed to occur for 20 min at RT. The reaction was quenched with 500 μL 1X RIPA buffer + 40 mM EDTA (final [EDTA] = 20 mM) + 5 mM PNBM (final [PNBM] = 2.5 mM), and then alkylated with PNBM for 1 hour at RT on a rotator. 40 μL of a 50% slurry of appropriate antibody-tagged magnetic beads (Bethyl labs) were to each sample and incubate 3–4 hours at 4°C on a rotator. Before use, beads were washed once with 1 mL 1X RIPA buffer and then resuspended in the original slurry volume and add to each sample. A magnet was used to collect the beads, washed 5 times w/1 mL 1X RIPA + protease inhibitor + phosphatase inhibitor, and then resuspended in 20 μL 1X RIPA + protease inhibitor + phosphatase inhibitor +1X Laemmli sample buffer. Samples were heated at 95°C for 2.5 min, and then loaded onto a SDS-PAGE gel. Western blotting was performed with anti-HA, anti-Myc, or anti-Thiophosphate Ester Rabbit Monoclonal Antibody (Epitomics). Assays with anti-KCC phospho-antibodies were performed essentially as described in (*26*), though the above Myc-tagged WNK1 plasmids and HA-tagged KCC2 were transiently transfected into N2A cells.

Statistical analysis

For the electrophysiology recordings each condition (i.e. shRNA WNK1) was studied during at least 4 experiments (transfections), 1-3 neurons per experiment. The same

802 experimental day at least two other conditions were analyzed (i.e. shRNA WNK1+CA and
803 scrambled shRNA). The population data were expressed as mean ± SEM, where n was
804 number of recorded neurons. For analysis of fluorescence, the mean value recorded from
805 multiple neurons located in the optical field was taken as single measurement (experiment).
806 The mean ± SEM values were results of analysis of indicated number (n) of experiments. The
807 one-way ANOVA or non-parametric Mann–Whitney tests were employed to examine the
808 statistical significance of the differences between groups of data, otherwise indicated.
809

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Figure 1. WNK1-dependent regulation of E_{GABA} in immature but not mature cultured hippocampal neurons

A. Typical gramicidin-perforated patch-clamp recordings of $GABA_A$ receptor-mediated currents (I) induced by short (50 ms) focal application of isoguvacine at different membrane potentials (focal I_{GABAA}). E_{GABA} was determined as the intercept of the I-V curve with the x-axis. Inset traces illustrate I_{GABAA} recorded from 7 div neurons expressing constitutively-active WNK1 (WNK1-CA).

B. Bar graph illustrating the mean ± SEM of E_{GABA} in neurons of different age transfected with eGFP + scrambled shRNA and recorded with or without bumetanide 10 µM. *p<0.05. Data represented is pooled from 5 separate experiments, 1-3 neurons per experiment.

C. E_{GABA} in neurons of different age transfected with eGFP plus the constructs indicated in the legend. Data represented is pooled from 4-5 experiments for each construct. 1-3 neurons per experiment. ***p< 0.001.

D. Examples of focal I_{GABAA} recorded at Vh=-80 mV before and after neuronal Cl^- loading achieved using combination of 10 s isoguvacine pulse and neuron depolarization to -20 mV. The kinetics of the recovery reflects neuronal Cl^- clearance.

E. Mean ± SEM half-time of I_{GABAA} recovery in neurons expressing indicated constructs. *p< 0.05; **p< 0.01.

Figure 2. WNK1-dependent regulation of Cl-Sensor ratiometric fluorescence in acute cortical slices prepared from rats of postnatal days 3-5 and 30. Rats were electroporated *in utero* at embryonic day 15 with Cl-Sensor plus scrambled shRNA (Sbl) or $shRNA_{WNK1}$ (shWNK1). Data represent results obtained from 5 rats per experimental condition. 2-3 slices were recorded per animal.

A. Typical images of Cl-Sensor fluorescence excited at 500 nm and taken at different optical magnifications. The dotted white line indicates the slice border. The ROI were drawn around the soma of cells located in the focal plane.

B, F. Typical ratiometric fluorescence ($R_{430/500}$) recordings from different neurons (encoded by different colors). Vertical bars indicate times of applications of ACSF containing isoguvacine (iso) or iso + 25 mM of KCl. Arrows and arrowheads indicate different types of responses described in Results section.

C, G. Mean ± SEM of basal levels of $R_{430/500}$ measured before iso application. ns – non significant, n=5.

D, H. Bar graphs illustrating the mean ± SEM of fluorescence change ΔR/R, where R is mean of 5 measurements before iso application and ΔR is difference between absolute maximum of iso-induces response and R. ns – non significant. $^{**}p < 0.01$, n=5.

E, I. Half-decay times of the fluorescence recovery after neuron's loading with Cl^-. ns – non significant. $^{**}p < 0.01$, n=5.

Figure 3. WNK-SPAK/OSR1 regulation and phosphorylation of endogenous KCC2 in immature and mature cultured hippocampal and cortical neurons.

A. Harvested lysates were subjected to immunoprecipitation (IP) with the indicated KCC2 total-, and Thr^{906} and Thr^{1007} phospho-antibodies. The immunoprecipitates were then immunoblotted with the indicated specific KCC2 antibody. Whole cell lysates were also subjected to immunoblot analysis with the indicated total and phospho-specific antibodies. The Western blots illustrate results obtained in three separate experiments. Both KCC2 dimers and KCC2 monomers are indicated with arrows. Molecular masses are indicated in kDa on the left-hand side of the Western blots.

B. The lower panel shows quantification of the results of the Western blots, as assessed by an unpaired t-test (n=3, error bars represent the mean ± SEM.) The quantification (ratio calculation) is based on (phospho-dimeric KCC2 + phospho-monomeric KCC2) / (total dimeric KCC2 + monomeric KCC2). ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; ns - non significant.

Figure 4. Genetic modulation of KCC2 Thr^{906} and Thr^{1007} phosphorylation affects E_{GABA} and the plasmalemmal surface expression of KCC2 in cultured hippocampal neurons.

A. E_{GABA} recorded in 10 div neurons that were transfected at 7 div with constructs as indicated. Recordings were made using gramicidin-perforated patch-clamp, as in Figure 1A. Numbers in columns indicate number of neurons recorded. Data are pooled from 5 separate experiments, with 1-3 neurons per experiment. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; ns - non significant.

B. Scheme of the multi-step immunolabelling protocol (see Materials and Methods for details).

C. Representative images illustrating membrane staining (F_m, left column) and internalized fluorescence (F_i, right column) of WT and mutated forms of KCC2 with external tag (KCC2-pH_{ext}) as indicated. Neuronal shape is shown in light green in each image respectively.

D. Normalized mean ± SEM of total protein, membrane staining (F_m) and internalized fluorescence (F_i) for each KCC2-pH_{ext} constructs (pooled data from 3 cultures, 5-8 cells per culture and condition). ***, $p<0.001$, **, $p<0.01$, ns - non significant.

Figure 5. Phospo-mimetic and non-phosphorylatable KCC2 Thr^{906}/Thr^{1007} mutants exhibit different Cl^--extrusion capacities and sensitivity to dominant-negative WNK1

A. Fluorescence signals recorded from N2a cells co-transfected with Cl-Sensor (green), GlyR (non-fluorescent) and mCherry-KCC2 (red). Scale bar = 20 µm.

B. Individual traces of Cl-Sensor fluorescence ratio changes measured in N2a cells expressing $KCC2^{E/E}$. Horizontal bar indicates the time of application of 100 mM KCl and 50 µM glycine to load cells with Cl^-. The ordinate axis indicates the ratio of Cl-Sensor fluorescence measured at 430 and 500 nm excitation wavelengths ($R_{430/500}$).

C. Normalized mean traces of Cl-Sensor ratiometric fluorescence change in N2a cells expressing different KCC2 constructs as indicated. The inset illustrates the full record of $R_{430/500}$ fluorescence from mock-transfected cells shown in the main plot.

D, E, F. Mean ± SEM of the half-decay time of Cl^- extrusion after glycine + KCl application in cells expressing different combinations of constructs as indicated. n = 4 to 5 experiments. **, $p<0.01$, *, $p<0.05$, ns: non-significant.

Figure 6. Chemical genetic inhibition of WNK1 stimulates KCC2 activity.

A. Engineering a chemical genetic switch of WNK1 kinase activity. Upper left panel depicts the ATP-binding site of WT Src kinase with ATP (green, left) and analog-sensitive (AS) Src-AS with N^6 Benzyl-ATP-γ-S (yellow, right). Mutation in the gatekeeper residue (blue) results in an affinity pocket where bulky ATP analogs (like Benzyl-ATP-γ-S) bind.

B, C, D. Representative normalized mean ± SEM traces of the ratiometric Cl-Sensor fluorescence recordings from 8-10 cells in the same experiments before and 10 min after cells incubation with 10 µM of 1-NA-PP1.

E. Difference between the half-decay times of Cl^- extrusion produced by application of 1-NA-PP1 in cells expressing different combinations of WNK1 and KCC2 related constructs as indicated. Mean ± SEM of 4 experiments in each series. ^^ (p<0.01) and ns (non-significant) indicate the effect of 1-NA-PP1 (paired *t*-test). ** (p<0.01) indicates the difference between groups (ANOVA test).

F. Chemical genetic inhibition of WNK1 kinase activity decreases KCC2 phosphorylation at P-Thr^{906} and Thr^{1007}. Myc-tagged WNK1-CA or WNK1-AS was transiently expressed with or without HA-KCC2 in N2a cells in the absence or presence of 1-NA-PP1 (10 µM for 2 hours) to inhibit WNK1-AS but not WNK1-CA activity. Lysates were harvested, subjected to SDS-PAGE and the phosphorylation at Thr^{906} and Thr^{1007} was assessed by Western blotting with phospho-specific antibodies directed against these residues as described in Materials and Methods. (*) Indicates statistically significant differences relative to control conditions as assessed by an unpaired t-test (p<0.01; n = 3). Error bars represent the mean ± SEM.

Figure 7. KCC2 interacts with WNK1 and SPAK *in vivo*.

A. 7 div and 14 div lysates of hippocampal cultures (Sample 2, as indicated in Figure 3) and cortical cultures (Sample 2) were immunoprecipitated (IP) with the indicated WNK1 and SPAK antibodies, and analyzed for their interacting partners using standard SDS-PAGE and immunoblotting (IB) techniques.

B. Whole-brain lysates obtained from P2 mice were immunoprecipitated (IP) with the indicated WNK1, SPAK and KCC2 antibodies and analyzed for their interacting partners using standard SDS-PAGE and immunoblotting (IB) techniques.

Figure 8. A model of WNK1-dependent control of neuronal Cl^- during development.

In immature neurons (left), the functional activity of KCC2 is low, such that NKCC1-mediated Cl^- loading predominates and the intraneuronal Cl^- concentration $[Cl^-]_i$ is relatively elevated. Consequently, $GABA_AR$ activation elicits membrane depolarization. In mature neurons (right), the functional activity of KCC2 is high, such that KCC2-mediated Cl^- extrusion predominates, $[Cl^-]_i$ is low, and $GABA_AR$ activation triggers membrane hyperpolarization. Our data suggest WNK1 kinase, complementing other mechanisms that determine KCC2 activity such as the regulation of protein expression, contributes to the depolarizing action of GABA in immature neurons by maintaining the inhibitory phosphorylation of KCC2 at Thr^{906} and Thr^{1007}. We therefore propose the antagonism of WNK1 kinase could be novel means to potentiate neuronal Cl^- extrusion in immature neurons, or even in mature neurons if KCC2 inhibitory phosphorylation and $[Cl^-]_i$ is pathologically elevated. Our data suggest that WNK1 regulates the phosphorylation of KCC2 via SPAK kinase at Thr1007, and via a yet unidentified kinase at Thr906.

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Supplementary Figure 1. Characterization of a specific WNK1 shRNA.

Rat PC-12 cells were transfected with control firefly (*FF*) luciferase (luc) shRNA (left lane) or rat *WNK1* shRNA according to Materials and Methods. Lysates were harvested and subjected to SDS-PAGE, and assessed by Western blotting with the indicated antibodies (see Materials and Methods). *WNK1* shRNA significantly depletes endogenous WNK1 protein in rat neuronal cells.

<u>Supplementary Figure 2</u>. Characterization of a WNK1 analog sensitive (AS) construct.

A. WNK1-AS utilizes Benzyl-ATP-γ-S with much greater efficiency than WNK1-CA. Empty vector (control), Myc-tagged WNK1-CA, or WNK1-AS was transiently expressed in N2a cells; cell lysate was prepared, and N6 substituted ATPγS (N^6 Benzyl-ATP-γ-S) was added to initiate the substrate labeling reaction. Thiophosphorylated proteins were alkylated using p-nitrobenzyl mesylate. The indicated WNK1 constructs were immunoprecipitated using an anti-Myc antibody, and lysates were subjected to SDS-PAGE and immunoblotted with a p-nitrobenzyl-thiophosphate ester specific antibody or the other indicated antibodies as described in Materials and Methods.

B. WNK1-AS catalyzes transfer of N^6 Benzyl-ATP-γ-S to a known substrate, OSR1. In N2a cells, Myc-tagged WNK1-CA, WNK1-AS, and WNK1-DN was transiently expressed with HA-OSR1 or HA-KCC2. Cell lysate was prepared and N^6 Benzyl-ATP-γ-S was added to initiate the substrate labeling reaction. Thiophosphorylated proteins were alkylated as in A, and HA-OSR1 or HA-KCC2 was immunoprecipitated from cell lysates with anti-HA antibody. Cell lysates or HA-bound immunoprecipitates were subjected to SDS-PAGE, and Western blots were performed with the indicated antibodies. WNK1-AS is able to catalyze the transfer of N^6 Benzyl-ATP-γ-S to OSR1 but not KCC2.

C. WNK1-AS is inhibited by 1-NA-PP1 in a dose-dependent manner. Myc-tagged WNK1-CA or WNK1-AS was transiently expressed with or without HA-OSR1 in N2a cells in the absence or presence of 1-NA-PP1 at the indicated concentrations. Cell lysate was prepared, and N^6 Benzyl-ATP-γ-S was added to initiate the substrate labeling reaction. Thiophosphorylated proteins were alkylated, and HA-OSR1 was immunoprecipitated from the whole cell lysate with anti-HA antibody. Whole cell lysates or OSR1 immunoprecipitates were subjected to SDS-PAGE. Western blots were performed with the indicated antibodies.

WNK1-AS is able to catalyze the transfer of N^6 Benzyl-ATP-γ-S to OSR1, and this is inhibited by 1-NA-PP1 in a dose-dependent manner.