

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

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30 **ABSTRACT**

31 Activation of Cl<sup>-</sup>-permeable GABA<sub>A</sub> receptors elicits synaptic inhibition in mature neurons  
32 but excitation in immature neurons, which is essential for brain maturation. This  
33 developmental “switch” in GABA function is dependent on a post-natal KCC2 cotransporter-  
34 mediated decrease in intraneuronal Cl<sup>-</sup> [Cl<sup>-</sup>]<sub>i</sub>, but the mechanisms regulating KCC2 in  
35 immature neurons are poorly understood. Here, we showed the serine-threonine kinase  
36 WNK1 forms a physical complex with KCC2 in the developing mouse brain, and dominant-  
37 negative mutation, genetic depletion, or chemical inhibition of WNK1 in immature neurons is  
38 sufficient to trigger a hyperpolarizing shift in GABA activity by facilitating KCC2-mediated  
39 Cl<sup>-</sup> extrusion. These effects resulted from removal of KCC2 phosphorylation at Thr<sup>906</sup> and  
40 Thr<sup>1007</sup>, a critical inhibitory motif of KCC2 activity we showed to be significantly up-  
41 regulated in immature neurons. Together, these data provide insights into the mechanism  
42 regulating Cl<sup>-</sup> homeostasis in immature neurons, and suggest changes in the WNK1-regulated  
43 inhibitory phosphorylation of KCC2 might play a role in the GABA excitatory/inhibitory  
44 developmental sequence.

45

46 **One Sentence Summary:** We elucidate a novel WNK1 kinase-dependent mechanism that  
47 regulates KCC2-mediated Cl<sup>-</sup> homeostasis and GABA activity in immature neurons.

48

49 **INTRODUCTION**

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

57 Human and mouse genetics have unequivocally demonstrated that the *SLC12A* family  
58 of cation- $\text{Cl}^-$  cotransporters (CCCs), including the  $\text{Cl}^-$ -intruding  $\text{Na}^+$ - $\text{K}^+$ - $2\text{Cl}^-$  cotransporters  
59 (N[K]CCs) and  $\text{Cl}^-$ -extruding  $\text{K}^+$ - $\text{Cl}^-$  cotransporters (KCCs), are primary determinants of  $[\text{Cl}^-]$   
60  $]$  in multiple cell types (2, 3, 6). The WNK (*with no lysine [K]*)-SPAK (SPS1-related  
61 proline/alanine-rich kinase) serine-threonine kinases are the master regulators of these  $\text{Cl}^-$   
62 transporters across evolution (7). While the WNK-SPAK signaling pathway has been  
63 extensively characterized in epithelial tissues active in water and solute transport, such as the  
64 kidney's distal nephron (8–11), its role in the central nervous system (CNS) is not well  
65 understood.

66 The emergence of inhibitory GABAergic signaling in the developing CNS  
67 demonstrates how changes in  $[\text{Cl}^-]_i$  can modulate GABA activity and consequently, the  
68 function of neurons and circuits.  $\text{GABA}_A$  receptors ( $\text{GABA}_A\text{Rs}$ ) are ligand-gated,  $\text{Cl}^-$ -  
69 permeable ion channels that allow the bidirectional flux of  $\text{Cl}^-$  ions, the direction of which is  
70 dictated by  $[\text{Cl}^-]_i$  and the membrane potential (12). In the adult brain,  $\text{GABA}_A\text{R}$  activation  
71 triggers membrane hyperpolarization and synaptic inhibition. Conversely, in the developing  
72 brain,  $\text{GABA}_A\text{R}$  activation triggers depolarization and in some instances excitation, which is  
73 critical for neuronal proliferation and migration, and synaptogenesis (13). This developmental

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

78 KCC2 protein levels increase during development in some neuronal populations (15),  
79 but it is unclear if protein level alone, versus changes in the functional regulation of the  
80 transporter, explain the overall increase in KCC2 activity (16). In hippocampi and cortices of  
81 both rats and mice, KCC2 protein expression begins in late embryonic stages and increases  
82 progressively during the first post-natal week (14, 15, 17, 18). However, the first signs of  
83 KCC2 function are not detectable until post-natal days 5-6 in hippocampi and days 6-8 in  
84 cortex (15, 18–20), and GABA remains depolarizing until post-natal days 8-13 (21, 22).  
85 Discordance between the level of KCC2 protein expression and KCC2 activity have also  
86 been reported in cultured immature hippocampal neurons (19). Together, these data suggest  
87 other regulatory factors may contribute to maintaining low KCC2 activity in immature  
88 neurons in the developing brain. Recent work has demonstrated that phosphorylation can  
89 significantly alter KCC2 activity, neuronal  $[Cl^-]_i$ , and GABA reversal potential ( $E_{GABA}$ ) (16,  
90 23–26). It has been found also that the level of KCC2 phosphorylation (residue Thr<sup>906</sup>) is  
91 relatively high in immature brain and decreases progressively during neuronal development  
92 (16).

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

99 dysregulation due to dysregulated  $\text{Cl}^-$  reabsorption (along with  $\text{Na}^+$  and/or  $\text{K}^+$ ) in the kidney's  
100 nephron (7). However, WNKs (including WNK2 and WNK3) are highly-expressed outside  
101 the kidney, including the developing and mature CNS (32–34), and each gene encodes  
102 multiple isoforms, some of which exhibit remarkable specificity in the brain or spinal cord  
103 (35, 36). The function of the WNK kinase pathway in the CNS, however, is largely unknown.  
104 Interestingly, mutations in WNK1 (WNK1/HSN2), one WNK1 isoform with particularly  
105 robust CNS expression, causes a severe autosomal recessive disease in humans characterized  
106 by congenital insensitivity to pain (OMIM# 201300 (35)), suggesting an essential but yet  
107 undefined role for the WNK kinases in the human CNS.

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

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

123 gene expression (*I4*), contributes to the depolarizing action of GABA in immature neurons  
124 by promoting the inhibitory phosphorylation of KCC2 at Thr<sup>906</sup> and Thr<sup>1007</sup>.

125

126 **RESULTS**127 **WNK1 kinase inhibition facilitates KCC2-dependent Cl<sup>-</sup> extrusion and causes a**  
128 **hyperpolarizing shift in E<sub>GABA</sub> in immature neurons**

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

140 In immature neurons, genetic silencing of WNK1 using specific shRNAs  
141 (**Supplementary Figure 1**) or dominant negative WNK1-DN produced a highly reproducible  
142 and significant ~15 mV hyperpolarizing shift of E<sub>GABA</sub> from -57.9±1.5 mV (n=14) to -  
143 73.1±2.7 mV (n=11) and -76.4±2.3 mV (n=17), respectively (**Figure 1C**, columns 1, 2  
144 and 3). The effect of WNK1 shRNA was specific, as it was rescued by the expression of a  
145 shRNA-resistant WNK1-CA mutant (**Figure 1C**, column 4). Critically, the hyperpolarizing  
146 shift of E<sub>GABA</sub> associated with shRNA-mediated WNK1 knockdown was dependent on KCC2  
147 expression, since neurons co-expressing WNK1 shRNA and KCC2 shRNA failed to elicit a  
148 E<sub>GABA</sub> depolarization (**Figure 1C**, column 5).

149 Conversely, constitutive activation of WNK1 in 6-7 div neurons achieved via  
150 expression of WNK1-CA did not produce a detectable change of E<sub>GABA</sub> as compared to

151 control neurons transfected with scrambled shRNA (**Figure 1C**, columns 1 and 6). These  
152 values were reminiscent of those recorded from immature neurons with knocked-down KCC2  
153 ( $-53.7 \pm 1.9$  mV, **Figure 1C**, column 7). These results show antagonism of WNK1 in  
154 immature neurons elicits a KCC2-dependent hyperpolarizing shift of  $E_{GABA}$ , whereas  
155 constitutive WNK1 activation has no detectable effect.

156 In more mature neurons (13-15 div) that were characterized by significantly more  
157 hyperpolarized  $E_{GABA}$  values ( $-83.1 \pm 1.7$  mV,  $n=9$ , **Figure 1C**, column 1), the expression of  
158 WNK1 shRNA or WNK1-DN did not elicit a further hyperpolarization of  $E_{GABA}$ ; the  
159 mean $\pm$ SEM values of WNK1 shRNA or WNK1-DN cells did not differ statistically from  
160 those transfected with scrambled shRNA (**Figure 1C**, columns 1, 2 and 3). Conversely,  
161 mature neurons (13-15 div) expressing WNK1-CA produced a depolarizing shift of  $E_{GABA}$  to  
162  $-62.4 \pm 3.7$  mV (**Figure 1C**, column 6); these values were, however, more negative than those  
163 measured in neurons with knocked-down KCC2 ( $-54.5 \pm 1.9$  mV,  $p=0.02$ ,  $n=12$ , **Figure 1C**,  
164 column 7).

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

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

173

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



176 Are the electrophysiological findings above relevant *in vivo*? We next studied whether  
177 WNK1 knockdown in immature neurons *in utero* affects  $[Cl^-]_i$  and GABA activity.  
178 Constructs encoding Cl-Sensor plus a scrambled shRNA (control) or Cl-Sensor plus WNK1  
179 shRNA were electroporated *in utero* in rats at E15 (see Materials and Methods for details).  
180 Transverse slices of cortex were prepared on postnatal days P3-P5 and P30. Electroporated  
181 slices harbored hundreds of neurons expressing Cl-Sensor in cortical layers III-V (**Figure**  
182 **2A**), which exhibited variable absolute values of  $R_{430/500}$  (**Figure 2B and 2C**). Cl-Sensor does  
183 not allow measurement of the exact concentration of  $[Cl^-]_i$  due to its sensitivity to  $[H^+]$  and  
184 some organic ions; however, it does allow the detection of even weak (2-4 mM) oscillations  
185 of resting  $[Cl^-]_i$  (e.g., in response to GABA<sub>A</sub>R or glycine receptor-channel activation), and is  
186 suitable for measurements of neuronal  $Cl^-$  extrusion capacity (39). We therefore avoided  
187 using ratiometric Cl-Sensor recordings to measure  $[Cl^-]_i$  or the magnitude of  $[Cl^-]_i$  changes,  
188 and designed all experiments to determine the *directionality* of  $Cl^-$ -dependent fluorescence  
189 changes in response to GABA<sub>A</sub>R activation, thereby analyzing the *kinetics* of fluorescence  
190 recovery after neuronal  $Cl^-$  loading.

191 Consistent with previously-described age-dependent depolarizing and hyperpolarizing  
192 actions of GABA in, respectively, immature and mature neurons (39), brief exposure of brain  
193 slices from control mice (animals electroporated with Cl-Sensor and scrambled shRNA) to  
194 isoguvacine (30  $\mu$ M, 3 minutes, min) produced bi-directional fluorescence responses  
195 dependent on the age of the animal: in slices from immature P3-P5 mice, most of neurons  
196 (80%) exhibited an uniform decrease of  $R_{430/500}$  (indicated with arrow in **Figure 2B**),  
197 reflecting an outward direction of  $Cl^-$  flux characteristic of GABA depolarizing activity. In  
198 slices from more mature (P30) animals, similar isoguvacine applications elicited no change  
199 (~60% of neurons) or an increase of  $R_{430/500}$  (indicated with arrow in **Figure 2F**), reflecting  
200 either  $Cl^-$  equilibrium or  $Cl^-$  influx, typical for GABA hyperpolarizing activity.

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

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

226

227 **KCC2 harbors significantly more inhibitory phosphorylation at Thr<sup>906</sup> and Thr<sup>1007</sup> in**  
228 **immature versus mature neurons**

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

236 We assayed KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation in dissociated rat hippocampal  
237 and cortical neurons that grow *in vitro* 6-7 and 14-15 days in the same conditions used for  
238 physiological experiments above. Briefly, we purified KCC2 from neurons using anti-KCC2  
239 antibody, and probed the resulting immunoprecipitates using phosphor-specific antibodies  
240 that recognize Thr<sup>906</sup> or Thr<sup>1007</sup> (26) (**Figure 3A**). Phosphorylation at both Thr<sup>906</sup> and Thr<sup>1007</sup>  
241 is significantly elevated in 6-7 div neurons compared to 14-15 div neurons. We calculated the  
242 phospho-KCC2: total KCC2 ratio at each time point, and this revealed a 2-6 fold increase in  
243 P-Thr<sup>906</sup>, and 5-12 fold increase in P-Thr<sup>1007</sup>, in 6-7 div versus 14-15 div neurons (**Figure**  
244 **3B**). We also measured the total levels, and phosphorylation status of WNK1 and SPAK at  
245 residues required for the activation of these enzymes (26), in the same cultures (**Figure 3A**  
246 **and 3B**). Phosphorylation of WNK1 Ser<sup>382</sup> and SPAK/OSR1 Ser<sup>373</sup>/Ser<sup>325</sup> were also  
247 significantly elevated in 6-7 div neurons compared to 14-15 div neurons. These results show  
248 that KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> inhibitory phosphorylation, and WNK1 pathway activating  
249 phosphorylation, is elevated in immature neurons relative to mature neurons.

250

251 **Alteration of KCC2 Thr<sup>906</sup>/Thr<sup>1007</sup> phosphorylation modulates KCC2 function and**  
252 **GABA activity**

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

270

271 **KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation regulates cotransporter activity by altering**  
272 **its surface expression**

273 How does KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation alter transporter activity?  
274 Phosphorylation could affect the intrinsic transport activity of molecules, or alternatively,  
275 could affect the transport of functional molecules to or from the cell surface. We assessed the

276 surface expression of WT KCC2, KCC2<sup>E/E</sup> and KCC2<sup>A/A</sup> using a previously described KCC2  
277 construct that harbors a tag (pHluorin) in an external loop of KCC2 (KCC2-pH<sub>ext</sub>, (42)). The  
278 multi-step immunolabelling protocol of living 10 div cultured hippocampal neurons  
279 expressing KCC2-pH<sub>ext</sub> mutants allowed visualization of the total KCC2-pH<sub>ext</sub> protein (F<sub>t</sub>,  
280 total fluorescence), transporter expressed at the cell surface (F<sub>m</sub>, membrane fluorescence),  
281 and transporter internalized during 2-hour period of incubation with primary anti-GFP  
282 antibody (F<sub>i</sub>, internalized fluorescence, see **Figure 4B** for scheme and Materials and Methods  
283 for details). Analysis revealed that while the levels of the intracellular expression of WT  
284 KCC2-pH<sub>ext</sub> and its KCC2<sup>E/E</sup>-pH<sub>ext</sub> and KCC2<sup>A/A</sup>-pH<sub>ext</sub> mutants were similar (**Figure 4C and**  
285 **4D**), the intensity of the surface-expressed pool of KCC2 Thr<sup>906</sup> and Thr1007 mutants  
286 strongly differed from that of WT KCC2. The phospho-mimetic KCC2<sup>E/E</sup>-pH<sub>ext</sub> mutant  
287 showed an almost three fold lower level of the surface expression than the non-  
288 phosphorylatable KCC2<sup>A/A</sup>-pH<sub>ext</sub> transporter mutant. Similar to electrophysiology studies  
289 shown in **Figure 4A**, WT KCC2-pH<sub>ext</sub> exhibited intermediate values. Analysis of the  
290 fluorescence intensities emitted by internalized clusters in neurons expressing phospho-  
291 mimetic KCC2<sup>E/E</sup>-pH<sub>ext</sub> revealed a prominent accumulation of latter in the region of soma,  
292 whereas the non-phosphorylatable KCC2<sup>A/A</sup>-pH<sub>ext</sub> mutant showed much less internalized  
293 clusters distributed along the length of dendrites (**Figure 4C**). Neurons expressing WT  
294 KCC2-pH<sub>ext</sub> exhibited an intermediate pattern of staining. Overall, the relative F<sub>i</sub> signal from  
295 KCC2<sup>E/E</sup>-pH<sub>ext</sub> expressing neurons was 4-fold stronger than KCC2<sup>A/A</sup>-pH<sub>ext</sub> mutant. The mean  
296 values of F<sub>i</sub> in WT KCC2-pH<sub>ext</sub>-expressing neurons were similar to those of KCC2<sup>A/A</sup>-pH<sub>ext</sub>  
297 mutant, although they showed higher degree of variability (**Figure 4D**). Related to cultured  
298 hippocampal neurons, in mouse Neuro2A (N2a) cells, immortalized neuronal-like cells  
299 without significant endogenous KCC2, expression of KCC2<sup>E/E</sup> exhibited a significantly lower  
300 Cl<sup>-</sup>-extrusion capacity, as measured using non-invasive Cl-Sensor (43), than cells expressing

301 KCC2<sup>A/A</sup> (**Figure 5A-5D**). The Cl<sup>-</sup>-extrusion ability of WT KCC2 in these cells was similar  
302 to that of KCC2<sup>E/E</sup> mutant (**Figure 5D**). Consistent with this finding, the phosphorylation of  
303 KCC2 at Thr<sup>906</sup> and Thr<sup>1007</sup>, assessed using specific phospho-antibodies that recognize these  
304 sites (26), revealed a robust level of baseline KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation (e.g.,  
305 see **Figure 6F**). These data suggest KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation reduces  
306 transporter activity by enhancing its internalization rate and decreasing its surface expression  
307

### 308 **Catalytic inhibition of WNK1 activates KCC2 and decreases KCC2 inhibitory** 309 **phosphorylation at Thr<sup>906</sup> and Thr<sup>1007</sup>**

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

321 We next utilized a chemical genetic approach (44, 45) to test whether specific  
322 inhibition of WNK1 kinase activity alters the activity and phosphorylation of KCC2 (**Figure**  
323 **6 and Supplementary Figure 2**). Many active site kinase inhibitors are relatively  
324 promiscuous due to residue conservation in the ATP binding site. The essential feature of the  
325 chemical genetic approach is that it combines a genetic change, in which a kinase-of-interest

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

336 WNK1-AS utilized N<sup>6</sup>-benzyl-ATP- $\gamma$ -S with much greater efficiency than WNK1-CA  
337 in autophosphorylation reactions (**Supplementary Figure 2A**) and catalyzed the transfer of  
338 benzyl-ATP- $\gamma$ -S to a known WNK1 substrate, OSR1, (**Supplementary Figure 2B**), but  
339 failed to transfer benzyl-ATP- $\gamma$ -S to KCC2 directly (**Supplementary Figure 2B**), suggesting  
340 WNK1 does not directly phosphorylate KCC2. WNK1-AS was also inhibited by 1-NA-PP1  
341 in a dose-dependent fashion (**Supplementary Figure 2C**). We exploited the sensitivity of  
342 WNK1-AS to inhibition with 1-NA-PP1 to test the effect of inhibiting WNK1 kinase activity  
343 on KCC2 function and Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation (**Figures 6**). We transfected N2a  
344 cells with WNK1-AS or WNK1-CA and measured KCC2-mediated Cl<sup>-</sup> extrusion in the  
345 presence or absence of 1-NA-PP1. The application of 1-NA-PP1 (10  $\mu$ M for 10 min) to cells  
346 co-expressing KCC2 and WNK1-CA had no effect on the KCC2-dependent fluorescence  
347 recovery after loading with Cl<sup>-</sup> (**Figure 6B and 6E**). However, in cells co-expressing KCC2  
348 and WNK-AS, 1-NA-PP1 produced a robust acceleration of KCC2-dependent fluorescence  
349 recovery by 3.4 $\pm$ 0.9 min (**Figure 6C and 6E**). Similar to experiments with WNK1-DN (see  
350 **Figure 5F**), KCC2 activation was reversed by substituting expression of phospho-mimetic

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

354 To directly test whether WNK1 inhibition alters KCC2 phosphorylation at Thr<sup>906</sup> and  
355 Thr<sup>1007</sup>, we chemically inhibited WNK1-AS with 1-NA-PP1 and individually assessed the  
356 phosphorylation status of KCC2 at Thr<sup>906</sup> and Thr<sup>1007</sup> using phospho-specific antibodies that  
357 recognize these sites (26) (**Figure 6F**). Lysates from N2a cells co-expressing HA-tagged  
358 KCC2 and WNK1-CA or WNK1-AS, with or without exposure to 1-NA-PP1 (10  $\mu$ M for 2  
359 hours), were harvested, subjected to SDS-PAGE, and the phosphorylation status of Thr<sup>906</sup> and  
360 Thr<sup>1007</sup> was assessed by Western blotting with phospho-specific antibodies. Chemical  
361 inhibition of WNK1 activity resulted in a significant decrease in the phosphorylation of both  
362 Thr<sup>906</sup> and Thr<sup>1007</sup>, with a mildly stronger effect on the Thr<sup>906</sup> residue (**Figure 6F**). This effect  
363 was not seen in cells expressing WNK1-AS without 1-NA-PP1 exposure, or in cells  
364 expressing WNK1-CA with 1-NA-PP1 exposure, suggesting a specific effect of inhibition of  
365 WNK1 catalytic activity. Together, these results show WNK1 regulates KCC2 Thr<sup>906</sup> and  
366 Thr<sup>1007</sup> inhibitory phosphorylation, and WNK1 inhibition activates KCC2 by decreasing this  
367 phosphorylation.

368

### 369 **WNK1 and SPAK kinase forms a physical complex with KCC2 in immature neurons** 370 **and the developing mouse brain**

371 WNK1 regulates KCC2 activity by modulating its phosphorylation at Thr<sup>906</sup> and  
372 Thr<sup>1007</sup>, but evidence suggests this is achieved via direct phosphorylation by other kinases.  
373 SPAK kinase is a known downstream kinase of WNK1, and its phosphorylation by WNK1 is  
374 required for its activation and phosphorylation (28). The KCC2a isoform processes an RFXV  
375 motif in its N terminus, which is an SPAK/OSR1 binding site; this RFXV motif is also



376 present in WNK1 (47). KCC2b isoform does not have this putative SPAK-binding site  
377 (RFxV), and there are 40 unique N-terminal amino acid residues difference between KCC2a  
378 and KCC2b (47). SPAK phosphorylates all KCC isoforms, including KCC2, at KCC2  
379 Thr<sup>1007</sup>, but the direct kinase phosphorylating Thr<sup>906</sup> is unknown (26). Since the WNK  
380 kinases often physically interact with the CCCs they regulate (48), we investigated whether  
381 WNK1 associated with KCC2. Controlled, reciprocal co-immunoprecipitation experiments  
382 with specific antibodies (26) revealed that WNK1, along with SPAK, form a physical  
383 complex with KCC2 in both immature cultured hippocampal and cortical neurons (**Figure**  
384 **7A**) and the developing mouse brain (**Figure 7B**). These results suggest WNK1 could  
385 regulate KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation by serving as scaffold that bridges SPAK  
386 to KCC2 for phosphorylation of Thr<sup>1007</sup> and potentially another yet undefined kinase that  
387 directly phosphorylates Thr<sup>906</sup>.

388

389 **DISCUSSION**

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

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

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

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

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

438 Previous studies suggested the existence of at least two distinct mechanisms of KCC2  
439 regulation during development, including the Neurotropic Factor (NF)–dependent up-  
440 regulation of *KCC2* transcription in immature neurons (reviewed by (51, 52), and the post-  
441 translational regulation of KCC2 activity via (de)phosphorylation (reviewed by (25, 52)). In  
442 immature neurons, KCC2 is phosphorylated and almost fully inactive despite clear neuronal  
443 expression of the KCC2 protein (15, 19, 20, 24); indeed, phosphorylation at Thr<sup>906</sup> is reduced  
444 33% by P3 and >90% by P21; in the adult, phosphorylation at Thr<sup>906</sup> is negligible (16) when  
445 the ion-transport activity of KCC2 is maximal (14, 19). Despite this inverse correlation  
446 between the level of KCC2 phosphorylation at these sites and transporter activity, little  
447 experimental data to date has linked these two phenomena. So far, only two studies showed  
448 that staurosporine, a broad kinase inhibitor, produces a rapid and potent stimulation of KCC2  
449 activity in immature but not mature cultured hippocampal neurons (19) and immature  
450 (E18.5), but not more mature (P7) cortical slices (24). Here, we identify a specific kinase  
451 (WNK1) that contributes to the developmental control of the KCC2 activity, reveal its likely  
452 mechanism of action (promoting Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation), and report a novel way  
453 of facilitating Cl<sup>-</sup> extrusion via KCC2 by developing a chemical genetic method of WNK1  
454 kinase inhibition.

455 What are the drivers contributing to maintenance of a high level of KCC2 inhibitory  
456 phosphorylation and low level of its activity? One could be ongoing neuronal activity.  
457 Immature neuronal networks in organisms from worms to primates are characterized by the  
458 existence of ongoing neuronal activity and synchronous oscillations of intracellular Ca<sup>2+</sup> that  
459 are result of synergistic depolarizing actions of both GABA and glutamate (39). During  
460 development, the strength of the depolarizing action of GABA decreases due to progressive  
461 KCC2-mediated Cl<sup>-</sup> extrusion, and synchronous oscillations of Ca<sup>2+</sup> and neuronal activity  
462 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<sup>906</sup> and Thr<sup>1007</sup> 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<sup>-</sup>  
469 cotransporters (CCCs) (7), and are sensors of intracellular Cl<sup>-</sup> concentration, extracellular  
470 osmolarity, and cell volume, transducing signals about changes in these parameters to the  
471 CCCs to regulate transporter activity. The *PRKWINK1* gene encodes multiple alternatively-  
472 spliced WNK1 isoforms. One isoform is mutated in Mendelian disease featuring impaired Cl<sup>-</sup>  
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<sup>1007</sup> but not Thr<sup>906</sup> (26). The kinase regulating KCC2 Thr<sup>906</sup>  
483 phosphorylation is currently unknown. However, WNK1 knockdown in HEK-293 cells  
484 decreases KCC3 phosphorylation at Thr<sup>991</sup>, a site homologous to Thr<sup>906</sup> 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<sup>906</sup> and Thr<sup>1007</sup> in a kinase-dependent manner. However, WNK1 is likely

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

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

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

509       Lastly, our results in neurons suggest inhibition of WNK signaling in the CNS might be  
510 a novel means of enhancing neuronal Cl<sup>-</sup> extrusion to restore GABAergic inhibition by  
511 stimulating KCC2. This might be of value neuropsychiatric conditions in which KCC2  
512 activity is suppressed and GABAergic disinhibition fosters the hyperexcitability of neurons

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

527

528

529 **MATERIALS AND METHODS**530 **Animals**

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

533

534 **Primary cultures and transfection of rat hippocampal neurons**

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

538 Hippocampi and cortices from 18-day-old rat embryos were dissected and then  
539 dissociated using trypsin and plated at a density of 70,000 cells cm<sup>-2</sup> in minimal essential  
540 medium (MEM) supplemented with 10% NU serum (BD Biosciences, Le Pont de Claix,  
541 France), 0.45% glucose, 1 mM sodium pyruvate, 2 mM glutamine and 10 IU ml<sup>-1</sup>  
542 penicillin–streptomycin as previously described (Buerli et al. 2007). On days 7, 10 and 13 of  
543 culture incubation, half of the medium was changed to MEM with 2% B27 supplement  
544 (Invitrogen). For physiology and immunocytochemistry experiments neurons were plated in  
545 35 mm culture dishes containing 14 mm coverslips. For Western Blot experiments neurons  
546 were plated in 60 mm dishes (4 dishes per culture and *in vitro* age).

547 Transfections of neuronal cultures were performed at 4 div (for recordings on 6-7 div)  
548 and 10 div (for recordings on 13-15 div) as described previously (Buerli et al. 2007). For  
549 transfection of cultures growing in 35 mm dishes, 300 µL of Opti-MEM media was mixed  
550 with 7 µL of Lipofectamine reagent 2000 (Invitrogen), 1 µL of Magnetofection CombiMag  
551 (OZ Biosciences, France) and 1–1.5 µg of different pcDNAs pre-mixed in desired  
552 proportions. The mixture was incubated for 20 min at room temperature (RT) and thereafter  
553 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<sup>+</sup> buffer was 95 mM  
575 NaCl, 50 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM Na<sub>2</sub>SO<sub>4</sub> and 20 mM  
576 HEPES (pH 7.4). Hypotonic high K<sup>+</sup> buffer was 80 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>,  
577 1mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM Na<sub>2</sub>SO<sub>4</sub> and 20 mM HEPES (pH 7.4). Isotonic buffer was 135 mM  
578 NaCl, 5 mM KCl, 0.5 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5mM Na<sub>2</sub>SO<sub>4</sub> and 15

579 mM HEPES (pH 7.5). Hypotonic low chloride buffer was 67.5 mM Na-gluconate, 2.5 mM  
580 K-gluconate, 0.25 mM CaCl<sub>2</sub>, 0.25 mM MgCl<sub>2</sub>, 0.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM Na<sub>2</sub>SO<sub>4</sub> and 7.5  
581 mM HEPES (pH 7.5).

582

### 583 **Phospho-antibody immunoprecipitations**

584 KCCs phosphorylated at the KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> equivalent residue were  
585 immunoprecipitated from clarified hippocampal and cortical culture lysates. The phospho-  
586 antibody was coupled with protein-G–Sepharose at a ratio of 1 mg of antibody per 1 mL of  
587 beads. A total of 2 mg of clarified cell lysate were incubated with 15 µg of antibody  
588 conjugated to 15 µL of protein-G–Sepharose in the presence of 20 µg/mL of lysate of the  
589 corresponding dephosphopeptide. Incubation was for 2 hours at 4°C with gentle agitation,  
590 and the immunoprecipitates were washed three times with 1 mL of lysis buffer containing  
591 0.15 M NaCl and twice with 1 mL of buffer A. Bound proteins were eluted with 1X LDS  
592 sample buffer.

593

### 594 **Immunoblotting**

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

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

608

### 609 **Electrophysiology recordings**

610 The gramicidin-perforated whole cell patch-clamp recordings from transfected neurons  
611 (eGFP-positive) were performed according to protocols described previously (41, 63). The  
612 external solution contained (in mM): 140 NaCl, 2.5 KCl, 20 HEPES, 20 D-glucose, 2.0  
613 CaCl<sub>2</sub>, 2.0 MgCl<sub>2</sub>, 0.001 tetrodotoxin and 0.0003 strychnine, pH 7.4. Coverslips with  
614 transfected neuronal cells were placed onto an inverted microscope and perfused via a fast  
615 perfusion system placed in front of the recording neuron to insure removal of trace amounts  
616 of gramicidin that could diffuse from the patch pipette. Patch pipettes (5 MΩ) were filled  
617 with a solution containing (in mM): KCl 150, HEPES 10, 20 μg·mL<sup>-1</sup> gramicidin A, pH 7.2.  
618 The gigaseals were formed by rapid 5-10 s approaching of the patch pipette to neuronal  
619 surface without applying positive pressure (to diminish leak of gramicidin). After sealing,  
620 series resistance (R<sub>s</sub>), membrane resistance (R<sub>m</sub>) and neuron capacitance (C) were monitored  
621 routinely at holding potential (V<sub>h</sub>) -80 with 5 mV hyperpolarizing pulses, typically taking 10-  
622 15 min for the series resistance to stabilize at 15–60 MΩ. Membrane potential values were  
623 corrected off-line for junction potential (V<sub>J</sub>) between the pipette and bath solutions (-4.0 mV)  
624 and series resistance as described (64, 65).

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

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

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

639

#### 640 ***In utero* electroporation**

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

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

657

### 658 **Cl-Sensor fluorescence recordings from brain slices**

659 Experiments were performed on acute transverse cortical slices (350  $\mu\text{m}$ ) that were cut  
660 into ice-cold (2–4°C) artificial cerebrospinal fluid (ACSF) composed of (mM): NaCl, 126;  
661 KCl, 3.5; CaCl<sub>2</sub>, 2; MgCl<sub>2</sub>, 1.3; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25; glucose, 11; pH 7.4, when  
662 equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> using a Vibratome (VT1000E; Leica, Nussloch,  
663 Germany). After cut slices were maintained in oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.3)  
664 ACSF at RT for at least 1 hour before use. Individual slices were transferred to a specially  
665 designed recording chamber where they were fully submerged and superfused with  
666 oxygenated ACSF (complemented with 1  $\mu\text{M}$  tetrodotoxin, 1  $\mu\text{M}$  strychnine and 10  $\mu\text{M}$   
667 NBQX to prevent spontaneous neuronal activity and non-controlled [Cl<sup>-</sup>]<sub>i</sub> changes) at 30–  
668 32°C at a rate of 2–3 mL/min. The acquisition of fluorescence images was performed using a  
669 customized imaging set-up and consecutive cells excitation at 430 and 500 nm as described  
670 previously (43). The frequency of acquisition was 0.05 Hz. The duration of excitation was  
671 selected to avoid use-dependent bleaching of the signal as described and was kept identical  
672 for all experiments (43). The applications of the ACSF solution containing isoguvacine (30  
673  $\mu\text{M}$ ) or KCl (25 mM) + isoguvacine (30  $\mu\text{M}$ ) were performed via a perfusion system. The  
674 recovery of fluorescence after Cl<sup>-</sup> overload produced by KCl + isoguvacine was recorded in  
675 ACSF containing 10  $\mu\text{M}$  Bicuculline in addition to mentioned above blockers to avoid Cl<sup>-</sup>  
676 efflux through GABA<sub>A</sub>R.

677

678 **Immunocytochemistry and quantitative immunofluorescence analysis of primary**  
679 **neuronal cultures**

680 For immunocytochemistry on living neurons, rabbit anti-GFP antibodies were diluted in  
681 culture media and applied above neurons for 2 hours at 37°C, 5% CO<sub>2</sub>. Neurons were then  
682 washed for 10 min (RT) in HEPES-buffered saline solution containing (in mM): NaCl 150,  
683 KCl 2.5, MgCl<sub>2</sub> 2.0, CaCl<sub>2</sub> 2.0, HEPES 20 and D-glucose 10, pH 7.4, labelled with anti-  
684 rabbit Cy3 antibody (dissolved in the HEPES-buffered saline) for 20 min at 13°C and fixed in  
685 Antigenfix (Diapath, Martingo, Italy). To reveal intracellular proteins, cells were  
686 subsequently permeabilized with 0.3% Triton X-100, blocked by 5% goat serum, labeled  
687 overnight (4°C) with mouse anti-GFP antibody and for 1 hour (RT) with anti-mouse Alexa-  
688 488 antibody. Cell nuclei were stained using 5-min staining with Hoechst 33258 (1µg/mL,  
689 Sigma-Aldrich).

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

700

701 **Constructs and materials**

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

712 shRNA KCC2 in mU6-pro vector was described previously (41) and targeted the  
713 following sequence in rat KCC2 mRNA GACATTGGTAATGGAACAACG (NP\_599190).  
714 A control construct with a scrambled sequence (GATGAACCTGATGACGTTC) lacked  
715 homology to any known mammalian mRNAs. shRNA WNK1 (OriGene Technologies, Inc,  
716 Rockville, MD USA in pRFP-C-RS retroviral vector) targeted the  
717 CATTTCAGATGTTGCTTCTGGTATGAGTGA sequence of rat WNK1 mRNA  
718 (NM\_001002823), and is predicted to knockdown known isoforms of *WNK1* in the brain,  
719 including *HSN2*.

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

727

728 **Antibodies**

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

734 Antibodies used for Western Blots included antibodies were raised in sheep and  
 735 affinity-purified on the appropriate antigen by the Division of Signal Transduction Therapy  
 736 Unit at the University of Dundee:]; KCC2a total antibody [residues 1-119 of human KCC2a];  
 737 KCC3a phospho-Thr<sup>991</sup> [residues 975-989 of human KCC3a phosphorylated at Thr<sup>991</sup>,  
 738 SAYTYER(T)LMMEQRSRR, corresponding to residues of rat KCC2 phosphorylated at  
 739 Thr<sup>906</sup>, SAYTYEK(T)LMMEQRSRR ]; KCC2a phospho-Thr<sup>906</sup> [residues 975-989 of human  
 740 KCC3a phosphorylated at Thr<sup>991</sup>, SAYTYER(T)LMMEQRSRR ]; KCC3a phospho-  
 741 Thr<sup>1039/1048</sup> [residues 1032-1046 or 1041-1055 of human KCC3a phosphorylated at Thr  
 742 <sup>1039/1048</sup>, CYQEKVHM(T)WTKDKYM, corresponding to residues of rat KCC2  
 743 phosphorylated at Thr<sup>1006</sup>, TDPEKVHLTW(T)KDKSV]. NKCC1 total antibody [residues 1-  
 744 288 of human NKCC1]; NKCC1 phospho-Thr<sup>203</sup>/Thr<sup>207</sup>/Thr<sup>212</sup> antibody [residues 198-217 of  
 745 human NKCC1 phosphorylated at Thr<sup>203</sup>, Thr<sup>207</sup> and Thr<sup>212</sup>,  
 746 HYYYD(T)HTN(T)YYLR(T)FGHNT]; WNK1-total antibody [residues 2360-2382 of  
 747 human WNK1]; WNK1phospho-Ser<sup>382</sup> antibody [residues 377-387 of human WNK1  
 748 phosphorylated at Ser<sup>382</sup>, ASFAK(S)VIGTP]; SPAK-total antibody [full-length GST-tagged  
 749 human SPAK protein]; SPAK/OSR1 (S-motif) phospho-Ser<sup>373</sup>/Ser<sup>325</sup> antibody [367–379 of  
 750 human SPAK, RRVPGS(S)GHLHKT, which is highly similar to residues 319–331 of human  
 751 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- $\beta$ -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 N<sup>6</sup>-  
770 substituted ATP $\gamma$ S (N<sup>6</sup> Benzyl-ATP- $\gamma$ -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-

777 expressed with HA-OSR1 or HA-KCC2 in N2A cells in 10-cm dishes. After 48 hours, cells  
778 were treated with 1-NA-PP1 (1  $\mu$ M or 10  $\mu$ M) or vehicle control (DMSO) for 2 hours before  
779 media was removed. Cells were then rinsed once with 5 mL cold PBS, harvested with a  
780 scraper, and lysed on ice in 500  $\mu$ L of 1X RIPA buffer + 1X protease inhibitor + 1X  
781 phosphatase inhibitor. Cells were then centrifuged at 10,000 x g for 10 min at 4°C to remove  
782 cell debris. Supernatant was saved, and to this was added 100  $\mu$ M N<sup>6</sup> Benzyl-ATP- $\gamma$ -S, 100  
783  $\mu$ M ATP, and 3 mM GTP to each sample to initiate the substrate labeling reaction.  
784 Thiophosphorylation of kinase substrates was allowed to occur for 20 min at RT. The  
785 reaction was quenched with 500  $\mu$ L 1X RIPA buffer + 40 mM EDTA (final [EDTA] = 20  
786 mM) + 5 mM PNBM (final [PNBM] = 2.5 mM), and then alkylated with PNBM for 1 hour at  
787 RT on a rotator. 40  $\mu$ L of a 50% slurry of appropriate antibody-tagged magnetic beads  
788 (Bethyl labs) were to each sample and incubate 3–4 hours at 4°C on a rotator. Before use,  
789 beads were washed once with 1 mL 1X RIPA buffer and then resuspended in the original  
790 slurry volume and add to each sample. A magnet was used to collect the beads, washed 5  
791 times w/1 mL 1X RIPA + protease inhibitor + phosphatase inhibitor, and then resuspended in  
792 20  $\mu$ L 1X RIPA + protease inhibitor + phosphatase inhibitor +1X Laemmli sample buffer.  
793 Samples were heated at 95°C for 2.5 min, and then loaded onto a SDS-PAGE gel. Western  
794 blotting was performed with anti-HA, anti-Myc, or anti-Thiophosphate Ester Rabbit  
795 Monoclonal Antibody (Epitomics). Assays with anti-KCC phospho-antibodies were  
796 performed essentially as described in (26), though the above Myc-tagged WNK1 plasmids  
797 and HA-tagged KCC2 were transiently transfected into N2A cells.

798

### 799 **Statistical analysis**

800 For the electrophysiology recordings each condition (i.e. shRNA WNK1) was studied  
801 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  $\pm$  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  $\pm$  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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984

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

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

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

996 C.  $E_{GABA}$  in neurons of different age transfected with eGFP plus the constructs  
997 indicated in the legend. Data represented is pooled from 4-5 experiments for each construct.  
998 1-3 neurons per experiment. \*\*\* $p$ < 0.001.

999 D. Examples of focal  $I_{GABAA}$  recorded at  $V_h=-80$  mV before and after neuronal  
1000  $Cl^-$  loading achieved using combination of 10 s isoguvacine pulse and neuron depolarization  
1001 to -20 mV. The kinetics of the recovery reflects neuronal  $Cl^-$  clearance.

1002 E. Mean  $\pm$  SEM half-time of  $I_{GABAA}$  recovery in neurons expressing indicated  
1003 constructs. \* $p$ < 0.05; \*\* $p$ < 0.01.

1004

1005

1006 **Figure 2. WNK1-dependent regulation of Cl-Sensor ratiometric fluorescence in acute**  
1007 **cortical slices prepared from rats of postnatal days 3-5 and 30.** Rats were electroporated  
1008 *in utero* at embryonic day 15 with Cl-Sensor plus scrambled shRNA (Sbl) or shRNA<sub>WNK1</sub>  
1009 (shWNK1). Data represent results obtained from 5 rats per experimental condition. 2-3 slices  
1010 were recorded per animal.

1011 A. Typical images of Cl-Sensor fluorescence excited at 500 nm and taken at different  
1012 optical magnifications. The dotted white line indicates the slice border. The ROI were drawn  
1013 around the soma of cells located in the focal plane.

1014 B, F. Typical ratiometric fluorescence ( $R_{430/500}$ ) recordings from different neurons  
1015 (encoded by different colors). Vertical bars indicate times of applications of ACSF containing  
1016 isoguvacine (iso) or iso + 25 mM of KCl. Arrows and arrowheads indicate different types of  
1017 responses described in Results section.

1018 C, G. Mean  $\pm$  SEM of basal levels of  $R_{430/500}$  measured before iso application. ns – non  
1019 significant, n=5.

1020 D, H. Bar graphs illustrating the mean  $\pm$  SEM of fluorescence change  $\Delta R/R$ , where R is  
1021 mean of 5 measurements before iso application and  $\Delta R$  is difference between absolute  
1022 maximum of iso-induced response and R. ns – non significant.  $**p < 0.01$ , n=5.

1023 E, I. Half-decay times of the fluorescence recovery after neuron's loading with Cl<sup>-</sup>. ns –  
1024 non significant.  $**p < 0.01$ , n=5.

1025

1026 **Figure 3. WNK-SPAK/OSR1 regulation and phosphorylation of endogenous KCC2 in**  
 1027 **immature and mature cultured hippocampal and cortical neurons.**

1028 A. Harvested lysates were subjected to immunoprecipitation (IP) with the indicated  
 1029 KCC2 total-, and Thr<sup>906</sup> and Thr<sup>1007</sup> phospho-antibodies. The immunoprecipitates were then  
 1030 immunoblotted with the indicated specific KCC2 antibody. Whole cell lysates were also  
 1031 subjected to immunoblot analysis with the indicated total and phospho-specific antibodies.  
 1032 The Western blots illustrate results obtained in three separate experiments. Both KCC2  
 1033 dimers and KCC2 monomers are indicated with arrows. Molecular masses are indicated in  
 1034 kDa on the left-hand side of the Western blots.

1035 B. The lower panel shows quantification of the results of the Western blots, as assessed  
 1036 by an unpaired t-test (n=3, error bars represent the mean ± SEM.) The quantification (ratio  
 1037 calculation) is based on (phospho-dimeric KCC2 + phospho-monomeric KCC2) / (total  
 1038 dimeric KCC2 + monomeric KCC2). \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; ns - non  
 1039 significant.

1040

1041 **Figure 4. Genetic modulation of KCC2 Thr<sup>906</sup> and Thr<sup>1007</sup> phosphorylation affects**  
 1042 **E<sub>GABA</sub> and the plasmalemmal surface expression of KCC2 in cultured hippocampal**  
 1043 **neurons.**

1044 A. E<sub>GABA</sub> recorded in 10 div neurons that were transfected at 7 div with constructs as  
 1045 indicated. Recordings were made using gramicidin-perforated patch-clamp, as in Figure 1A.  
 1046 Numbers in columns indicate number of neurons recorded. Data are pooled from 5 separate  
 1047 experiments, with 1-3 neurons per experiment. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; ns - non  
 1048 significant.

1049 B. Scheme of the multi-step immunolabelling protocol (see Materials and Methods for  
 1050 details).

1051 C. Representative images illustrating membrane staining (F<sub>m</sub>, left column) and  
 1052 internalized fluorescence (F<sub>i</sub>, right column) of WT and mutated forms of KCC2 with external  
 1053 tag (KCC2-pH<sub>ext</sub>) as indicated. Neuronal shape is shown in light green in each image  
 1054 respectively.

1055 D. Normalized mean  $\pm$  SEM of total protein, membrane staining ( $F_m$ ) and internalized  
1056 fluorescence ( $F_i$ ) for each KCC2-pH<sub>ext</sub> constructs (pooled data from 3 cultures, 5-8 cells per  
1057 culture and condition). \*\*\*,  $p < 0.001$ , \*\*,  $p < 0.01$ , ns - non significant.

1058 **Figure 5. Phospo-mimetic and non-phosphorylatable KCC2 Thr<sup>906</sup>/Thr<sup>1007</sup> mutants**  
1059 **exhibit different Cl<sup>-</sup>-extrusion capacities and sensitivity to dominant-negative WNK1**

1060 A. Fluorescence signals recorded from N2a cells co-transfected with Cl-Sensor (green),  
1061 GlyR (non-fluorescent) and mCherry-KCC2 (red). Scale bar = 20  $\mu$ m.

1062 B. Individual traces of Cl-Sensor fluorescence ratio changes measured in N2a cells  
1063 expressing KCC2<sup>E/E</sup>. Horizontal bar indicates the time of application of 100 mM KCl and 50  
1064  $\mu$ M glycine to load cells with Cl<sup>-</sup>. The ordinate axis indicates the ratio of Cl-Sensor  
1065 fluorescence measured at 430 and 500 nm excitation wavelengths ( $R_{430/500}$ ).

1066 C. Normalized mean traces of Cl-Sensor ratiometric fluorescence change in N2a cells  
1067 expressing different KCC2 constructs as indicated. The inset illustrates the full record of  
1068  $R_{430/500}$  fluorescence from mock-transfected cells shown in the main plot.

1069 D, E, F. Mean  $\pm$  SEM of the half-decay time of Cl<sup>-</sup> extrusion after glycine + KCl  
1070 application in cells expressing different combinations of constructs as indicated. n = 4 to 5  
1071 experiments. \*\*,  $p < 0.01$ , \*,  $p < 0.05$ , ns: non-significant.

1072

1073 **Figure 6. Chemical genetic inhibition of WNK1 stimulates KCC2 activity.**

1074 A. Engineering a chemical genetic switch of WNK1 kinase activity. Upper left panel  
1075 depicts the ATP-binding site of WT Src kinase with ATP (green, left) and analog-sensitive  
1076 (AS) Src-AS with N<sup>6</sup> Benzyl-ATP- $\gamma$ -S (yellow, right). Mutation in the gatekeeper residue  
1077 (blue) results in an affinity pocket where bulky ATP analogs (like Benzyl-ATP- $\gamma$ -S) bind.

1078 B, C, D. Representative normalized mean  $\pm$  SEM traces of the ratiometric Cl-Sensor  
1079 fluorescence recordings from 8-10 cells in the same experiments before and 10 min after cells  
1080 incubation with 10  $\mu$ M of 1-NA-PP1.

1081 E. Difference between the half-decay times of Cl<sup>-</sup> extrusion produced by application of  
1082 1-NA-PP1 in cells expressing different combinations of WNK1 and KCC2 related constructs  
1083 as indicated. Mean  $\pm$  SEM of 4 experiments in each series. ^^ ( $p < 0.01$ ) and ns (non-  
1084 significant) indicate the effect of 1-NA-PP1 (paired  $t$ -test). \*\* ( $p < 0.01$ ) indicates the  
1085 difference between groups (ANOVA test).

1086 F. Chemical genetic inhibition of WNK1 kinase activity decreases KCC2  
1087 phosphorylation at P-Thr<sup>906</sup> and Thr<sup>1007</sup>. Myc-tagged WNK1-CA or WNK1-AS was  
1088 transiently expressed with or without HA-KCC2 in N2a cells in the absence or presence of 1-  
1089 NA-PP1 (10  $\mu$ M for 2 hours) to inhibit WNK1-AS but not WNK1-CA activity. Lysates were  
1090 harvested, subjected to SDS-PAGE and the phosphorylation at Thr<sup>906</sup> and Thr<sup>1007</sup> was  
1091 assessed by Western blotting with phospho-specific antibodies directed against these residues  
1092 as described in Materials and Methods. (\*) Indicates statistically significant differences  
1093 relative to control conditions as assessed by an unpaired  $t$ -test ( $p < 0.01$ ;  $n = 3$ ). Error bars  
1094 represent the mean  $\pm$  SEM.

1095

1096 **Figure 7. KCC2 interacts with WNK1 and SPAK *in vivo*.**

1097       A. 7 div and 14 div lysates of hippocampal cultures (Sample 2, as indicated in Figure 3)  
1098 and cortical cultures (Sample 2) were immunoprecipitated (IP) with the indicated WNK1 and  
1099 SPAK antibodies, and analyzed for their interacting partners using standard SDS-PAGE and  
1100 immunoblotting (IB) techniques.

1101       B. Whole-brain lysates obtained from P2 mice were immunoprecipitated (IP) with the  
1102 indicated WNK1, SPAK and KCC2 antibodies and analyzed for their interacting partners  
1103 using standard SDS-PAGE and immunoblotting (IB) techniques.

1104

1105 **Figure 8. A model of WNK1-dependent control of neuronal Cl<sup>-</sup> during development.**

1106 In immature neurons (left), the functional activity of KCC2 is low, such that NKCC1-  
1107 mediated Cl<sup>-</sup> loading predominates and the intraneuronal Cl<sup>-</sup> concentration [Cl<sup>-</sup>]<sub>i</sub> is relatively  
1108 elevated. Consequently, GABA<sub>A</sub>R activation elicits membrane depolarization. In mature  
1109 neurons (right), the functional activity of KCC2 is high, such that KCC2-mediated Cl<sup>-</sup>  
1110 extrusion predominates, [Cl<sup>-</sup>]<sub>i</sub> is low, and GABA<sub>A</sub>R activation triggers membrane  
1111 hyperpolarization. Our data suggest WNK1 kinase, complementing other mechanisms that  
1112 determine KCC2 activity such as the regulation of protein expression, contributes to the  
1113 depolarizing action of GABA in immature neurons by maintaining the inhibitory  
1114 phosphorylation of KCC2 at Thr<sup>906</sup> and Thr<sup>1007</sup>. We therefore propose the antagonism of  
1115 WNK1 kinase could be novel means to potentiate neuronal Cl<sup>-</sup> extrusion in immature  
1116 neurons, or even in mature neurons if KCC2 inhibitory phosphorylation and [Cl<sup>-</sup>]<sub>i</sub> is  
1117 pathologically elevated. Our data suggest that WNK1 regulates the phosphorylation of KCC2  
1118 via SPAK kinase at Thr1007, and via a yet unidentified kinase at Thr906.

1119

1120





1122 **Supplementary Figure 1. Characterization of a specific WNK1 shRNA.**

1123         Rat PC-12 cells were transfected with control firefly (*FF*) luciferase (*luc*) shRNA (left  
1124 lane) or rat *WNK1* shRNA according to Materials and Methods. Lysates were harvested and  
1125 subjected to SDS-PAGE, and assessed by Western blotting with the indicated antibodies (see  
1126 Materials and Methods). *WNK1* shRNA significantly depletes endogenous WNK1 protein in  
1127 rat neuronal cells.

1128

1129

**1130 Supplementary Figure 2. Characterization of a WNK1 analog sensitive (AS) construct.**

1131           A. WNK1-AS utilizes Benzyl-ATP- $\gamma$ -S with much greater efficiency than WNK1-  
1132 CA. Empty vector (control), Myc-tagged WNK1-CA, or WNK1-AS was transiently  
1133 expressed in N2a cells; cell lysate was prepared, and N<sup>6</sup> substituted ATP $\gamma$ S (N<sup>6</sup> Benzyl-ATP-  
1134  $\gamma$ -S) was added to initiate the substrate labeling reaction. Thiophosphorylated proteins were  
1135 alkylated using p-nitrobenzyl mesylate. The indicated WNK1 constructs were  
1136 immunoprecipitated using an anti-Myc antibody, and lysates were subjected to SDS-PAGE  
1137 and immunoblotted with a p-nitrobenzyl-thiophosphate ester specific antibody or the other  
1138 indicated antibodies as described in Materials and Methods.

1139           B. WNK1-AS catalyzes transfer of N<sup>6</sup> Benzyl-ATP- $\gamma$ -S to a known substrate, OSR1.  
1140 In N2a cells, Myc-tagged WNK1-CA, WNK1-AS, and WNK1-DN was transiently expressed  
1141 with HA-OSR1 or HA-KCC2. Cell lysate was prepared and N<sup>6</sup> Benzyl-ATP- $\gamma$ -S was added  
1142 to initiate the substrate labeling reaction. Thiophosphorylated proteins were alkylated as in A,  
1143 and HA-OSR1 or HA-KCC2 was immunoprecipitated from cell lysates with anti-HA  
1144 antibody. Cell lysates or HA-bound immunoprecipitates were subjected to SDS-PAGE, and  
1145 Western blots were performed with the indicated antibodies. WNK1-AS is able to catalyze  
1146 the transfer of N<sup>6</sup> Benzyl-ATP- $\gamma$ -S to OSR1 but not KCC2.

1147           C. WNK1-AS is inhibited by 1-NA-PP1 in a dose-dependent manner. Myc-tagged  
1148 WNK1-CA or WNK1-AS was transiently expressed with or without HA-OSR1 in N2a cells  
1149 in the absence or presence of 1-NA-PP1 at the indicated concentrations. Cell lysate was  
1150 prepared, and N<sup>6</sup> Benzyl-ATP- $\gamma$ -S was added to initiate the substrate labeling reaction.  
1151 Thiophosphorylated proteins were alkylated, and HA-OSR1 was immunoprecipitated from  
1152 the whole cell lysate with anti-HA antibody. Whole cell lysates or OSR1 immunoprecipitates  
1153 were subjected to SDS-PAGE. Western blots were performed with the indicated antibodies.

1154 WNK1-AS is able to catalyze the transfer of N<sup>6</sup> Benzyl-ATP- $\gamma$ -S to OSR1, and this is  
1155 inhibited by 1-NA-PP1 in a dose-dependent manner.