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Trends in Neurosciences

CellPress OPEN ACCESS

Review

The Multifaceted Roles of KCC2 in Cortical Development

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KCC2, best known as the neuron-specific chloride-extruder that sets the strength and polarity of GABAergic currents during neuronal maturation, is a multifunctional molecule that can regulate cytoskeletal dynamics via its C-terminal domain (CTD). We describe the molecular and cellular mechanisms involved in the multiple functions of KCC2 and its splice variants, ranging from developmental apoptosis and the control of early network events to the formation and plasticity of cortical dendritic spines. The versatility of KCC2 actions at the cellular and subcellular levels is also evident in mature neurons during plasticity, disease, and aging. Thus, KCC2 has emerged as one of the most important molecules that shape the overall neuronal phenotype.

KCC2 in the Fundamental Machinery Underlying Neuronal Development, Signaling, and Structure

Fast synaptic transmission relies on ion fluxes through ligand-gated ion channels. Although the default state of a cell is to have a high intracellular chloride concentration ($[Cl^-]_i$), mature neurons in the CNS have evolved a unique ability to maintain a low $[Cl^-]_i$, which is needed for the generation of hyperpolarizing Cl^- currents across $GABA_A$ and glycine receptors ($GABA_A$Rs and GlyRs) [1]. This specialization comes at a high energy cost [2], and is brought about by upregulation of the neuron-specific K-Cl cotransporter KCC2 during neuronal maturation [1,3,4].

KCC2 belongs to the evolutionarily ancient family of *SLC12* cation/chloride cotransporters (CCCs) (Box 1; for **KCCs** and **NKCCs**, see Glossary) that have their roots in a single gene in Archaea, from which numerous duplication events in both archaeans and eukaryotes have led to the divergence and neofunctionalization of the paralogous CCC subfamilies [5]. Possibly because of a primordial role in cellular volume regulation, CCCs have evolved to communicate with the actin cytoskeleton. Indeed, KCC2 has emerged as an important player in controlling actin dynamics during neuronal development and plasticity [6–9].

The functional upregulation of KCC2-mediated K-Cl cotransport in hippocampal and neocortical neurons underlies the hyperpolarizing shift in GABAergic currents which takes place postnatally in rats and mice [3], but it has become clear that KCC2 is expressed at low but functionally significant levels pre- and perinatally. KCC2 acts in an ion transport-independent manner as an antiapoptotic factor in projection neurons in the prenatal mouse neocortex [10]; in the perinatal mouse and rat hippocampus transport-functional KCC2 decreases the depolarizing driving force of $GABA_A$R-mediated currents (DF_{GABA}), thereby influencing spontaneous network events at their developmental onset [11].

In this review we highlight the developmental paths from KCC2 protein expression to its multiple functions, and discuss the wide variety of post-translational mechanisms which control these phenomena. The versatility of KCC2 regulation at the cellular and subcellular levels is also evident in mature neurons during plasticity, disease, and aging. Thus, KCC2 has emerged as one of the most important molecules that shape the overall neuronal phenotype (Figure 1).

Highlights

KCC2 is a neuron-specific chloride-extruder that shapes cortical development.

Changes in the strength of GABAergic synapses brought about by up- and downregulation of Cl^- extrusion by KCC2 exert a major effect on neuronal plasticity and network functions.

The structural interactions of the C terminus of KCC2 regulate signaling pathways involved in early neuronal survival, spinogenesis, and glutamatergic synaptic transmission.

The recently discovered tertiary and quaternary structures of KCC2 and other cation/chloride cotransporter (CCC) members provide first insights into their functions at an atomic level.

The multisite phosphorylation code of KCC2 controls its membrane expression and stability, as well as its intrinsic transport rate during development, plasticity, and disease.

KCC2 mutations linked to epilepsy and autism spectrum disorder (ASD) disrupt both ion transport-dependent and -independent KCC2 functions, suggesting a novel molecular basis for CNS disorders related to the structural roles of KCC2.

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Box 1. Molecular Structure of CCCs

Despite the fact that CCCs were cloned >20 years ago, their tertiary and quaternary structures remained unknown until very recently. The development of cryogenic electron microscopy (cryo-EM) and single-particle image processing brought several reports describing high-resolution structures of zebrafish [126] and human [127] NKCC1, mouse KCC4 [128], as well as human KCC1 [129,130], KCC2/KCC3 [131], and KCC2/KCC3/KCC4 [132].

All these studies determined a common transmembrane domain (TMD) in KCCs and NKCCs that consists of 12 transmembrane (TM) helices, the first 10 of which form the ion-translocating path in all CCCs. The NKCC and KCC structures show remarkable conservation of the K^+ binding site [126,129,133] that is coordinated by five amino acids (N131, I132, Y216, P429, and T432 in hKCC1 [129]). The positions of the Cl^- binding sites were resolved in hKCC1 [129], mKCC4 [128], hKCC4 [132], and DrNKCC1 [126]. Surprisingly, two Cl^- sites were discovered in hKCC1 and hKCC4: one that is directly involved in K-Cl cotransport, and a second that allosterically facilitates the occupancy of the former. In hKCC1, the Cl^- in the transport site is coordinated by main-chain amide groups of G134, V135, I136, the hydroxyl group of S430, and K^+ ion, whereas the allosteric coordination center consists of the amide groups of G433, I434, M435, and the hydroxyl group of Y589 from TM10 [129].

The N-terminal domains of CCCs yield poor resolution by EM, indicating high conformational flexibility. Interestingly, the human KCC2/KCC3 [131] and KCC2/KCC4 [132] structures have a ~25 amino acid N-terminal loop that blocks the cytoplasmic entry of the translocation pore. Consistent with the function of this loop as a modulator of the transporter, its deletion [131] or mutation [132] activated ion transport.

tWith the unique exception of the monomeric mKCC4 structure [128], all CCCs showed a dimeric assembly, as had been predicted before. The dimer interface comprises TMDs and C-terminal domains (CTDs) in a swapped conformation in which the CTD of one subunit is placed under the TMD of the other (Figure 2B). The swapping occurs in the 'scissor helix' region that connects TMD and CTD within each subunit, as shown for all hKCCs [129,130] and DrNKCC1 [126]. The extracellular domains stabilize hKCC1 [129,130] but not hKCC2, hKCC3, or hKCC4 dimers [131,132]. It is worth pointing out here that the SDS-resistant higher homo-oligomers of KCC2 in western blots are obviously a result of the well-known aggregation of membrane proteins subjected to heating or acidic pH [134]. In particular, the amount of KCC2 oligomers in SDS gels bears no relation to the presence of functional dimers in the original tissue.

As a whole, the accumulating knowledge on the molecular structure of KCC2 provides information that can be used in studies on the molecular mechanisms and modulation of ion transport by KCC2 inhibitors (reviewed in [133]) and activators [123,124], and by interacting molecules, including those depicted in Figure 1 in the main text.

Modulation of KCC2 Activity in the Perinatal Period and Beyond

During the development of various parts of the vertebrate CNS, $GABA_A$Rs and GlyRs mediate a depolarizing effect [1,12] which depends on the high $[Cl^-]_i$ concentration maintained by the Na-K-2Cl cotransporter NKCC1 [12–14]. In immature pyramidal neurons of rats and mice, the GABAergic depolarization is seen prenatally and during the first postnatal week [11,12,15,16]. The depolarizing actions of GABA have long been known to play an important role in cell proliferation and survival, migration, differentiation, and early network wiring, but direct evidence for depolarizing GABA signaling *in vivo* is only starting to emerge [17,18]. It is important to note that depolarizing GABAergic transmission in immature networks *in vivo* can be functionally excitatory, as seen in mouse hippocampus [18], or inhibitory, as demonstrated in mouse neocortex [17–19].

KCC2 levels in the hippocampus and cortex rise steeply during the first 2 postnatal weeks, paralleled by enhanced Cl^- extrusion and by a shift from depolarizing to hyperpolarizing in the reversal potential of GABAergic currents ($\mathbf{E_{GABA}}$) [20,21] (Box 2). This developmental E_{GABA} shift, which is an excellent indicator of the stage of maturation of distinct neuronal populations [3], has by now been described in most types of central neurons [1,22]. In the human neocortex, massive upregulation of KCC2 expression is observed during the second half of gestation [23,24].

Recent work in rat and mouse hippocampal slices has shown that KCC2 already becomes transport-active at the time when the first **early network events** (the so-called giant depolarizing potentials, GDPs, in *in vitro* preparations) are observed [11]. Notably, the specific KCC2 inhibitor VU0463271 [25] causes a pronounced enhancement of GDP activity in field-potential recordings, an enhancement of the depolarizing drive of GABAergic interneurons that facilitates spiking of CA3 pyramidal neurons, and collapse of an experimentally induced somatodendritic Cl^- gradient [11] that provides a quantitative read-out of Cl^- extrusion efficacy ([26]; also [27,28]).

An important but often ignored aspect of KCC2 regulation is that the *Slc12a5* gene generates two splice variant proteins, KCC2a and KCC2b (Figure 2), via the use of alternative promoters. In fact, one of the earliest attempts to generate a KCC2 knockout (KO) mouse [29] resulted (as discovered later [30,31]) in a KCC2b-specific KO mouse that has seizures but can survive for up to 2 weeks. Both KCC2 isoforms have an influence on respiratory pattern generation after birth [32,33], and the full KCC2 KO mouse is unable to breathe and dies at birth [20]. Notably, KCC2a and KCC2b expression show a twofold and 20-fold increase, respectively, during cortical development in the mouse [34]. Thus, although KCC2b is the predominant isoform in the mature brain, KCC2a may have functions that have so far gone unnoticed (Box 3).

In mature neurons, the various postsynaptic structures of GABAergic synapses are highly dynamic and display short-term and long-term plasticity [35]. However, DF_{GABA} (and thus the efficacy of inhibition) can also undergo large changes depending on the cellular Cl^- load and the efficacy of Cl^- extrusion (originally termed by us **ionic plasticity** [36]). The fastest effects of ionic plasticity on DF_{GABA}, at timescales of seconds, are caused by activity-dependent redistribution of ions, whereas longer-lasting adjustments, from minutes to days and even years, are caused by changes in post-translational and genomic regulation ([37] for review). In addition to these temporal domains of ionic plasticity in GABA-mediated signaling, there are basically two types of mechanisms that can lead to spatial gradients of Cl^- in neurons: (i) under steady-state conditions in quiescent neurons, Cl^- gradients do exist, and these are attributable to nonhomogeneous distribution of KCC2 and NKCC1 [38–41] ([42] for discussion on NKCC1 expression in the brain). (ii) Moreover, in the living brain, neuronal activity is likely to impose fast changes in the local Cl^- 'loads' originating from $GABA_A$Rs and other Cl^--permeable channels (see earlier) because of abrupt and large changes in the membrane potential (V_m) and, consequently, in the driving force for Cl^- ($DF_{Cl} = V_m - E_{Cl}$). Current imaging techniques do not permit direct observation of these fast Cl^- shifts, but that they must exist can be readily deduced on the basis of what is known of the electrophysiology of GABAergic transmission and fast ionic plasticity ([1] for discussion).

Downregulation of KCC2 has been reported in neuronal trauma and various CNS diseases [43–48]. These changes often lead to a depolarizing shift in steady-state $GABA_A$R responses, consistent with a general trauma-induced dedifferentiation at the level of transcription and translation, including loss of spines. Furthermore, several types of conditions ranging from neuroendocrine adaptations [49] to psychiatric disorders [50] are associated with marked changes in KCC2 expression and function.

Regulation of KCC2 (De)Phosphorylation

A large amount of evidence points to (de)phosphorylation cascades as major modulators of KCC2 functionality. In general, NKCCs are activated whereas KCCs are inhibited by phosphorylation [51,52] (but see below for protein kinase C (PKC)). An interesting aspect of their molecular evolution is that the C terminus of KCC2 and the N terminus of NKCC1 have a similar four amino acid phosphorylation motif [53] that is possibly involved in these opposite effects. In KCC2, the

Glossary

Developmental apoptosis: a form of programmed cell death that occurs naturally in embryonic development. It sculpts the organism by removing unwanted structures and by controlling the number and quality of cells that are initially produced in excess.

Early network events: spontaneous synchronous network activity during the period of initial network wiring. In the hippocampus, depolarizing GABAergic transmission promotes intrinsic bursting of the glutamatergic network. As the neuronal network matures, the discrete events become replaced by continuous oscillations (second postnatal week in rat and mouse neocortex, and around the time of full-term birth in human cortical electroencephalography).

E_{GABA}: the membrane potential at which the current through open $GABA_A$Rs reverses its polarity (reversal potential). E_{GABA} depends on the intracellular and extracellular concentrations of Cl^- and HCO_3^- according to the Goldman–Hodgkin–Katz equation, and intracellular HCO_3^- therefore has a very small effect on E_{GABA} in neurons with a high intracellular Cl^- concentration. However, in mature neurons with a low $[Cl^-]_i$, the bicarbonate-mediated current component can be larger than that mediated by Cl^-.

Ionic plasticity: a change in the strength of GABAergic or glycinergic synapses, that is brought about by temporal and spatial changes in the driving force of the $GABA_A$R-mediated current. These are caused by (i) activity-dependent Cl^- redistribution and, in a wider time span, by (ii) changes in the membrane expression of functional Cl^- transporters and Cl^- permeable channels.

KCCs: secondary active K-Cl cotransporters that under physiological conditions actively extrude Cl^- from the cell, using the energy of the plasmalemmal K^+ gradient. There are four different isoforms (KCC1–4), of which KCC2 is the only neuron-specific transporter.

NKCCs: secondary active Na-K-2Cl cotransporters that move K^+ and Cl^- into the cell using energy derived from the plasmalemmal Na^+ gradient. There are two isoforms (NKCC1 and NKCC2), and NKCC1 is expressed in various tissues and cell-types, including CNS neurons and glia. In developing neurons, NKCC1

major sites for (de)phosphorylation include two C-terminal threonines (T906 and T1007; Box 3). These residues are targeted by kinases of the WNK (With-No-Lysine) family, which act as sensors of $[Cl^-]_i$ [54]. WNK kinases are unique in that Cl^- inhibits their autophosphorylation and consequent kinase activation [54]. Mature neurons express mainly WNK1 and, to a lesser extent, WNK3 [55]. Although WNK1 mRNA levels remain stable during cortical and hippocampal development, WNK3 mRNA levels decrease during the developmental period of KCC2 upregulation [52]. When active (i.e., at low $[Cl^-]_i$), WNKs inhibit KCC2 activity by increasing KCC2 C-terminal phosphorylation via STE20/SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress response kinase 1 (OSR1) kinases (T1007 site), or via another kinase that remains to be identified (T906 site) [56,57]. Although most of the available literature suggests that (de)phosphorylation of T906/T1007 regulates KCC2 membrane expression [58–60], possibly through changes in KCC2 membrane diffusion [55], some studies suggest that (de)phosphorylation of T906/T1007 would instead regulate its intrinsic transport activity [53,61–63].

In line with the developmental E_{GABA} shift, WNK1-mediated T906/T1007 KCC2 phosphorylation decreases with neuronal maturation *in vitro* [60]. Mass spectrometric analysis of whole mouse brains has shown a decrease in the proportion of KCC2 molecules that are phosphorylated at T906 from ~20% at postnatal day (P)0 to ~10% by P3, and to undetectable levels after P21 [53]. Transgenic KCC2 mice with mutations affecting T906/T1007 phosphorylation have confirmed the importance of the threonine sites for KCC2-mediated chloride transport. Homozygous knockin mutant mice with phosphomimetic threonine to glutamate substitutions (T906E/T1007E) are unable to breathe and die shortly after birth [61] in a manner similar to the full constitutive KCC2 KO mice [20].

A large number of phosphorylation sites have been described in different domains of KCC2 [57,61,63–65], including the alternatively spliced N-terminal region (T6 [57,65]). Most work on the activation and inhibition of CCCs in neurons has focused on the role of kinases. It is therefore important to note that, in contrast to the mechanisms described earlier, KCC2 regulation via serine 940 phosphorylation by PKC and PP1 (protein phosphatase 1) [66–68] enhances the stability (amount) of plasmalemmal KCC2, and thereby increases the efficacy of K-Cl cotransport [28,66–68].

Functional regulation of KCC2 by (de)phosphorylation can be very fast. Chloride influx and subsequent WNK inhibition promotes KCC2 membrane diffusion within tens of seconds [55], and the broad-spectrum kinase inhibitor staurosporine enhances KCC2-mediated K-Cl cotransport activity in cultured hippocampal neurons within a few minutes [26], which can be attributed to impaired WNK-SPAK/OSR1-mediated phosphorylation of KCC2 [64].

The phosphorylation cascades are modulated in an activity-dependent manner, as seen in experiments in adult mice in which pentylenetetrazole-induced seizures activated both WNK1 and SPAK/OSR1 to increase KCC2 T906 and T1007 phosphorylation [55]. In P5–P7 rats, a kainate-induced seizure episode enhanced Cl^- extrusion in CA1 neurons in <30 minutes, with an apparently TrkB-dependent increase in KCC2 surface expression and no change in total KCC2 protein [69]. An interesting question concerns whether distinct (de)phosphorylation mechanisms acting in subcellular compartments might contribute to the variation of steady-state E_{GABA} values that have been observed along the axo-somato-dendritic axis [38–41,70].

Based on the aforementioned studies, it seems that (de)phosphorylation-controlled changes in KCC2 functionality are largely attributable to mechanisms that act at the level of (i) subcellular targeting, membrane trafficking, and stability of the transporter protein. (ii) Allosteric regulation

maintains the high intracellular Cl^- concentration needed for depolarizing GABAergic transmission, whereas in healthy mature neurons NKCC1 actions seem to be mainly confined to the axon initial segment and probably also to axons.

Shunting inhibition: in standard textbooks, 'hyperpolarizing postsynaptic inhibition' mediated by $GABA_A$Rs implies that $E_{GABA} < V_m$ at rest, which is maintained by KCC2, whereas 'shunting inhibition' refers to short circuiting of the membrane by the $GABA_A$R-mediated conductance to the resting V_m level ($E_{GABA} \approx V_m$) as typically measured in brain slices. However, this kind of distinction is not valid under *in vivo* conditions, in which resting V_m is ill-defined and the activity-dependent intraneuronal Cl^- load is high. Thus, *in vivo* the stability of shunting inhibition also requires active Cl^- extrusion by KCC2 to maintain the membrane potential at a more negative level than the action potential threshold.

Spinogenesis: the generation of dendritic spines. In pyramidal neurons, excitatory synapses are predominantly located on dendritic spines – small protrusions on neuronal dendrites. Newly formed spines often have a small head diameter and may lack synaptic contacts.

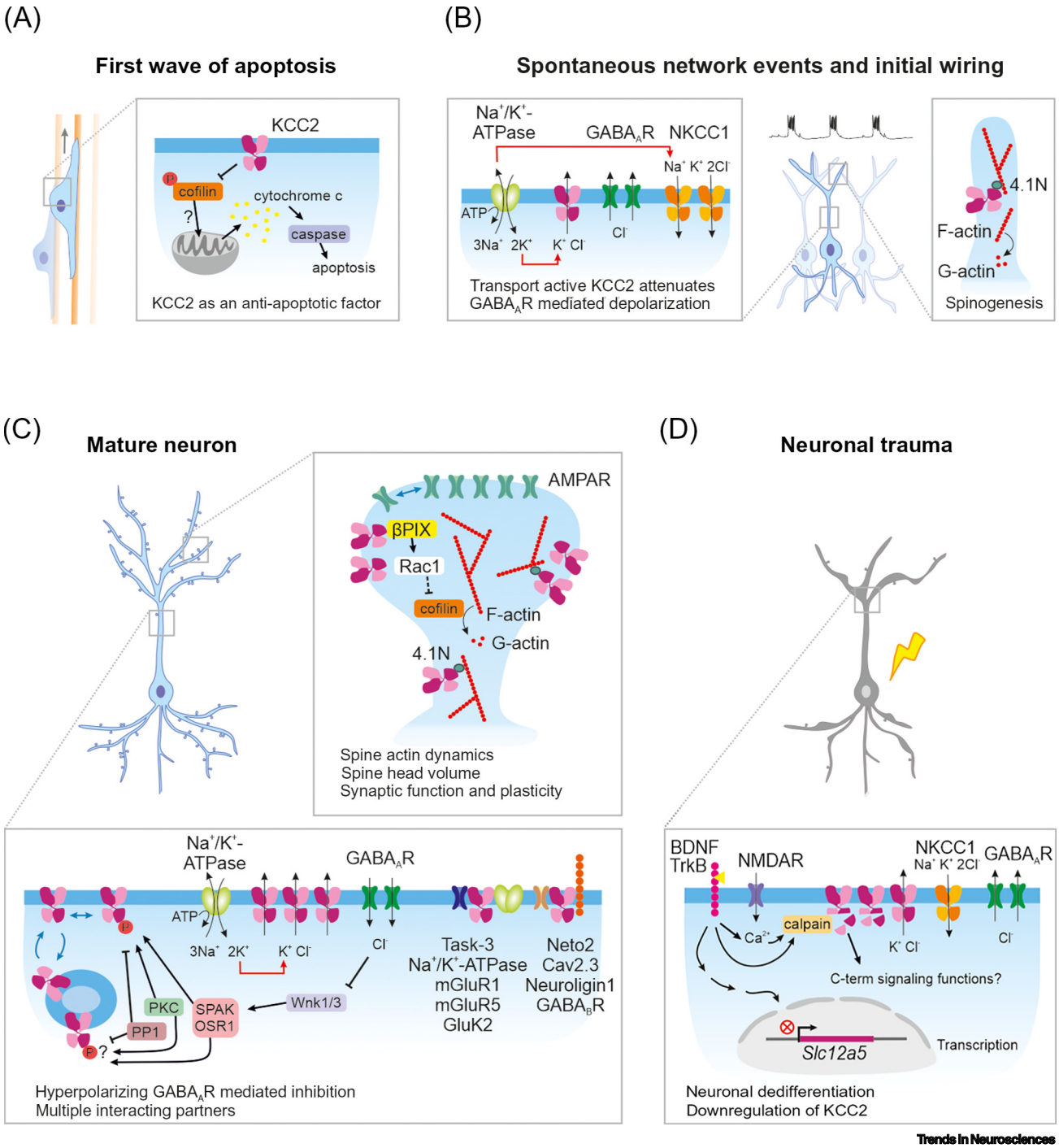

Figure 1. Multiple Functions of KCC2 in Cortical Development and Trauma. (A) KCC2 promotes the survival of migrating pyramidal neurons during the first wave of apoptosis independently of its functions as a K-Cl cotransporter. Signaling cascades triggered by the C terminus act to prevent cofilin hyperphosphorylation, which controls cytosolic release of cytochrome *c* and apoptosis. (B) KCC2 shapes the formation of emerging neuronal circuits through its ion transport-dependent and -independent mechanisms. In the perinatal rat and mouse hippocampus, KCC2-mediated Cl^- extrusion is already functional and delimits the depolarizing $GABA_A$ receptor (R)-mediated transmission and synchronous network events (left panel). KCC2 also plays a transport-independent structural role in dendritic spinogenesis

(Figure legend continued at the bottom of the next page.)

Box 2. The 'Why's of KCC2 Developmental Upregulation

(i) Cl^- extrusion is a specialized feature of mature neurons. During the early development of cortical structures, the principal neurons are not yet coupled into functional networks, and they do not need a specific Cl^- extrusion mechanism. This is because the immature neurons communicate via gap junctions and paracrine mechanisms, and, in the near-absence of electrical activity such as glutamatergic excitatory postsynaptic potentials (EPSPs) and trains of action potentials, there is little Cl^- uptake driven by depolarizing V_m fluctuations [2]. Cl^- levels are therefore maintained by NKCC1 and the time-average V_m which set E_{Cl} and consequently E_{GABA} at a depolarizing level. A rough calculation, based on an ion turnover rate of 4000/s for NKCC1 [135] and a standard $GABA_AR$ conductance of 28 pS, shows that, to maintain a constant 20–40 mV driving force of a depolarizing Cl^- current, the number of plasmalemmal NKCC1 transporters must be up to 500–1000-fold higher than that of open $GABA_AR$ channels.

(ii) In the early stages of network formation and the establishment of electrical synaptic contacts, cortical networks generate sparse and discontinuous 'events' (e.g., GDPs observed in slice preparations) in which depolarizing GABA plays a permissive (or facilitatory) role, whereas the glutamatergic pyramidal neurons have an instructive role in the temporal patterning of these events [136]. During this developmental time-window, transport-functional KCC2 expression commences to deal with the enhanced, activity-dependent Cl^- load. However, E_{GABA} remains depolarizing despite the observable [11] net Cl^- extrusion via KCC2. This is a transitional stage, which is perinatal in rats and mice, but in many species, including guinea pigs, monkeys and human, occurs prenatally.

(iii) During further maturation, the network events become more frequent, and KCC2 becomes progressively upregulated. At this stage, E_{GABA} shows a shift to more negative levels, paralleling the steep increase in cellular connectivity based on the fast maturation of both glutamatergic and GABAergic synapses. This increase in network connectivity is manifest in the electroencephalogram (EEG), in which the discrete network events are gradually replaced by ongoing oscillations. The depolarizing-to-hyperpolarizing E_{GABA} shift is restricted to the prenatal period in guinea pigs and monkeys [3,137]; in humans, it occurs before and around full-term birth [23,24], whereas in rats and mice it takes place postnatally [3].

(iv) Once the neuronal connectivity and ongoing network activity are becoming fully mature, the time-average Cl^- load, the expression level of KCC2, and the hyperpolarizing E_{GABA} shift reach their peak levels. Here, it should be also noted that much of the inhibitory effect of GABAR activation is based on **shunting inhibition**. In parallel, neuronal ionic trafficking and energy metabolism reach their maxima, and this has numerous physiological consequences, including the characteristically high dependence of neuronal survival on the availability of oxygen. Notably, neuronal damage leads to loss of KCC2 and to 'reversal' of the developmental E_{GABA} shift, which may be part of a wider picture of dedifferentiation that is necessary for neuronal survival under adverse conditions.

of the intrinsic ion-turnover rate of the transporter, as has been suggested for NKCC1 [71], is also thought to have occurred in several studies on KCC2 [53,61–63], and the data suggest that the intrinsic transport activity of KCC2 is increased by phosphorylation of T934 or S937 [63], and decreased by phosphorylation of T906 and T1007 [53]. Clearly, if the mechanisms underlying (i) and (ii) above are shared during a (de)phosphorylation event, it will be very difficult to establish their relative contributions to net changes in KCC2 function.

In addition to the above, a large number of mechanisms, including pronounced modulation of *KCC2* gene transcription by miRNAs [72], KCC2 clustering in lipid rafts [68,73,74], and KCC2 degradation by calpain [68,75,76] are known to contribute to what is currently lumped together into the ambiguous 'KCC2 activation/inactivation' terminology. Notably, however, KCC2 mutations can have an effect on all aspects of KCC2 functions, including intrinsic ion-transport efficacy, membrane trafficking, and the role of KCC2 in spine formation and **developmental apoptosis** (Box 4).

(right panel). (C) During further neuronal maturation, robust upregulation of KCC2 expression and the consequent increase in Cl^- extrusion lead to hyperpolarizing $GABA_AR$-mediated chloride currents (lower panel). Membrane expression of KCC2 is regulated by activity-dependent post-translational modifications including (de)phosphorylation cascades, which control the exo- and endocytosis and lateral diffusion of KCC2. KCC2 interacts directly with a variety of plasmalemmal molecules, as shown in the illustration. In dendrites of pyramidal neurons, KCC2 plays a structural, ion transport-independent role in regulating actin dynamics within spines through cofilin phosphorylation (upper panel). KCC2 also binds to the actin cytoskeleton through 4.1N, which regulates the lateral diffusion of KCC2 and AMPA receptors (AMPARs) near excitatory synapses [27]. (D) KCC2 downregulation upon neuronal trauma leads to a depolarizing shift in $GABA_AR$ responses, consistent with the observed trauma-induced dedifferentiation [147,148]. KCC2 is C-terminally cleaved by the Ca^{2+}- and brain-derived neurotrophic factor (BDNF)-activated protease calpain [68,75], and KCC2 transcription is downregulated through TrkB signaling [43]. Abbreviations: NMDAR, N-methyl-D-aspartate receptor; PKC, protein kinase C; PP1, protein phosphatase 1.

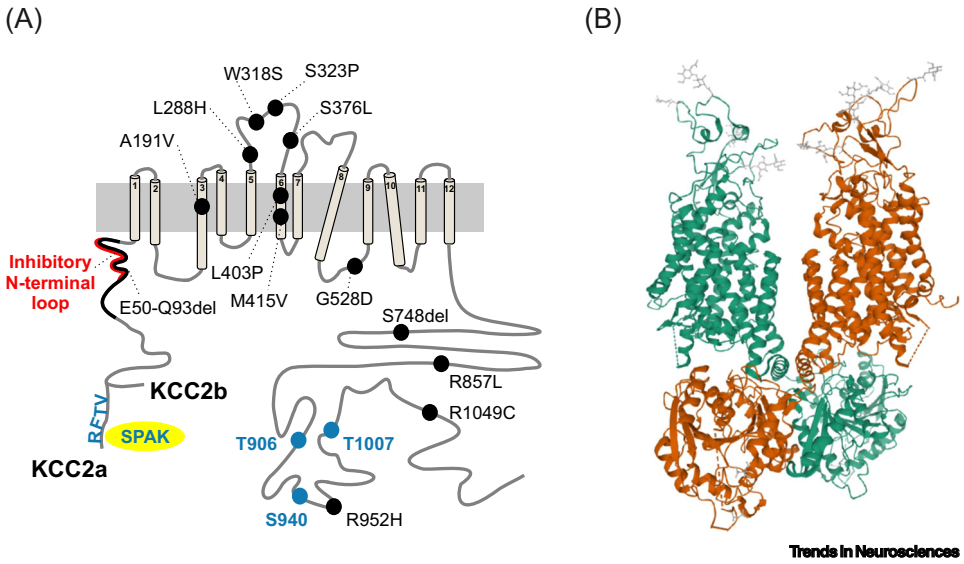

Figure 2. KCC2 Structure, Illustrated Along with Important Regulatory Phosphoresidues and Known Human Mutations. (A) Schematic representation of KCC2 secondary structure, with known human mutations (black) and some of the important phosphorylation sites (blue). KCC2 has two variants, KCC2a and KCC2b, which are generated by transcription from independent promoters and differ in their first exon. Only the KCC2a isoform contains the conserved SPAK/OSR1 binding site (RFTV). The autoinhibitory N-terminal loop (A66–N83 in KCC2b [131] and V81–N107 in KCC2a [132], Box 1) (red) in the entry of the cytosolic vestibule functions as a blocker. The amino acid numbering in the figure is according to the human KCC2b isoform. (B) Cryo-electron microscopy (cryo-EM) structure of the human KCC2 dimer in which the two monomers are indicated in different colors; glycans are shown in gray. The dimer has a domain-swapped architecture in which the C terminus of one monomer is located beneath the transmembrane domain of the other one. The image was created with the Mol* molecular graphics tool [149] using human KCC2 atomic coordinates reported previously [131] and deposited in the Protein Data Bank (Research Collaboratory for Structural Bioinformatics [150]) under identification number 6M23. Broken lines represent regions that are poorly resolved in the cryo-EM structure.

Structural Role of KCC2 in the Generation and Function of Dendritic Spines

The steepest phase of cortical KCC2 upregulation in rats and mice as well as in humans coincides with the most intense period of both glutamatergic [77,78] and GABAergic synaptogenesis [78,79]. There is also a remarkable spatial coincidence between KCC2 upregulation, **spinogenesis**, and glutamatergic innervation [27,68,80], and it is now well established that KCC2 plays a structural, transport-independent role in the generation of dendritic spines and likely acts as a synchronizing factor in development of functionally excitatory and inhibitory synapses.

Depletion and overexpression of KCC2 in developing cortical and hippocampal pyramidal neurons have both been shown to perturb the formation of functional synaptic contacts, and to cause an aberrant, often filopodia-like spine morphology [6,9,81]. Importantly, the effects on spine morphology and functionality are mediated by the C-terminal domain (CTD) because the effects of full-length KCC2 are mimicked by overexpression of KCC2 constructs consisting only of the CTD [9,81] or by full-length KCC2 lacking the N-terminal domain (ΔNTD [6,9]). However, the effect of CTD overexpression depends on the experimental paradigm: in some cases, the structural effects of full-length KCC2 can be mimicked by CTD overexpression [9,10], whereas in other cases the CTD can act in a dominant negative manner, mimicking KCC2 downregulation [6,8,27]. Neither the CTD nor the ΔNTD construct is expected to be delivered to the plasma membrane. In fact, the KCC2 NTD is required for KCC2 membrane insertion [82], suggesting that the ΔNTD construct may be retained in intracellular vesicles. Thus, the structural effects of ΔNTD and CTD may be mediated by the intracellularly exposed CTD in vesicles, or by free cytosolic CTD, respectively.

Box 3. Phosphorylation of KCC2: Role of Splice Variants

As explained in the main text, the activity of K-Cl cotransporters is strongly inhibited by phosphorylation of two C-terminal threonine residues (T906 and T1007 according to human KCC2b notation). KCC2 T906 phosphorylation site is located within a consensus sequence that is found in all KCCs. Interestingly, a similar sequence is also present in the N terminus of NKCC1, but, in contrast to KCCs, phosphorylation increases the transport activity. Phosphorylation of the N-terminal threonines in NKCC1 is mediated by the WNK-SPAK/OSR1 signaling cascade and depends on the N-terminal SPAK-binding motif positioned upstream of the phosphorylation site [138].

The cytoplasmic N-terminal parts of the K-Cl cotransporters are subject to extensive alternative splicing. KCC2a and KCC2b splice variants differ only in their first exons that encode the most N-terminal regions (40 amino acids in KCC2a versus 17 amino acids in KCC2b), and thus the SPAK-binding motif is present in KCC2a but not KCC2b. Accordingly, in the heterologous HEK293 system, SPAK inhibits the activity of KCC2a [57,65,139] via binding to the KCC2a variant but not to KCC2b [139]. Thus, a surprising and largely overlooked fact is that, although the SPAK/OSR1 binding motif is conserved in the N-termini of most CCCs, it is not present at all in the major KCC2 splice variant, KCC2b. This raises the obvious question whether the N-terminal SPAK motif is dispensable for the regulation of KCC2 by the WNK-SPAK pathway.

As confirmed by recent cryo-EM [126–132] and X-ray crystal structure [140], CCCs exist as dimers, and both the cytoplasmic C-terminal and transmembrane domains are involved in dimerization. Thus, KCC2a heterodimerization with KCC2b *in vivo* [34] (and the tight interaction of the C-termini within dimers) might allow SPAK/OSR1 to phosphorylate the C-terminal threonines of both KCC2 variants in the heterodimer. This question should be addressed in future work.

Because of their independent promoters, the expression levels of KCC2a and KCC2b differ during development. The contribution of KCC2a to total KCC2 protein expression shows a dramatic decrease especially in the neocortex – in mice from ~65% at E14 to ~5% in the adult [29,31,34]. This fall in the relative KCC2a expression appears to be closely paralleled by the developmental decrease in T906/T1007 phosphorylation. Thus, it is tempting to speculate that KCC2a–KCC2b heterodimerization might act as an important mechanism to maintain K-Cl cotransport activity at low levels during early stages of neocortical development, at the time when KCC2 exerts its structural, antiapoptotic effects and promotes the survival of migrating projection neurons.

The molecular mechanisms underlying the structural role of KCC2 seem to converge on regulation of the actin cytoskeleton. In KCC2-deficient neurons, dendritic spines exhibit a large pool of stable actin, resulting in reduced spine motility [7]. The effect of KCC2 on actin dynamics is mediated by the interaction of KCC2 and the β isoform of Rac/Cdc42 guanine nucleotide exchange factor β-PIX, which through Rac1 GTPase, controls the phosphorylation of one of the major actin-regulating proteins, cofilin-1 [7,8]. Cofilin is well known for its ability to sever actin filaments and increase filament turnover [83], and cofilin-mediated changes in actin dynamics are permissive for the insertion of AMPA receptors (AMPARs) during chemically induced long-term potentiation (LTP) [84]. In line with this, KCC2 downregulation prevents LTP by decreasing activity-driven membrane delivery of AMPARs through enhanced cofilin phosphorylation [8].

KCC2 also interacts with the cytoskeleton-associated protein 4.1N [6], and this interaction hinders lateral diffusion of KCC2 [68] and AMPARs [27] in the vicinity of glutamatergic synapses, indicating that 4.1N may serve to tether KCC2 to the submembrane actin cytoskeleton. Further insights into the roles of interactions between KCC2 and 4.1N come from studies on a KCC2 mutant (C586A) that is unable to interact with 4.1N and lacks ion-transport activity. These experiments indicate that, although some of the structural effects of KCC2 overexpression, such as the increased spine density in cortical neurons [9,81] and perturbed early neural development of knockin mice [85] depend on this site, actin regulation through cofilin phosphorylation is independent of 4.1N [7].

KCC2 physically interacts with a variety of membrane proteins, including the GluK2 subunit of kainate receptors [86,87] and its auxiliary subunit Neto2 [88], Task-3 (KCNK9) leak potassium channels [89], and $GABA_B$ receptors [90], adding to the variety of ways in which KCC2 can influence synaptic function and neuronal excitability. KCC2 also interacts with the Na-K-ATPase

Box 4. KCC2 Mutations in Epilepsy

Seizures represent an outcome of highly synchronized neuronal activity which is often (but not always) associated with impaired inhibition. Mutations in the human *KCC2* gene, often biallelic heterozygous, have been linked to cases of febrile seizures (FS) [28], idiopathic generalized epilepsy (IGE) [122], and epilepsy of infancy with migrating focal seizures (EIMFS) [141–143].

Most KCC2 mutations identified are single amino acid substitutions scattered through KCC2 domains (see Figure 2A in main text). Discovered in patients with FS and IGE, the C-terminal substitutions R952H [28,122] and R1049C [122] (using human KCC2b notation) both reduce Cl^- extrusion, but seemingly via different mechanisms: R952H decreases KCC2 surface expression, whereas R1049C affects intrinsic transport rate without changes in surface expression [28,122]. Importantly, the R952H variant is unable to support the two known ion transport-independent functions of KCC2 – the maintenance of dendritic spines [28] and the antiapoptotic effect on cortical projection neurons in embryonic neocortex [10]. Interestingly, histidine is also found at position 952 of the naked mole rat KCC2 [144], where it may have been favored by natural selection as one of the striking energy-metabolic adaptations [145] of this eusocial mammal.

Eleven KCC2 mutations in total have been described in EIMFS patients [141–143]. Four are in the large extracellular loop 3, of which three (L288H, W318S, S323P) are located in a region comprising four cysteines that are indispensable for Cl^- extrusion [146] because they stabilize the structure via disulfide bond formation. This region has also several glycosylation sites which are important for membrane trafficking [4]. Some of the mutants (e.g., L288H) indeed demonstrate reduced glycosylation and surface expression [141], whereas others (e.g., S323P) do not [142].

Three more EIMFS mutations that impair Cl^- extrusion, but not surface expression, are found in the transmembrane segments TM3 (A191V) and TM6 (L403P, M415V) [141,142], which together with TM1 and TM10 coordinate the K^+ and Cl^- binding sites. Thus, these three mutations most likely affect the ion translocation process. Notably, Cl^- extrusion activity is completely blocked in the L403P mutant. The only other EIMFS mutation with such dramatic loss of function of K-Cl transport activity is G528D, which resides in the intracellular loop between TM8 and TM9 [141].

One of the EIMFS variants (E50-Q93del), that lacks 44 amino acids including the N-terminal inhibitory loop (Box 1), demonstrates strongly suppressed Cl^- extrusion activity, but intact surface expression. Moreover, two EIFMS mutations (S748del [142] and R857L [143]) have been found in the C terminus, but their impact on the surface expression and transport activity of KCC2 remains to be studied.

From an etiological point of view, it is noteworthy that the IGE and EIMFS mutations so far identified in the *KCC2* gene are also associated with autism and schizophrenia [121]. An intriguing idea which arises here is that loss of transport-independent functions of KCC2 might contribute to the pathogenesis of neurodevelopmental disorders by increasing apoptosis and interfering with spinogenesis during early cortical development.

[91,92] that may play a role in joint subcellular targeting (*cf* Figure 1) and perhaps in the formation of an ion-transport metabolon [37]. However, the identity of the Na-K-ATPase isoform (for instance whether it is α3, rather than α2 as suggested in [37]) has not been rigorously demonstrated.

The effects of KCC2 downregulation on spine morphology seem to depend on the stage of maturity, and only an increase in spine head diameter was seen following KCC2 knockdown in mature hippocampal neurons [27]. In the immature brain, spines are highly motile and often short-lived [93], whereas in adults they become more stable with a larger head diameter and a pronounced postsynaptic density [94]. It is thus likely that KCC2-regulated actin dynamics promote the development of network wiring in immature neurons, and promote network plasticity at later stages. Indeed, inducible overexpression of KCC2 in adult mice leads to enhanced spine formation rate during motor learning in layer V pyramidal neurons of the motor cortex [95].

In recent years it has become evident that a substantial fraction of spines are targets of GABAergic synapses [79,96]. These observations suggest that KCC2 may be involved in the regulation of intraspine chloride levels. Given the large cation fluxes that take place in spines during glutamatergic transmission [97], it would not be surprising to observe parallel Cl^- shifts mediated by KCC2 (see Outstanding Questions).

Transport-Inactive KCC2 Promotes Neuronal Survival during Early Neocortical Development

Two waves of developmental apoptosis sweep the neocortex: the first peaks at around embryonic day (E)14 in mice and regulates the survival of neuronal progenitors and neuroblasts, and the second at P2–P9 modulates the survival of differentiating neurons [98]. Although knockdown of KCC2 in migrating cortical interneurons does not alter their rate of apoptosis [99], little information has been available, until recently, on the possible involvement of KCC2 in apoptosis of migrating neocortical pyramidal neurons.

In cortico-hippocampal regions of mice, the earliest KCC2 mRNA and protein expression has been reported at E15.5 in neocortical pyramidal neurons using RNA-seq analysis [10] and western blots [21], and by immunohistochemistry of E18.5 pyramidal neurons and interneurons in the CA3 region [11,21,100]. In humans, KCC2-immunoreactive cells can be seen in the subplate as early as 16 PCW (postconception weeks) and in the cortical plate at 20 PCW [23,101].

Our group recently used a novel conditional KCC2 mouse model (*Kcc2*$^{lox/lox}$) where *in utero* electroporation of Cre recombinase results in deletion of *Kcc2* in individual migrating pyramidal neurons [10]. Conditional deletion of *Kcc2* at E14.5 in a subpopulation of layer II–IV pyramidal neurons increased the fraction of apoptotic neurons at E16.5 as detected by TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling) staining and cleaved caspase-3 (Cas-3) immunoreactivity. By contrast, no effect was seen on neuronal proliferation or migration. Strikingly, the KCC2-ablated neurons could be rescued from increased apoptosis equally well with full-length KCC2 and with transport-deficient truncated KCC2 constructs (CTD and ΔNTD; see earlier). Thus, plasmalemmal expression of KCC2 is not necessary for the CTD-mediated antiapoptotic effect, as is the case also with spinogenesis. As discussed earlier, in mature neurons loss of KCC2 results in enhanced phosphorylation and subsequent deactivation of cofilin [7,8], increasing the stability of actin filaments in dendritic spines. Similarly, the apoptosis elicited by KCC2 deletion was prevented by blocking cofilin hyperphosphorylation using *in utero* electroporation of a non-phosphorylatable cofilin mutant [10]. Changes in the phosphorylation and oxidation of cofilin are a prerequisite for its mitochondrial translocation, which triggers mitochondrial permeabilization and release of cytochrome *c* into the cytosol [102], a 'point of no return' in apoptosis.

Interestingly, neocortical Cajal–Retzius neurons (CRNs), that disappear almost entirely during the second wave of apoptosis, are devoid of KCC2 and persistently express NKCC1 [14,103]. Their apoptosis is promoted by GABAergic depolarization [104], whereas an opposite effect is seen following reduction of $[Cl^-]_i$ by genetic deletion or pharmacological blockade of NKCC1 [104] as well as by expression of the inward rectifier Kir2.1 [103]. This dependence of CRN apoptosis on activity is opposite to that of both projection neurons and interneurons, in which suppression of spontaneous network activity exacerbates apoptosis [105,106].

Concluding Remarks and Future Perspectives

The versatility of KCC2 is reflected by its wide range of functions that span both its canonical role as a K-Cl cotransporter and its noncanonical roles as a structural and antiapoptotic factor. It has become increasingly evident that this multifunctionality shows striking cell type- and developmental stage-dependent variations, including the signaling cascades that target KCC2, some of which are depicted in Figure 1. For instance, the molecular mechanisms whereby KCC2 is involved in the formation and dynamics of dendritic spines are not identical in the neocortex and hippocampus [81], and apparently are not present at all in cerebellar Purkinje neurons [107]. There are also cell type-specific differences with regard to the ion-transport functions of KCC2; notably, some CNS neurons, such as dopaminergic neurons in the substantia nigra, are devoid of KCC2 and regulate their $[Cl^-]_i$ by means of other transporters, albeit with lower efficacy [108].

Outstanding Questions

Does the (calpain-cleaved) C-terminal fragment of KCC2 have signaling functions of its own?

Most KCC2 antibodies target the C terminus. Is it possible that much of the intracellular KCC2 immunoreactivity reported in the literature reflects the presence of the C-terminal fragments, and not the full KCC2 molecule?

Do spines have transport-active KCC2? If they do, a high Na^+ load that replaces intraspine K^+ might reverse local K-Cl cotransport to an uptake mode.

Is plasmalemmal KCC2 able to switch between transport-active and transport-inactive (structural) modes?

To what extent is KCC2 activation based on trafficking or allosteric modulation of its intrinsic transport rate by (de)phosphorylation? How does this depend on context, such as developmental stage, disease, and type of neuron?

What is the physiological significance of the KCC2a splice isoform during brain development?

What are the modes of action of drugs known as 'KCC2 activators', such as CLP257? Do these drugs have an effect on spines or apoptosis?

Is downregulation of KCC2 associated with CNS disorders a maladaptive (disease-promoting) or an adaptive (protective) response? Are these two alternatives context-dependent (e.g., early versus chronic trauma)?

KCC2 is a candidate for gene therapy. Does enhanced Cl^- extrusion induced by KCC2 overexpression in cortical or hippocampal neurons lead to changes in spine number and morphology?

SLC12A5 belongs to the top 4.5% of human genes that are intolerant to genetic variation (http://chgv.org/GenicIntolerance/). Thus, *SLC12A5* mutations are likely to contribute to severe CNS disease phenotypes. What is the overall significance of the structural role of KCC2 in epilepsy phenotypes (Box 4) related to *SLC12A5* mutations?

Changes in the capacity for Cl^- extrusion and consequent maintenance of effective GABAergic inhibition during development and disease have received considerable attention, as described in this review. However, we would argue that solely qualitative observations on the up- and downregulation of KCC2 functionality – that abound in the literature – are limited in paving the path to significant conceptual insights into the roles of KCC2. Basic 'design principles' in physiological systems imply that, during evolution and ontogeny, capacities are matched to loads in a dynamic manner [109]. Thus, for instance, measurements of the steady-state E_{GABA} in quiescent neurons in slice preparations do not provide a meaningful estimate of the efficacy of Cl^- extrusion. This conclusion is based on *a priori* considerations of leak–pump relations [110] and on data obtained under conditions of experimental [26–28] or endogenous Cl^- load or endogenous Cl^- load.

In neurons *in vivo*, momentary changes in DF_{GABA} are controlled by the dynamics of cellular leak–pump relationships related to Cl^- and HCO_3^-, including the redistribution of these two anions across $GABA_A$Rs [1]. This type of short-term ionic plasticity can have important consequences for neuronal network functions. For instance, a recent study has shown that, in the spinal dorsal horn, a heightened synaptic drive which imposes a high Cl^- load leads to a rapid reduction in inhibition in lamina I neurons. This, in turn, brings about potentiation of upstream excitatory synapses, resulting in a modality-specific sensitization to thermal and mechanical stimuli [111]. At an even higher level of neuronal integration, in substantia nigra pars reticulata neurons, dynamic changes in DF_{GABA} have been implicated in behavioral decision making and olfactory learning [112,113]. Under pathophysiological conditions such as seizures, activity-dependent ionic shifts involving intraneuronal Cl^- and HCO_3^- as well as extracellular K^+ become major determinants of network events [1,37,114,115].

It has been recently observed that changes in the cellular mechanisms supporting learning and memory in the aging brain involve reorganization of excitatory signaling, whereby high synapse specificity is replaced by multi-input synapses [116]. A key factor here might be functional downregulation of KCC2 [117], and this would fit with the general principle that the dynamic ranges of physiological capacities (see earlier) tend to become narrower during aging and senescence. Age-dependent KCC2 downregulation may also explain the improvement of cognition that is achieved by reducing neuronal hyperexcitability with anticonvulsant agents in elderly patients with mild cognitive impairment (MCI) [118]. Whether KCC2 downregulation is involved in the age-dependent loss of dendritic spines [119] in cortical neurons is not known. Nevertheless, KCC2 may well turn out to be an important factor in the chain of events which leads from MCI to major neurodegenerative diseases, including Alzheimer's (e.g., [120]).

From a translational point of view, we call for wider recognition of the multifaceted roles of KCC2 in neurodevelopmental disorders (see Outstanding Questions). Recent studies point to malfunctions of both K-Cl cotransport as well as the actin-mediated effects of KCC2 in autism, schizophrenia, and epilepsy [28,121,122]. Clearly, the multifunctionality of KCC2 deserves more attention in work on putative therapeutic strategies based on KCC2-targeting drugs [123,124] and gene manipulation [125].

Acknowledgments

We thank Profs Biff Forbush and Juha Voipio for helpful discussions, Profs Biff Forbush and Christian A. Hübner for critical reading of an early version of the manuscript, and Dr Martin Puskarjov for constructive comments on an early draft. Our original research work was supported by grants from the Academy of Finland (grants 319237 and 294375 to K.K., 321698 to M.A.V.), the European Research Council (ERC-2013-AdG 341116 to K.K), the Sigrid Jusélius Foundation (to K.K.),

the Human Frontier Science Program (RGP0022/2013 to J.C.P.), the Fondation pour la Recherche Médicale (DEQ20140329539 to J.C.P.), European Research Area Network (ERANET)-Neuron (funded by the Agence Nationale de la Recherche, to J.C.P.), and the Fondation Française pour la Recherche sur l'Epilepsie – Fédération pour la Recherche sur le Cerveau (to J.C.P.).

Declaration of Interests

The authors declare no conflicts of interest.

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