

Modulation of neuronal activity by phosphorylation of the K–Cl cotransporter KCC2

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The K–Cl cotransporter KCC2 establishes the low intraneuronal Cl[−] levels required for the hyperpolarizing inhibitory postsynaptic potentials mediated by ionotropic γ -aminobutyric acid receptors (GABA_AR) and glycine receptors (GlyRs). Decreased KCC2-mediated Cl[−] extrusion and impaired hyperpolarizing GABA_AR- and/or GlyR-mediated currents have been implicated in epilepsy, neuropathic pain, and spasticity. Recent evidence suggests that the intrinsic ion transport rate, cell surface stability, and plasmalemmal trafficking of KCC2 are rapidly and reversibly modulated by the (de)phosphorylation of critical serine, threonine, and tyrosine residues in the C terminus of this protein. Alterations in KCC2 phosphorylation have been associated with impaired KCC2 function in several neurological diseases. Targeting KCC2 phosphorylation directly or indirectly via upstream regulatory kinases might be a novel strategy to modulate GABA- and/or glycinergic signaling for therapeutic benefit.

Modulation of KCC2 activity underlies dynamic changes in neuronal Cl[−] homeostasis and GABAergic signaling during development and in disease

Fast synaptic inhibition in the adult central nervous system (CNS) is chiefly mediated via GABA_AR and GlyRs, which are ligand-gated Cl[−] channels *cf.* [1]. The homeostasis of intraneuronal Cl[−] concentration [Cl[−]]_i, established by the dynamic functional regulation of Cl[−] channels, transporters, and exchangers, is a prime determinant of GABA and glycine function, and endows GABA_AR and GlyRs with a unique functional plasticity [2,3] (Box 1). Although

there have been many investigations of GABA_AR modulation as a determinant of inhibitory synaptic strength [4–7], less attention has been paid to the functional effects of Cl[−] transporter regulation. The molecular mechanisms that regulate [Cl[−]]_i and the dynamic changes in GABA_AR and GlyR signaling during CNS development, synaptic activity, and in neurological diseases [8,9] are only beginning to be understood.

The ability of neurons to maintain low [Cl[−]]_i is dependent upon the neuron-specific K–Cl cotransporter KCC2, the principal Cl[−] extruder in adult neurons [8,10]. KCC2, a member of the cation-chloride cotransporter (CCC) *SLC12A* gene family, utilizes the energetically favorable plasmalemmal K⁺ concentration gradient to extrude Cl[−] beyond electrochemical equilibrium values. Because immature central neurons are characterized by a lower functional expression of KCC2 relative to the Na–K–2Cl cotransporter isoform NKCC1, which mediates Cl[−] uptake, [Cl[−]]_i remains high and GABA_AR activation results in a depolarizing rather than a hyperpolarizing response in these cells [8,11–13]. These depolarizing GABA_AR-mediated responses affect early network activities [14] and activity-dependent synaptic changes required for neuronal migration [15,16] and circuit formation [17–19]. During postnatal brain development, increased functional expression of KCC2 is associated with a change in GABA- and/or glycinergic signaling from depolarizing to hyperpolarizing [8,10]. Indeed, without KCC2, the inhibitory strength of GABAergic signaling is compromised and may result in increased membrane excitability [20–22]. Genetic deficits in KCC2 expression, as seen in *Caenorhabditis elegans* [23], *Drosophila melanogaster* [24,25], and *Mus musculus* [21,26–28], result in the development of network hyperexcitability.

In the mature CNS, depolarizing GABA_AR-mediated responses are often associated with pathological enhancement of excitability [29], although some populations of neurons exhibit these responses normally [30–33], even in the absence of an activity-induced intracellular Cl[−] load

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Box 1. Role of neuronal Cl⁻ homeostasis in GABA/glycinergic signaling

Fast synaptic inhibition in the adult CNS is largely mediated via ionotropic GABA_ARs and GlyRs, ligand-gated anion channels permeable to Cl⁻, *cf.* [1]. Upon receptor binding and channel opening, the electrochemical driving force determines whether the current carried by Cl⁻ is hyperpolarizing or depolarizing. The strength of the inhibitory action of GABA_ARs and GlyRs is determined not only by the respective conductances, but also by the efficacy of Cl⁻ extrusion. Therefore, regarding the role of ion transport in controlling the efficacy of inhibition, the determining factor is the capability of Cl⁻-extruding mechanisms to maintain the reversal potentials of GABA_AR- and GlyR-mediated responses, E_{GABA} and E_{Gly}, at a sufficiently negative level to prevent the neuron from firing action potentials [1,8]. In the somatic and dendritic compartments of many adult neurons at rest, [Cl⁻]_i is maintained at only a few millimoles [2,19,99–101]. Therefore, E_{GABA} and E_{Gly} are sensitive to small changes in [Cl⁻]_i and must be tightly regulated to maintain the strength of inward Cl⁻ flux upon GABA_AR activation. Accordingly, an increasingly recognized form of regulation of GABA_A function, known as 'ionic plasticity' [8,102,103], is based on short- and long-term changes in neuronal [Cl⁻]_i, and is thus highly sensitive to changes in the functional expression of KCC2 [8].

[8,34]. Deficits in KCC2-mediated cotransport, together with a decreased efficacy of GABAergic inhibition and emergence of depolarizing GABA_AR-mediated currents [22,35], have been documented in certain types of epilepsy (e.g., temporal lobe epilepsy) [29,36–38], in neuropathic pain (including hyperalgesia and allodynia related to peripheral inflammation or nerve injury) [39–41], and following traumatic brain and spinal cord injury [42–45].

(De)phosphorylation is a potent but poorly understood mechanism of KCC2 regulation with therapeutic implications

The changes in KCC2 activity responsible for the alterations in GABA_AR- and GlyR-mediated responses that occur normally in development and in the aforementioned disease states have largely been attributed to differences in the spatiotemporal control of KCC2 mRNA transcription and/or translation (e.g., [46–49]). Although KCC2 cotransport functionality is often altered under these conditions, it seems improbable that expression levels of KCC2 mRNA or total protein alone explain fast changes in KCC2 activity. Recent work has demonstrated that KCC2 mRNA translation is not a major rate-limiting step in the regulation of KCC2 function, and that the degradation rate of KCC2 may be enhanced under pathophysiological states characterized by increased excitability [50,51]. Given the known rapid timescale (minutes to hours) of activity-dependent functional down- or upregulation of KCC2 [35,50,52,53], the fast functional modulation of KCC2 is likely to be mediated by several interdependent mechanisms, with acute regulation of transporter activity achieved via multiple intracellular second messenger systems triggering post-translational covalent modifications. However, this does not exclude long-term consolidation of changes in the functional expression of KCC2 at the level of transcription [37].

Mounting evidence indicates that protein (de)phosphorylation is an important regulator of KCC2-mediated Cl⁻ extrusion [35,54–64], altering transporter function

by modulating the intrinsic transport velocity, transporter affinity for ionic substrates, and the number of functional transporters expressed on the plasmalemmal surface via effects on endocytosis, proteolytic cleavage, degradation, and other mechanisms [8,50,51, 58,61]. The half-life of membrane-associated KCC2 appears exceptionally fast (approximately 5 min in HEK-293 cells [58]). Such rapid (de)phosphorylation-controlled membrane recycling of KCC2 is likely to enable dynamic post-translational regulation of the relative amount and the intrinsic functional properties of KCC2 molecules located in the plasma membrane and transport vesicles. Also, the work often cited for rapid plasmalemmal turnover of KCC2 protein in hippocampal (HC) slices [65] demonstrates a net decrease of plasmalemmal KCC2, with a time constant of approximately 20 min, under conditions of constitutively increased KCC2 degradation, *cf.* [50]. Thus, post-translational modulation is in a key position to rapidly determine the level of functional expression of KCC2 and, consequently, neuronal Cl⁻ extrusion capacity both under physiological and pathophysiological conditions.

Here, we review recent data that illustrate the importance of regulatory serine, threonine, and tyrosine phosphorylation of KCC2 for its transporter function (Figure 1). Regulatory changes in KCC2 phosphorylation may have significant consequences for the efficacy of synaptic inhibition mediated by GABA_ARs and GlyRs, with implications for neurodevelopment, network excitability, and neurological disease. A better understanding of the mechanisms underlying the functional regulation of KCC2 via phosphorylation may enable selective modulation of KCC2 activity, thereby providing a novel means of manipulating GABAergic and glycinergic signaling for the treatment of neurological diseases.

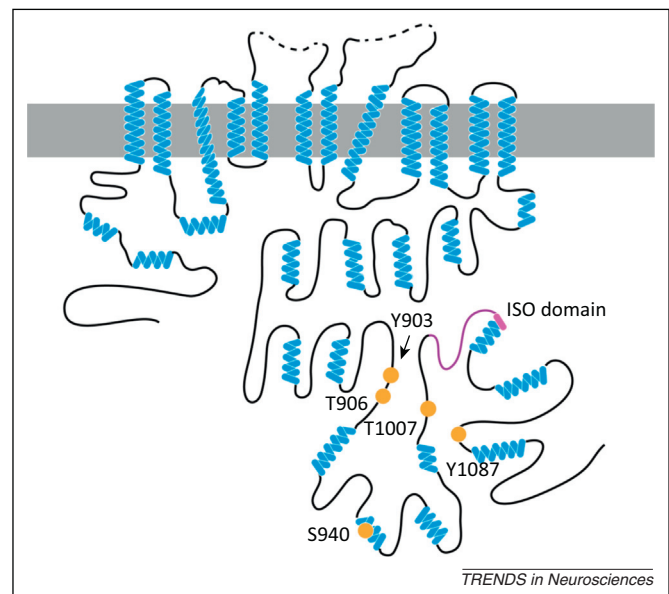


Figure 1. Schematic representation of important regulatory phosphoresidues of KCC2, the neuron-specific K-Cl cotransporter. Orange dots indicate the positions of phosphoresidues in the cytoplasmic C terminus of the transporter that are critical for functional regulation of KCC2, including tyrosine 903 (Y903), threonine 906 (T906), serine 940 (S940), threonine 1007 (T1007), and tyrosine 1087 (Y1087). The pink region denotes the KCC2 'ISO' domain, required for hyperpolarizing GABAergic transmission [90].

Protein kinase C-dependent phosphorylation of KCC2 serine 940: identification and implications for the stress response of the body

Lee *et al.* [58] identified serine 940 (S940) in the C-terminal tail of KCC2 as a major site of protein kinase C (PKC) phosphorylation. *In vitro* kinase assays and radioactive metabolic labeling experiments established that PKC directly phosphorylates S940, which rapidly enhances KCC2 cell surface stability and increases ion transport. Independent peptide mapping studies and assays with S940 phospho-specific antibodies corroborated that the major site of PKC-dependent phosphorylation is S940 [35,58]. Mutation of S940 to a nonphosphorylatable alanine (S940A) was shown to slow the internalization rate of KCC2, and prevented the PKC-dependent increase in K–Cl flux [58].

Support for the physiological relevance of PKC-dependent modulation of KCC2 function was obtained using HC slices. Gramicidin-perforated patch-clamp revealed that tonic activation of group I metabotropic glutamate receptors (mGluR1s) regulates inhibitory synaptic strength via downstream activation of a PKC-dependent pathway in CA3 pyramidal cells [66]. Pharmacological activation of PKC was shown to mimic the effect of DHPG, a specific group I mGluR agonist, resulting in a hyperpolarizing shift in E_{GABA} , which was reversed by the addition of a Ca^{2+} -dependent PKC inhibitor, Gö6976. Together, these data implicated a PKC-mediated pathway regulating E_{GABA} downstream of group I mGluR. Application of the KCC2 (and NKCC1) blocker furosemide resulted in a depolarizing shift in E_{GABA} , whereas PHCCC, an mGluR1 antagonist, had no additional effect [66]. Conversely, treatment with the NKCC1 inhibitor bumetanide had no effect on E_{GABA} , suggesting the mGluR-dependent hyperpolarizing shift in E_{GABA} was due to functional expression of KCC2 [66]. Together, these data suggest that, in CA3 pyramidal cells under basal conditions, group I mGluRs modulate KCC2 function and Cl^- extrusion, which can be tuned up or down within minutes.

It is well known that the hypothalamic–pituitary–adrenal (HPA) axis, which mediates the response of the body to stress, is regulated largely by GABAergic inputs to corticotropin-releasing hormone (CRH)-releasing neurons. Sarkar *et al.* demonstrated that dephosphorylation of KCC2 at S940 in the hypothalamic paraventricular nucleus (PVN) takes place following acute stress in adult mice. This resulted in decreased KCC2 functional expression and was associated with emergence of excitatory actions of GABA on CRH-releasing neurons and subsequent activation of the HPA axis [67] (see also [68]). These findings suggest that phosphoregulation of KCC2 at S940 has a role in control of the hormonal stress response of the body. This could serve as a novel therapeutic target for diseases associated with hyperexcitability of the HPA axis (e.g., Cushing's syndrome and neuropsychiatric diseases, such as anxiety, major depression, and post-traumatic stress disorder, *cf.* [69,70]).

NMDA receptor-dependent KCC2 serine 940 dephosphorylation: implications for seizures

Lee *et al.* demonstrated using dissociated HC neurons that an increase in NMDA receptor (NMDAR) activation,

experimentally induced by elevating ambient glutamate, triggers a Ca^{2+} -dependent KCC2 dephosphorylation at S940 and downregulation of both the surface expression and function of KCC2. Both of these events were sensitive to the protein phosphatase 1 (PP1) inhibitor okadaic acid, which prevented the loss of hyperpolarizing GABAergic inhibition triggered by glutamate exposure [35]. Thus, PKC-dependent phosphorylation of S940 enhances [58], whereas PP1-mediated dephosphorylation of S940 appears to inhibit KCC2 [35]. Lee *et al.* proposed that the KCC2 functionality is strongly influenced by the phosphorylation state of S940 which is, in turn, controlled by the relative activities of PKC and PP1 [35] (Figure 2). These results are compelling, considering the occurrence of elevated glutamate signaling in the CNS during numerous pathophysiological states associated with a decrease in the functional expression of KCC2 [38]. Lee *et al.* [35] also highlighted the therapeutic potential of NMDAR antagonists to limit damage to the Cl^- homeostatic mechanism during the acute phase of neuronal injury. In this context, it is not unlikely that the recently reported C-terminal truncation and functional inactivation of KCC2 by the Ca^{2+} -dependent cysteine protease calpain triggered by sustained NMDAR activation, observed in HC and spinal cord neurons [50,51] is regulated by a KCC2 phosphorylation state-dependent mechanism (Figure 2B). For example, the GlyR and GABA_AR scaffolding protein gephyrin [71] and the canonical calpain substrate spectrin [72] are notable examples of substrate phosphorylation state-dependent targeting to calpain-mediated cleavage.

PKC-dependent activation of KCC2 by 5-HT_{2A} receptor signaling: implications for spinal cord injury-associated spasticity

A recent investigation of a mouse model of spinal cord injury indicated that PKC-mediated regulation of KCC2, most likely involving the principal site for serine phosphorylation, S940 (*cf.* [35,58]), may be involved in spasticity, a debilitating neurological condition commonly exhibited by patients following traumatic brain and spinal cord injury [73]. Bos *et al.* [74] recently demonstrated that prolonged application of 2,5-dimethoxy-4-iodoamphetamine (DOI), a 5-HT_{2A/2B/2C} receptor agonist, resulted in a long-lasting hyperpolarizing shift of inhibitory postsynaptic potential (IPSP) reversal potential (E_{IPSP}) values of neonatal rat spinal cord motoneurons. Of note, KCC2 is expressed at earlier ages in the spinal cord [75,76]. Importantly, rats pups that had undergone spinal cord injury (SCI) exhibited significantly more depolarized E_{IPSP} values in their motoneurons compared to age-matched control rats. Acute 5-HT_{2A/2B/2C} receptor antagonism with DOI resulted in a hyperpolarizing shift of E_{IPSP} in SCI animals, and chronic treatment of these animals with DOI restored E_{IPSP} to values comparable to those of untreated non-SCI rats. Immunohistochemical analysis revealed a reduced localization of KCC2 to the somatodendritic cell surface of lumbosacral motoneurons in SCI rats. Chronic treatment with DOI restored KCC2 expression to that of control animals. Using a combination of specific 5-HT_{2A/2B/2C} agonists and antagonists, the authors

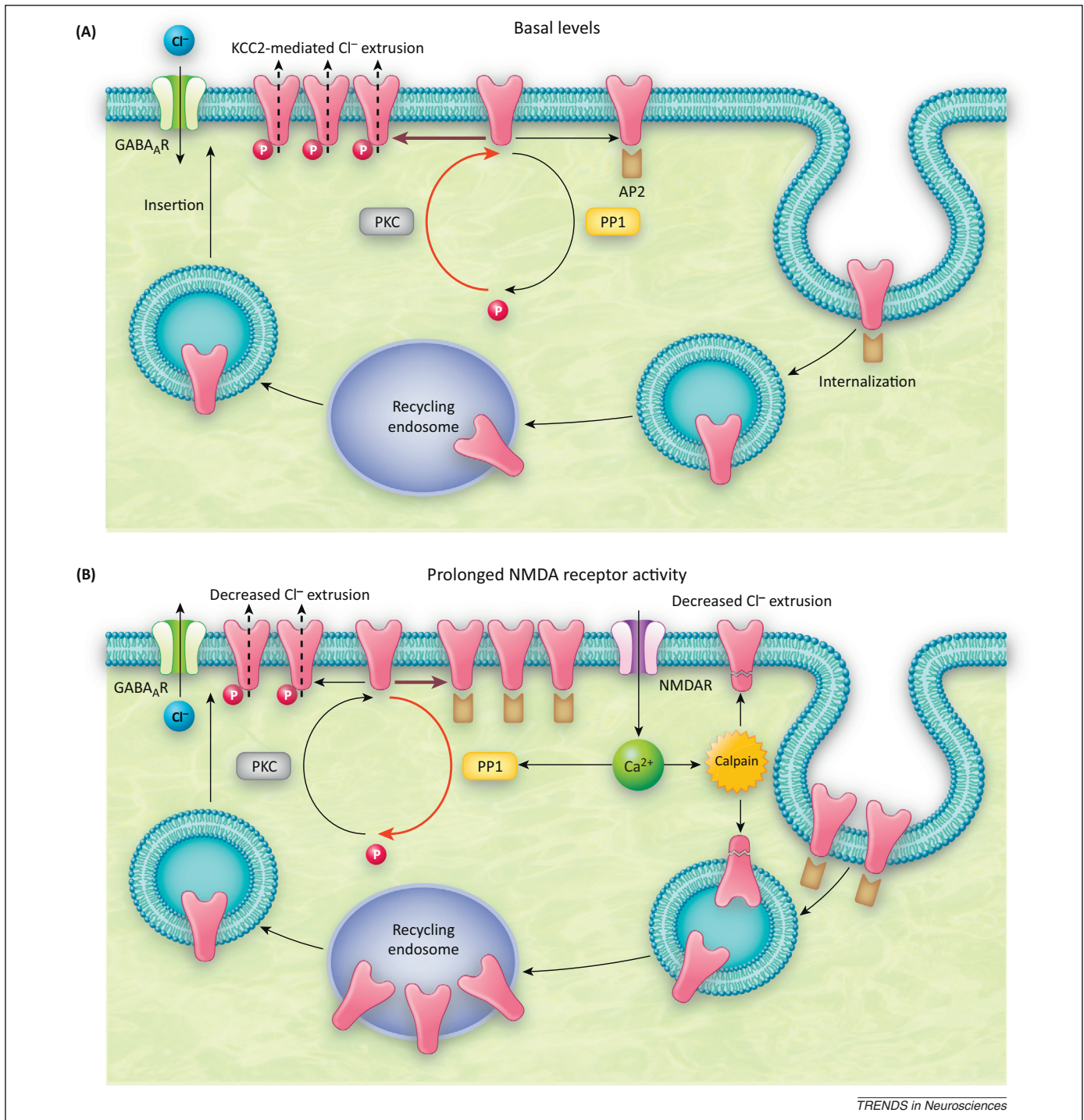


Figure 2. Serine 940 (de)phosphorylation and KCC2 function. **(A)** Under control conditions, constitutive membrane recycling of KCC2 is determined in part by the relative phosphorylation state of serine 940 (S940), which is phosphorylated by protein kinase C (PKC) and dephosphorylated by protein phosphatase 1 (PP1). Phosphorylation of S940 limits adaptor-protein 2 (AP2)-mediated endocytosis of KCC2 molecules, resulting in a stable population of membrane-localized KCC2. **(B)** Prolonged exposure to glutamate, as might be seen during specific pathophysiological conditions (e.g., seizures and neuropathic pain) leads to increases in NMDA-receptor activity (NMDAR), thereby triggering a Ca^{2+} -dependent cascade that favors PP1-dependent dephosphorylation of S940 and endocytosis of KCC2, leading to a functional downregulation of KCC2 transport activity [35]. Such NMDAR-dependent changes in KCC2 phosphorylation status may regulate targeting of KCC2 for activity-dependent cleavage by calpain [50,51,71].

concluded that the DOI effects are mediated through the 5-HT_{2A} receptor [74].

Further analysis in cultured motoneurons revealed that the 5-HT_{2A} high-affinity agonist, TCB-2, hyperpolarized E_{IPSP} values and increased the plasma membrane expression of KCC2. Application of the recently identified KCC2 inhibitor VU0240551 [77] depolarized E_{Gly} values

and occluded the effect of TCB-2. However, because this KCC2 inhibiting compound has been shown to possess serious off-target actions, including inhibition of several G protein-coupled receptors and Ca^{2+} channels [78,79], these results should be interpreted with caution. Application of Gö6976 revealed that the actions downstream of 5HT_{2A} are partly mediated by a Ca^{2+} -independent PKC

isoform. In intact spinal cord preparations, TCB-2 significantly accelerated the rate-dependent depression of monosynaptic responses and reduced polysynaptic responses, which was occluded by KCC2 inhibition. Intraperitoneal injection of TCB-2 in paraplegic spastic adult rats (post SCI) decreased the Hoffman wave amplitude specifically when high frequency stimulation was used. These data compellingly suggest that compromised inhibitory transmission in the spastic spinal cord is due to perturbations in Cl^- homeostasis and impaired KCC2 functional expression, and can be repaired by promoting PKC-dependent phosphorylation of KCC2 via 5-HT_{2A} receptor activation. This area of research holds promise for rapid clinical translation, given the paucity of effective agents for this debilitating and common condition.

Overall, the above studies suggest that PKC-dependent phosphorylation of KCC2 at S940 is important for the modulation of the strength of $\text{GABA}_{A\text{R}}$ and GlyR-mediated

synaptic inhibition. Decreased S940 phosphorylation may underlie the altered regulation of KCC2 in disease states associated with enhanced network excitability or increased HPA axis activation, including seizures, ischemia, and general forms of brain and spinal cord injuries. The precise mechanism of how S940 phosphorylation elicits changes in KCC2 activity is currently unclear; perhaps phosphorylation determines the accessibility (or inaccessibility) of cotransporter sites to regulatory elements such as the endocytic machinery, or induces specific structural configurations that enable the binding or release of other regulatory proteins, notably, kinases, phosphatases, and/or proteases, such as calpain.

Activation of KCC2 via threonine 906 and 1007 dephosphorylation: implications for neuronal development

Kahle *et al.* first demonstrated a potent kinase-dependent reciprocal switch of KCC2 (and NKCC1) activity *in vitro*

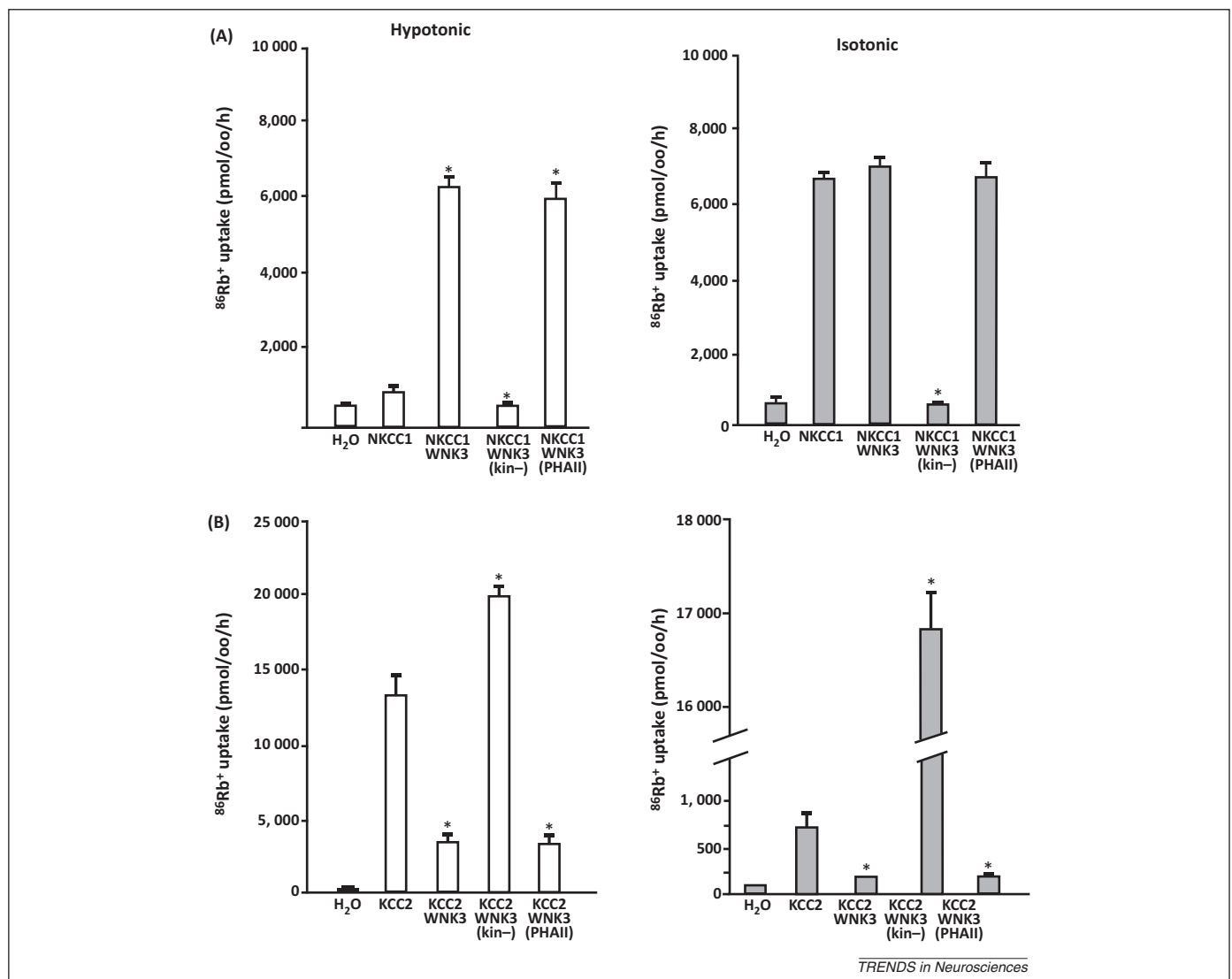


Figure 3. WNK3 kinase reciprocally modulates NKCC1 and KCC2 activity. (A) As assessed using $^{86}\text{Rb}^+$ flux-measurements in oocytes, NKCC1 normally exhibits minimal activity in hypotonic conditions (180 mOsm), and is partially active under isotonic conditions (200 mOsm). Active WNK3 maximally increases NKCC1 activity in both conditions. Conversely, kinase-inactive WNK3 strongly inhibits NKCC1 activity (*, $P < 0.0001$ versus NKCC1 alone). (B) Compared with NKCC1, KCC2 is partially active in isotonic conditions (as is seen in the brain) and is induced in hypotonic conditions in oocytes. Expression of active WNK3 inhibits KCC2 under both conditions. By contrast, kinase-inactive WNK3 (kin^-) strongly activates KCC2 under both hypotonic and isotonic conditions (*, $P < 0.0001$ versus KCC2 alone). 'PHAI1' denotes a point mutation with no effect on WNK3 activity. Reproduced, with permission, from [57].

using active and dominant-negative forms of WNK3, a serine-threonine kinase that is sensitive to cell volume changes [57] (Figure 3). WNK3 overexpression in *Xenopus* oocytes increased Cl^- influx via NKCC1, but inhibited Cl^- efflux via KCC2 under isotonic conditions, thus increasing $[\text{Cl}^-]_i$ [57]. By contrast, a kinase-inactive variant of WNK3 had the opposite effects, inhibiting NKCC1 and robustly activating KCC2 in a PP1-dependent pathway to decrease $[\text{Cl}^-]_i$ [55,57]. Notably, however, the functional effect of PP1 inhibition on KCC2 observed in oocytes [55,57] is opposite to what has been observed in cultured hippocampal neurons [35]. The effects of WNK3 are imparted via altered phosphorylation and surface expression of its targets, indicating that WNK3 can modulate the level of intracellular Cl^- via opposing actions on uptake and extrusion pathways [80]. Rinehart *et al.* identified residues in the KCCs modulated by WNKs to alter transporter activity [56]. Two threonine residues in the related KCC3 cotransporter (T991 and T1048) were shown to be rapidly dephosphorylated in hypotonic (cell swelling) conditions in parallel with increased transport activity, as seen in HEK-293 cells. Alanine substitutions at these sites in KCC3 resulted in a robust, constitutively active K-Cl cotransport in conditions that are usually inhibitory for KCC3 [56]. Homologous threonines were shown to be conserved and phosphorylated in all human KCCs, including KCC2 (T906 and T1007) (Figure 4). Intriguingly, these residues on KCC2 were shown to be partially phosphorylated in neonatal mouse brain and dephosphorylated in parallel with brain maturation [56].

Currently, the functional significance of the (de)phosphorylation of KCC2 at residues T906/T1007 is unknown; however, we suggest that it is an important determinant of GABA_A signaling by altering neuronal $[\text{Cl}^-]_i$ during development, given the robust downregulation of inhibitory T906 phosphorylation seen from day P0 to P20 in mouse brain [56]. In this context, it is interesting that endogenous taurine appears to modulate the phosphorylation of KCC2 T906/T1007 by triggering the phosphorylation (and activation) of WNK1, and the substrates of WNK1, SPAK and OSR1, and that cerebral radial migration was altered by a taurine-insensitive nonphosphorylatable form of KCC2, KCC2-T906A/T1007A [81]. SPAK phosphorylation is also elevated in embryonic versus neonatal brains, and is sensitive to taurine transporter inhibition *in vivo* [81]. Importantly, the mammalian KCC2 gene is N-terminally spliced, producing two neuron-specific isoforms, KCC2a and KCC2b, with comparable cotransport properties when expressed in HEK cells [82]. KCC2b is the major isoform, contributing approximately 90% of total KCC2 protein in the murine cortex [82,83]. The N terminus of KCC2a contains a binding motif for the Ste20-type kinases SPAK/OSR1 that is absent in KCC2b, suggesting differential mechanisms of phosphoregulation of the two KCC2 isoforms [82,83].

In the human brain, of the WNK kinase family members WNK1–4, the mRNA of WNK3 is developmentally regulated in a reciprocal fashion compared with KCC2 expression (Figure 5; *cf.* [84]). This striking expression profile, coupled with the known kinase-dependent inhibition of KCC2 activity [57], suggests that a higher level of

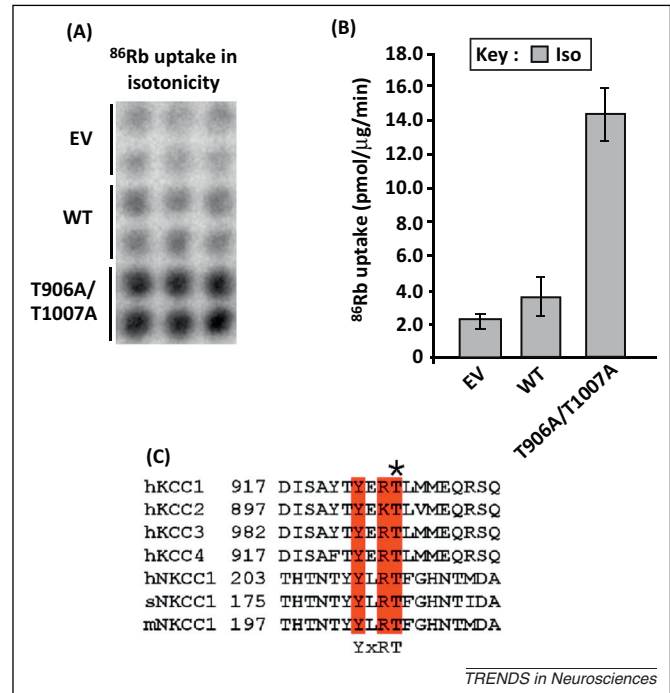


Figure 4. Threonine 906 and 1007 dephosphorylation activates KCC2. Rinehart *et al.* identified regulatory phospho-threonines in K-Cl cotransporters that are modulated by cell swelling and are dependent on WNK1 kinase activity in HEK-293 cells [56]. In KCC2, T906 and T1007 are the critical homologous threonine residues. Simultaneous phosphorylation of both residues inhibits KCC2 activity, and dephosphorylation at these residues activates KCC2 in isotonic conditions. In the neonatal mouse brain, T906 is partially phosphorylated, but largely dephosphorylated in the adult brain in parallel with KCC2 activation [56]. (A) An autoradiogram showing ⁸⁶Rb⁺ flux in HEK-293 cells, with increased ⁸⁶Rb⁺ flux in wild type (WT) KCC2-transfected cells compared with controls transfected with an empty vector (EV), and a robust increase in ⁸⁶Rb⁺ flux relative to wild type KCC2 in cells transfected with the KCC2 T906A/T1007A, whose double alanine mutations mimic dephosphorylation at these sites. (B) Results of ⁸⁶Rb⁺ flux assays for each of the indicated transfected constructs. Note that wild type KCC2 exhibits low activity under isotonic conditions (mediated by the ISO domain [90], *cf.* to other KCCs that exhibit no isotonic activity [104]), whereas KCC2 T906A/T1007A exhibits robust activation relative to wild type KCC2 in the same conditions [56]; (*P* < 0.0001). (C) Homologous phospho-threonines are conserved and phosphorylated in all human KCCs. The C termini of all the human KCCs, and the homologous region of the N terminus of NKCC1 [human (h), mouse (m), and shark (s)], are aligned. The asterisk (*) indicates the conserved threonine residue common to all the *SLC12A* CCCs, with the conserved phospho-motif highlighted in red (YXXRT^P). Notably, T212 in NKCC1 (human) is required for NKCC1 activation and is phosphorylated downstream of the WNK/SPAK(OSR1) kinase signaling pathway [85,105–107]. Given the homology of KCC2 T906 to NKCC1 T212, a reciprocal phosphorylation mechanism downstream of WNK/SPAK kinase that concurrently stimulates NKCC1 and inhibits KCC2 (when activated, promotes threonine phosphorylation), but inhibits NKCC1 and stimulates KCC2 (when inhibited, promotes threonine dephosphorylation) could be important for dynamic regulation of neuronal Cl^- homeostasis [56,57,62,80]. Reproduced, with permission, from [56].

WNK3 activity in part determines the elevated level of inhibitory KCC2 T906 phosphorylation and associated decrease in KCC2 functional expression early in development. At present, it is unclear whether WNK3 (or other WNK kinases) directly phosphorylate KCC2 at T906/T1007; WNKs might regulate other kinases, such as SPAK or OSR1, which serve as direct KCC2 phosphorylators, in a manner analogous to the regulation of the WNK/SPAK pathway on NKCC1 [85]. Given the homology of the KCC2 phosphomotif, including T906, to that of the critical N terminal regulatory region of NKCC1 known to be a phosphorylated by the WNK/SPAK pathway, we propose the existence of a reciprocal phosphorylation mechanism at specific phosphorylation sites, downstream of WNK/SPAK

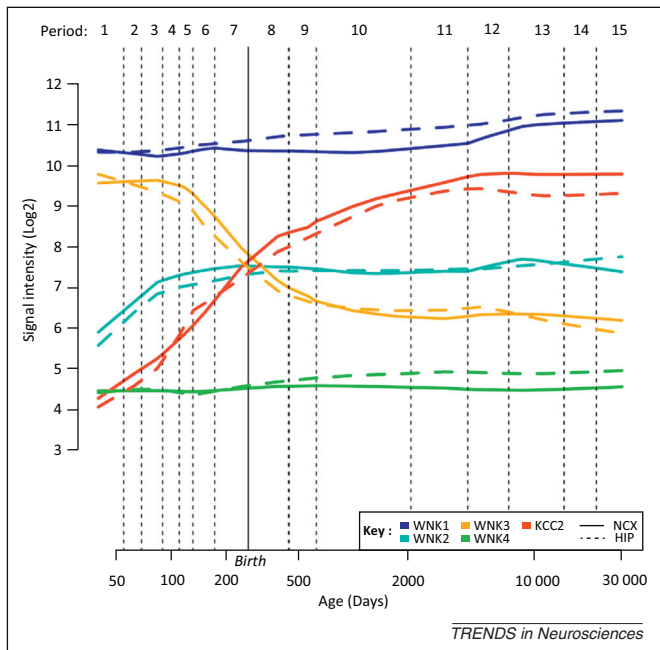


Figure 5. *WNK3* transcript expression is developmentally regulated in the human hippocampus (HIP) and neocortex (NCX) in a reciprocal fashion relative to *KCC2*. Among *WNK1–4*, the mRNA levels of especially *WNK1* and *WNK3* are expressed at high levels in the human brain. However, *WNK3* mRNA is subject to downregulation during the phase of robust *KCC2* upregulation. Acquisition of data and the developmental periods are described in [84]. Adapted, with permission, using data from the human brain transcriptome data bank described in [84] and accessible at <http://hbatlas.org>.

that raises *NKCC1* activity and lowers *KCC2* activity, thereby favoring the accumulation of $[Cl^-]$. Given their expression level (Figure 5) and known effect on the CCCs *in vitro* (e.g., [54]), other *WNKs* could also have major roles in the CNS via CCC regulatory phosphorylation. Indeed, expression of *WNK1* and *WNK2* mRNA is sustained throughout development in the human neocortex and HC, whereas that of *WNK4* appears to be expressed at a low level (Figure 5; cf. [84]). Interestingly, *WNK1/HSN2*, an isoform expressed most highly in the HC and spinal cord dorsal horn [86], is mutated in a Mendelian syndrome featuring congenital insensitivity to pain [87]. An interesting question for future work is whether *WNK1/HSN2* is implicated in modulation of pain sensation via regulation of *KCC2* function in the dorsal horn [88]. Given the physical and catalytic interaction between different members of the *WNK* family [89], the question of redundancy, and compensation may complicate the analysis of knockout (KO) models.

Additionally, it will be an interesting topic of future work to examine the relation between the T906/T1007 phosphorylation motif and the ISO domain in the C terminus of *KCC2*, which is required for *KCC2* activity under isotonic conditions and for hyperpolarizing inhibition by GABA in adult neurons [90]. Interestingly, when *KCC2* lacks the ISO domain (Box 2), it still retains its swelling-activated transport property [90], which suggests that there are distinct molecular determinants of isotonic and swelling-induced K–Cl cotransport in neurons.

One exciting avenue for the potential manipulation of neuronal Cl^- gradients and GABA_A or GlyR signaling is the

Box 2. Swelling-regulated K–Cl cotransport

K–Cl cotransport was initially identified in red blood cells [100–110], where, and as later discovered in most other cells of the body, it is robustly activated by hypotonic cell swelling and mediates regulatory volume decrease through an efflux of K^+ , Cl^- and osmotically obliged water. Cell swelling can be induced either by (extracellular) hypotonic stress or by increased cytoplasmic (intracellular) osmolarity. In contrast to most other cells, physiologically-induced swelling of neurons results from activity-dependent ionic loads, not from hypotonic stress [42]. Thus, intense synaptic activity under physiological and pathophysiological conditions will lead to neuronal swelling caused by an enhanced cellular ionic influx, which is accompanied by net movement of water. By contrast, under hypotonic conditions, the intracellular solute level is reduced [111], and the volume of glial cells (not neurons) is immediately affected, likely because of the apparent lack of aquaporins in neurons [112]. *KCC2*, similar to the three other K–Cl cotransporter family members, is dephosphorylated at T906 and T1007 that increases transporter activity in response to swelling. However, compared with the other *KCC* isoforms, the neuron-specific isoform *KCC2* is unique in that it is capable of constitutive K–Cl cotransport under isotonic conditions [90,113–115]. The molecular determinant of this important feature of *KCC2* has been pinpointed to a stretch of amino acid residues located in the C terminal region that is known as the ‘iso’ domain [114]. Deletion of this domain in neurons leads to loss of Cl^- extrusion under isotonic conditions while sparing the swelling-activation mechanism, suggesting that there are distinct molecular mechanisms of isotonic and swelling-induced neuronal *KCC2* activity [90].

indirect modulation of *NKCC1* and/or *KCC2* activity via the targeting of upstream phosphoregulators [80]. As discussed, stimulation of *NKCC1* and inhibition of *KCC2* are both mediated via threonine phosphorylation at homologous motifs residing in N or C termini of the transporters, which suggests that a common cascade regulates both *NKCC1* and *KCC2* in a reciprocal fashion. The fact that phosphorylation at these sites in both *NKCC1* and *KCC2* is dependent on *WNK1* kinase *in vitro* suggests that kinase inhibitors that inhibit *WNK* isoforms in the nervous system are particularly attractive targets for therapeutic intervention. For example, inhibition of the kinase activity of *WNK3* may be a particularly potent means of decreasing neuronal $[Cl^-]_i$ by concurrently inhibiting *NKCC1* activity and promoting *KCC2* activity via changes in transporter phosphorylation at critical regulatory residues [55,57].

However, caution should be taken in interpreting these preliminary findings, and further experimental evidence is needed. To investigate these hypotheses further, the development of robust tools such as phospho-specific antibodies, is required to enable the identification of changes in phosphorylation state of these residues in rodent models of epilepsies, neuropathic pain, and of other disease states. A thorough study of the phosphorylation state of these residues is also warranted in different regions during brain and spinal cord development, because the current techniques used to monitor *KCC2* phosphorylation have utilized whole brain [56].

KCC2 tyrosine 1087 phosphorylation: implications for development and neuronal stress

Kelsch *et al.* were the first to suggest that kinetic activation of *KCC2* by tyrosine phosphorylation is required for the increase in neuronal Cl^- extrusion capacity during

development [91] (although see [92]). Later work by Stein *et al.* demonstrated that the amount of tyrosine-phosphorylated KCC2 increased in the mouse cortex between P3 and P30 [76]. Interestingly, Vale *et al.* observed that the KCC2 protein was highly expressed in both P1 and P40 neurons of the cochlear nucleus (see also [93,94]), whereas the level of phosphotyrosine was almost absent at birth and became significantly higher at P40 [95].

Inhibition of tyrosine kinase activity has been shown to change the surface distribution pattern of KCC2 from punctate to diffuse [60]. Similarly, tyrosine kinase inhibition has been shown to trigger a positive shift in E_{GABA} values and an associated decrease in KCC2 tyrosine phosphorylation that is reversible within minutes. A KCC2 Y1087D mutant exhibited a more diffuse staining pattern and more depolarized E_{GABA} values [60], although it is unclear whether this mutation simulates increased phosphorylation or dephosphorylation of this specific site. Furthermore, inhibition of tyrosine phosphatase activity increased the association of KCC2 with lipid rafts in neurons [60]. This result, although controversial, is intriguing because association with membrane rafts has been suggested to inactivate KCC2 and activate NKCC1 [96]. Together, these results suggest that tyrosine kinase activity has rapid and reversible effects on KCC2 clustering in the membrane and associated transporter activity.

Wake *et al.* examined the biochemical and functional effects of cellular stress on KCC2 tyrosine phosphorylation in cultured HC neurons [59]. Using a nonspecific phosphotyrosine antibody, the ratio of phosphotyrosine KCC2 to total KCC2 was robustly decreased by short (<2 h) exposure to H_2O_2 , brain-derived neurotrophic factor (BDNF), or 0-Mg²⁺ conditions. These effects preceded reductions in the total KCC2 levels. H_2O_2 exposure also caused a progressive depolarizing shift in E_{GABA} as measured using gramicidin-perforated patch-clamp. Although it was unclear from these experiments which tyrosine residue exactly was phosphorylated, the authors proposed that neuronal stress induces a loss of tyrosine phosphorylation of KCC2 that correlates with its internalization and reduced transporter activity [59].

Subsequent experiments have shown that the principal sites of tyrosine phosphorylation in KCC2 are residues Y903 and Y1087 [61]. Although tyrosine phosphorylation was absent under basal conditions in HEK-293 cells, exposure to the tyrosine phosphatase inhibitor Na_3VO_4 for 30 min robustly increased phosphotyrosine levels, and of these nearly 75% was abolished by simultaneous mutation of Y903 and Y1087 to phenylalanine, resulting in internalization and degradation of KCC2 that was blocked by combined nonphosphorylatable amino acid substitutions of each tyrosine residue. Interestingly, prolonged activation of muscarinic acetylcholine receptors (mAChRs) in neurons enhanced KCC2 tyrosine phosphorylation at these sites and promoted its degradation [61]. Further investigation with the muscarinic receptor agonist pilocarpine revealed that 1 h of *status epilepticus* (SE) promoted KCC2 tyrosine phosphorylation and subsequent degradation of KCC2 [61]. To the best of our knowledge, this is the earliest time point after drug-induced SE that KCC2 surface

expression has been analyzed in adult animals (*cf.* [52]), although several other studies have also observed decreased KCC2 levels for more prolonged periods after SE induction, *cf.* [37,48,97]. Importantly, the effects of global tyrosine phosphatase inhibition on the biochemical profile of KCC2 found by Lee *et al.* [61] were not consistent with the observations of Wake *et al.* [59]. It is possible that this discrepancy is due to the different basal levels of phosphorylation observed among the separate investigations. Such differences can largely be attributed to specific culturing conditions, which are known to vary widely (see also [92]).

Concluding remarks and future directions

Recent evidence suggests that rapid and reversible phosphorylation and/or dephosphorylation of critical serine, threonine, and tyrosine residues in the KCC2 cytoplasmic carboxyl terminus constitutes a potent and dynamic set of mechanisms to modulate KCC2 activity. PKC-dependent S940 phosphorylation increases KCC2 activity and plasma membrane accumulation by slowing transporter endocytosis. By contrast, WNK kinase-dependent phosphorylation of T906 and T1007 appears to reduce the intrinsic rate of KCC2-mediated ion transport, and dephosphorylation of these residues is an important activator of KCC2. Y1087 phosphorylation promotes KCC2 activity by increasing the membrane insertion of transporters. The role of Y903 in KCC2 regulation is currently unclear, but it is intriguing that this residue is positioned close to T906, raising the possibility of tyrosine kinase modulation of Y903 having an impact on adjacent threonine phosphorylation, or vice versa.

It is evident from the data reviewed above (see also Table 1) that the classical model, derived from work on non-neuronal K–Cl cotransport (for original work see e.g., [98]), in which ‘phosphorylation inactivates’ and ‘dephosphorylation activates’ K–Cl cotransport, does not have predictive value with regard to functional regulation of KCC2. Rather, the functional effect of (de)phosphorylation of the transporter is determined by the identity of the residue in question.

Alterations in the phosphorylation state of these residues are variably altered across neurodevelopment and in models of different neurological diseases. Normally, these phosphoevents probably function not so much as on/off switches of KCC2 activity, but more as modulators, conferring quantitatively graded changes in KCC2 activity in response to physiological signals and perturbations, thus matching fluctuations in neuronal Cl⁻ loads, or increased metabolic demands [34]. Selective modulation of these phosphoresidues, either directly or indirectly, could represent novel therapeutic strategies for the treatment of epilepsy, neuropathic pain, and spasticity, conditions that have been linked to the functional downregulation of KCC2 (or to enhancement in the functional expression of NKCC1), and, in some instances, have shown an altered phosphorylation profile.

An important future direction will be to relate functional modulation, produced by phosphorylation, to changes in the 3D structure of KCC2, which has 12 transmembrane domains (Figure 1). Unfortunately, structural studies of

Table 1. Reported effects of KCC2 (de)phosphorylation on its total, surface, and functional expression

| KCC2 (de)phosphorylation | Reported effect on KCC2 | Model | Trigger | Refs |
|---|----------------------------|-----------------------------|--|---------|
| Serine | | | | |
| Phosphorylation (S940 P-Ab) | Surface: ↑ ^b | HEK-293 | PDBu (PKC activator) | [35,58] |
| | Total: no Δ | | | |
| | Function: ↑ | | | |
| | Function: ↓ | Rat HC cultured neurons | Glutamate | [35] |
| | Surface: ↓ | | | |
| | Total: ↓ | Mouse PVN slices | Acute restraint stress | [67] |
| Surface: ↓ | | | | |
| Total: ↓ | | | | |
| Threonine | | | | |
| Dephosphorylation (T906 P-Ab; T1007 P-Ab) | Function: ↑ | HEK-293 | T906/T1007A | [56] |
| Tyrosine | | | | |
| Dephosphorylation ^a | Surface: ↑ | HEK-293 | Y903/1087F | [61] |
| | Total: ↑ | | | |
| | Surface: no Δ | HEK-293 | Y903F | [61] |
| | Total: no Δ | | | |
| | Surface: no Δ | HEK-293 | Y1087F | [61] |
| | Total: no Δ | | | |
| | Function ↓ ^c | Rat HC cultured neurons | Genistein (Y-kinase inhibitor) | [60] |
| | Surface: no Δ ^d | | | |
| | Total: no Δ | | | |
| | Function: ↓ | Rat HC cultured neurons | H ₂ O ₂ , BDNF, and 0-Mg ²⁺ | [59] |
| | Surface: ↓ | | | |
| | Total: ↓ | | | |
| Phosphorylation ^a | Surface: ↓ | HEK-293 | Na ₃ VO ₄ (Y-phosphatase inhibitor) | [61] |
| | Total: ↓ | | | |
| | Surface: ↓ | Rat HC cultured neurons | Na ₃ VO ₄ and carbachol | [61] |
| | Total: ↓ | | | |
| | Surface: ↓ | Mouse HC slices | Pilocarpine status epilepticus | [61] |
| | Function: ↓ ^c | Mouse organotypic HC slices | Axotomy | [63] |
| Total: no Δ | | | | |

^aNonspecific Y-phospho-antibody (P-Ab) used.

^b↑, increase; ↓, decrease; no Δ, no change.

^cUpregulation of Cl⁻ importers may account for the observed effect.

^dRedistribution in the membrane.

membrane proteins are difficult and, to date, there is no high-resolution structure available for KCC2. Another major area of focus in the near future will be a proteomic analysis of other KCC2-associated proteins, including protein phosphatases. Moreover, upstream signaling elements, including hormones, peptides, membrane receptors, scaffolding proteins, kinases, and phosphatases that transmit signals to effect changes in KCC2 (de)phosphorylation at the important residues described here are essentially unknown. Understanding the normal and pathological cues that trigger the phosphorylation events to elicit changes in KCC2 function could be an important step in facilitating or preventing these processes for therapeutic benefit.

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