Cation–chloride co-transporters in neuronal communication, development and trauma

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Electrical signaling in neurons is based on the operation of plasmalemmal ion pumps and carriers that establish transmembrane ion gradients, and on the operation of ion channels that generate current and voltage responses by dissipating these gradients. Although both voltage- and ligand-gated channels are being extensively studied, the central role of ion pumps and carriers is largely ignored in current neuroscience. Such an information gap is particularly evident with regard to neuronal Cl^- regulation, despite its immense importance in the generation of inhibitory synaptic responses by GABA- and glycine-gated anion channels. The cation–chloride co-transporters (CCCs) have been identified as important regulators of neuronal Cl^- concentration, and recent work indicates that CCCs play a key role in shaping GABA- and glycine-mediated signaling, influencing not only fast cell-to-cell communication but also various aspects of neuronal development, plasticity and trauma.

The cation–chloride co-transporters (CCCs) were first widely studied in terms of their physiological roles in the recovery of cell volume after swelling or shrinkage in hypotonic or hypertonic media [1]. In the CNS, the CCCs play a key role in intracellular Cl^- regulation [2]. In order for Cl^- to mediate currents across the resting membrane through GABA- or glycine-gated anion channels, intracellular Cl^- must be maintained away from electrochemical equilibrium. Cl^- transport mediated by CCCs is performed without any net charge movement across the membrane (i.e. the CCCs are electroneutral) and the transport cycles are driven without the direct hydrolysis of ATP (i.e. the CCCs are secondarily active). The energy for net transport is mainly derived from the cation gradients generated by the Na^+/K^+-ATPase. Other secondarily active transport proteins can also participate in overall neuronal Cl^- homeostasis, including Na^+-dependent and Na^+-independent anion exchangers (NDAE and AE, respectively), which exchange Cl^- for HCO_3^- [3,4] (Fig. 1).

The CCC gene family consists of three broad groups (Fig. 2a): Na^+–Cl^- co-transporters (NCCs), Na^+–K^+–$2Cl^-$ co-transporters (NKCCs) and K^+–Cl^- co-transporters (KCCs). Under normal physiological

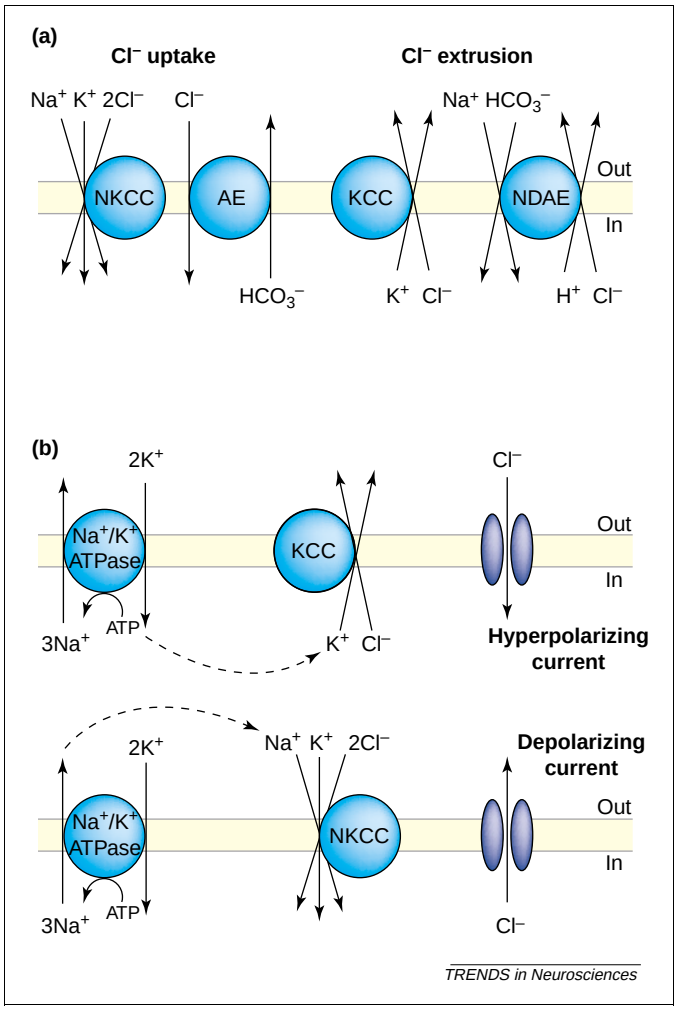

Fig. 1. Secondarily active Cl^- transporters and their basic modes of operation. (a) Under physiological conditions, Cl^- uptake is mediated by Na^+–K^+–$2Cl^-$ co-transporters (NKCCs) and Na^+-independent anion exchangers (AEs), whereas K^+–Cl^- co-transporters (KCCs) and Na^+-dependent anion exchangers (NDAEs) extrude Cl^-. As HCO_3^- is a substrate for AEs and NDAEs, these two transporters are also directly involved in the regulation of intracellular pH. Arrows indicate the direction of net transport. (b) The transport of Cl^- by secondarily active transporters is driven by the concentration gradients of cations. The K^+ gradient generated by the Na^+/K^+-ATPase fuels the extrusion of Cl^- by KCC, which results in an inwardly directed electrochemical gradient for Cl^- that generates hyperpolarizing currents across anion-permeable channels (top). Uptake of Cl^- by NKCCs (bottom) is driven mainly by the energy taken from the Na^+ gradient, and the resulting outwardly directed Cl^- electrochemical gradient permits depolarizing Cl^- currents.

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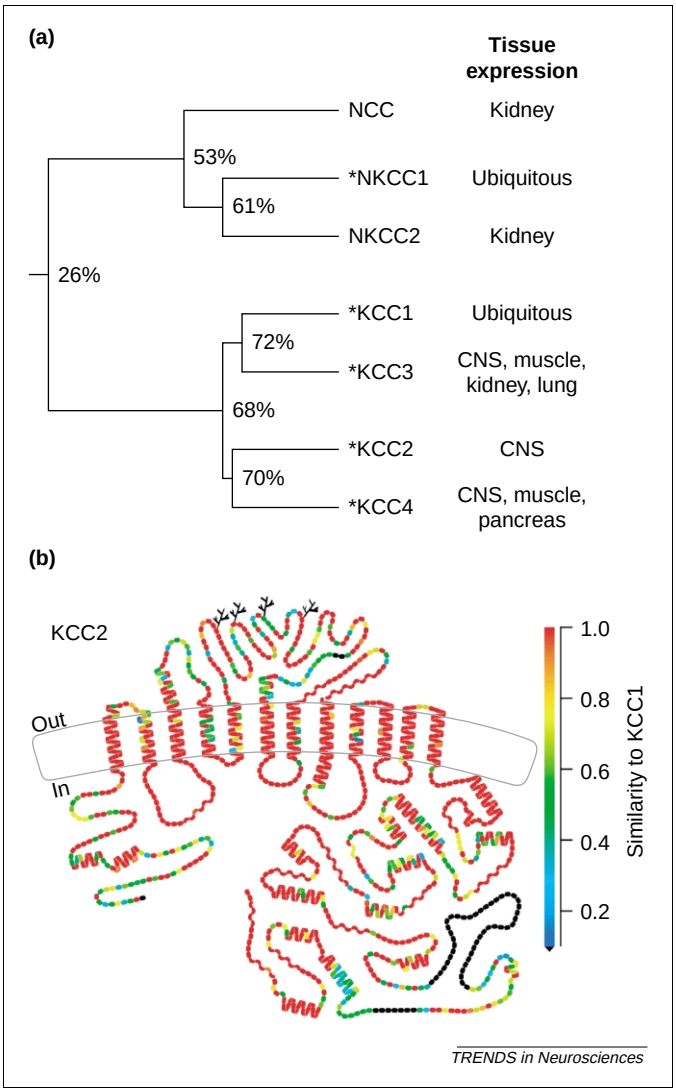

Fig. 2. Phylogenetic tree of human cation–chloride co-transporters (CCCs) and structure of the K^+–Cl^- co-transporter 2 (KCC2). (a) The percentage of identical residues between aligned protein sequences is shown at branch points. Human protein sequences were aligned and the phylogenetic tree constructed using CLUSTALW [100]. The predominant tissue expression is listed for each CCC and those identified in nervous tissue are indicated by asterisks. (b) Model of the KCC2 protein and similarity to KCC1. Putative transmembrane segments were predicted on the basis of hydropathy analysis using the Kyte–Doolittle algorithm [18,19]. Secondary structural elements were predicted using the PHD server [101] (helices represent α structures; wavy lines represent β structures). Rat KCC2 and rabbit KCC1 are compared with one another: red residues are identical between KCC2 and KCC1; black residues are absent from KCC1. Potential asparagine-linked glycosylation sites between putative transmembrane segments 5 and 6 are indicated with branched lines. This figure was kindly prepared by Bliss Forbush of Yale University, using DNAPLOT [18].

conditions, NCC and NKCC function in active Cl^- accumulation, whereas KCC operates in active Cl^- extrusion. As electroneutral transporters they are bi-directional and can carry out net ion influx or efflux, depending on the concentration gradients of the transported ions. NCC has not been found in nervous tissue [5].

In neurons, 'classical' synaptic inhibition mediated by anion-permeable $GABA_A$ and glycine receptors is associated with Cl^- influx, which tends to hyperpolarize the membrane [2]. Under some physiological (e.g. development) [6,7] and pathophysiological (e.g. trauma [8–11], epilepsy [12,13]) conditions, however, $GABA_A$ and glycine receptors mediate a depolarizing Cl^- efflux. Clearly, such long-term changes require profound alterations in neuronal Cl^- transport mechanisms. The reversal potential of $GABA_A$-mediated current and voltage responses (E_{GABA}) has often been taken as an estimate of the Cl^- equilibrium potential (E_{Cl}). However, this approach leads to substantial errors, especially in neurons with a low intracellular Cl^- concentration {$[Cl^-]_i$}, as neuronal pH regulation produces an electrochemical HCO_3^- gradient that drives a depolarizing bicarbonate current across $GABA_A$-receptor channels [2,14,15].

CCC isoforms and tissue distribution

The CCC proteins are glycoproteins with apparent molecular weights in the range of 120–200 kDa. They have a relatively small intracellular N terminus followed by 12 putative transmembrane segments, and a large intracellular C terminus that constitutes about half the protein (Fig. 2). One NCC, two NKCC and four KCC isoforms have been identified to date [5,16–22].

NKCC and KCC are of particular interest with regard to neuronal Cl^- homeostasis. NKCC1 is prominently expressed in the CNS where it is found not only in neurons, but also in glial cells, and in the choroid plexus and vascular endothelial cells [23–26]. NKCC1 is also widely expressed outside the CNS, whereas NKCC2 is principally expressed in the kidney [27]. A unique KCC isoform (KCC2) is exclusively expressed in mature neurons [19,28–33]. Indeed, there is ample evidence that KCC2 is largely responsible for the low $[Cl^-]_i$ in these cells. KCC1, KCC3 and KCC4 have been found in the nervous system, with a much more limited expression in neurons [18,19,28,32,34,35].

The role of CCCs in neuronal function has been studied using knockout mice [34,36,37]. Disruption of the gene encoding NKCC1 leads to a negative shift in E_{GABA} in dorsal root ganglion cells [38], as might be expected to happen upon a loss of active Cl^- accumulation [39,40]. Besides this, no major neuronal phenotype is evident in NKCC1-null mice. However, the KCC2 knockout mice die immediately after birth because of anomalous excitatory actions of GABA and glycine, which lead to deficits in motor functions, including those that control respiration [34] (Fig. 3). 'Hypomorphic' KCC2 gene-targeted mice with 20–30% of normal KCC2 levels are viable and have no obvious behavioral abnormalities [41], whereas mice with only 5–10% of KCC2 display spontaneous, generalized seizures and die shortly after birth [42].

Ion selectivity and pharmacology of CCCs

The Na^+ transport site of the CCCs can accept either Na^+ or Li^+, and the K^+ transport site can accept K^+, Rb^+, NH_4^+ or Cs^+, albeit with significantly different apparent affinities [43,44]. Complete replacement of intracellular K^+ by Cs^+ inhibits net Cl^- efflux via KCC2, and available data [45,46] indicate a selectivity sequence $K^+ \approx Rb^+ \gg Cs^+$. Thus, the frequent practice of using Cs^+ in whole-cell clamp experiments is bound to interfere with KCC2 function [45]. As to anions, only Br^- has been shown to replace Cl^- partially on NKCC and KCC [43,47].

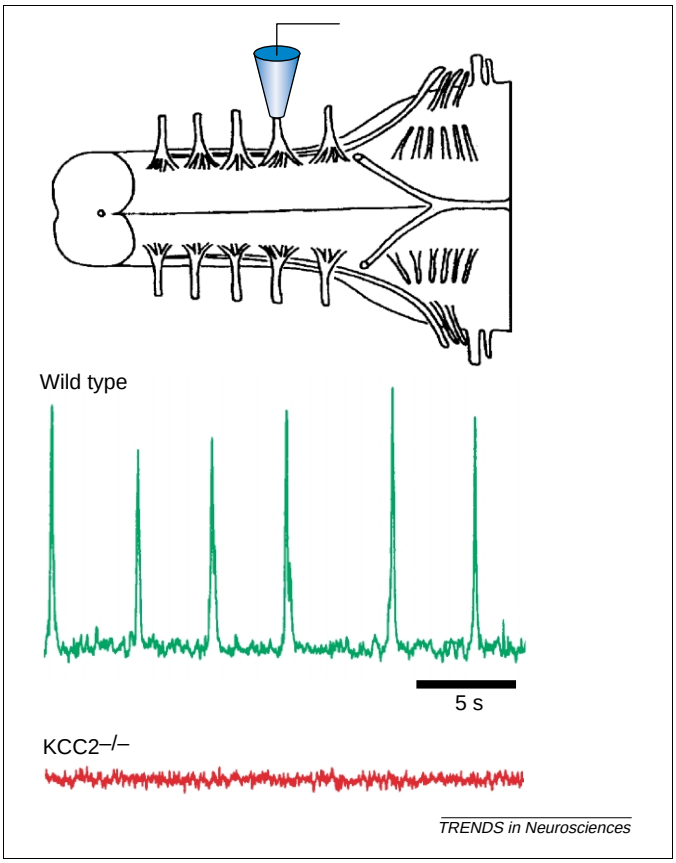

Fig. 3. Failure of hyperpolarizing inhibition abolishes respiratory rhythmogenesis in K^+–Cl^- co-transporter 2 (KCC2)-deficient mice. Rhythmic discharges related to 'virtual inspiration' can be recorded with an extracellular suction electrode from the ventral (motor) rootlets of the isolated perinatal brainstem–spinal cord preparation (top) [102]. In the KCC2-null mice, neuronal Cl^- extrusion is ineffective and the normally inhibitory transmission mediated by GABA and glycine is functionally excitatory, which prevents the generation of synchronous, patterned motor activity (compare the rectified and integrated traces in the middle and bottom panels). Traces are adapted, with permission, from Ref. [34].

The 'loop' diuretics furosemide and bumetanide inhibit NKCC and KCC. Furosemide has about equal potency for both NKCC and KCC ($K_i \sim 25–50\ \mu M$), whereas bumetanide has ~500-fold greater affinity for NKCC ($K_i \sim 0.1\ \mu M$) than for KCC ($K_i \sim 25–50\ \mu M$), and therefore low concentrations of bumetanide (2–10 μM) can be used to inhibit NKCC without significantly affecting KCC [18,22,48].

Neuronal Cl^- and volume regulation

Cl^- regulation

Regulation of neuronal $[Cl^-]_i$ by the CCCs requires that their net transport flux change appropriately in response to altered $[Cl^-]_i$. A mechanism to achieve rapid changes in transporter activity with changes in $[Cl^-]_i$ is to alter the phosphorylation state of the co-transporter protein or an accessory protein – this is often referred to as biochemical (allosteric, kinetic) regulation. For example, NKCC1 is very sensitive to changes in cell $[Cl^-]$ such that a fall in $[Cl^-]_i$ below a 'set-point' promotes direct phosphorylation and activation of the transporter protein, leading to restoration of $[Cl^-]_i$ [49–52]. By contrast, activation of red cell KCC involves a dephosphorylation event [53] and, once biochemically activated, KCC is highly dependent upon $[Cl^-]_i$, exhibiting higher transport rates at higher $[Cl^-]_i$ levels (see below) [54]. It is not known whether KCC2 is subject to significant biochemical regulation via phosphorylation-dependent mechanisms. The fact that NKCC1 and KCC2 are co-expressed in specific neurons [55–57] implies that these two proteins are functionally coupled in the control of neuronal $[Cl^-]_i$.

A co-transporter can also be sensitive to $[Cl^-]_i$ via an alteration in the thermodynamic force driving net transport. This type of 'thermodynamic regulation' has been hypothesized for KCC2 [58], which requires, first, that KCC2 be constitutively active. Second, KCC2 must be near thermodynamic equilibrium (Fig. 5). Third, the apparent affinity of KCC2 for intracellular Cl^- should be near the physiological level. Given these three conditions, the transport rate of KCC2 will be highly sensitive to changes in $[Cl^-]_i$. Indeed, the constitutive activity of KCC2 is in line with findings showing that the plasma membrane-associated KCC2 protein has a high turnover rate [59] that permits physiological and pathophysiological modulation of neuronal $[Cl^-]_i$ to take place via changes at the level of KCC2 expression.

Volume regulation

Biochemical regulation of K^+–Cl^- co-transport is a well-characterized mechanism that mediates regulatory volume decrease in response to swelling induced by hypotonic media [1]. However, physiologically induced neuronal swelling results from activity-dependent net uptake of ions, not from hypotonia. The important difference here is that $[Cl^-]_i$ decreases with hypotonic swelling but increases with activity-induced swelling.

Volume changes are known to modulate intrinsic neuronal firing properties [60], NMDA receptors [61] and ephaptic coupling (signaling via extracellular field effects) [62]. Several studies have reported dramatic swelling of dendrites in response to intense excitatory input [61,63,64]. Because charging the membrane capacitance to any relevant voltage requires very small movements of net charge, activity-induced swelling must reflect a simultaneous influx of both cations and anions (i.e. Na^+ and Cl^-; compare with Ref. [65]), as takes place during simultaneous activation of postsynaptic $GABA_A$ and glutamate receptors. In addition, extrasynaptic $GABA_A$ receptors, which are sensitive to 'ambient' (μM) levels of GABA [66] are also likely to mediate a large Cl^- load during neuronal excitation, which could explain the finding that in the rat hippocampus KCC2 is expressed at high levels close to excitatory inputs [67]. Irrespective of the underlying mechanism, a swelling-associated increase in $[Cl^-]_i$ provides a favorable driving force for net extrusion of Cl^- and K^+ by KCC2, which will assist in the recovery from swelling. The isoform KCC4 (initially referred to as 'KCC3' in Ref. [21]) is genuinely volume sensitive – that is, biochemically activated by swelling, but it shows little expression in the mature brain [47]. Isoform KCC3 is not volume sensitive [68].

Modulation of GABA-mediated transmission by CCCs

GABA-mediated responses in the adult CNS are not static, and they can undergo pronounced changes in amplitude

and polarity in response to both physiological and pathophysiological influences, which lead to alterations in neuronal $[Cl^-]_i$ and $[HCO_3^-]_i$ [2]. The time-frame of this type of ion-based plasticity of GABA-mediated transmission can vary tremendously.

Development

In immature neurons, $GABA_A$ receptor-mediated responses are depolarizing and lead to activation of voltage-dependent Ca^{2+} channels (VDCCs) and $[Ca^{2+}]_i$ transients that have a central role in neuronal differentiation, growth and maturation [7,69–71]. In rat hippocampal pyramidal neurons, $GABA_A$ receptor-mediated currents are depolarizing (often excitatory) at birth, but E_{GABA} becomes progressively more negative during early postnatal maturation, indicating a decrease in $[Cl^-]_i$ during development [6,72]. NKCC1 is present in early development and appears to be the primary transporter responsible for the elevated $[Cl^-]_i$ and depolarizing action of GABA in neonatal neurons [10,26,33,73–75]. By contrast, KCC2 exhibits low levels of expression in hippocampal, cortical and retinal neurons in rat neonates, but shows a steady increase in expression until the end of the second postnatal week (Fig. 4), which leads to the negative shift in E_{GABA} [28,31,75,76].

Various CCCs exhibit distinct spatiotemporal patterns of expression in the embryonic rodent brain, suggesting distinct roles for the various transporters in neuronal proliferation, migration and maturation [33] (see also Ref. [68]). Howard *et al.* [37] have recently shown that mutations in the SLC12A6 gene locus (solute carrier family 12, member 6), which encodes KCC3, causes peripheral sensorimotor neuropathy with agenesis of the corpus callosum in humans and mice.

A major question surrounding the developmental negative shift in GABAergic responses is what controls the transformation. A recent report argues that GABA itself controls developmental expression of KCC2 [77]. It has also been shown that the activation of KCC2 in cultured neurons is dependent upon growth factor-mediated tyrosine kinase phosphorylation [78]. An intriguing issue to

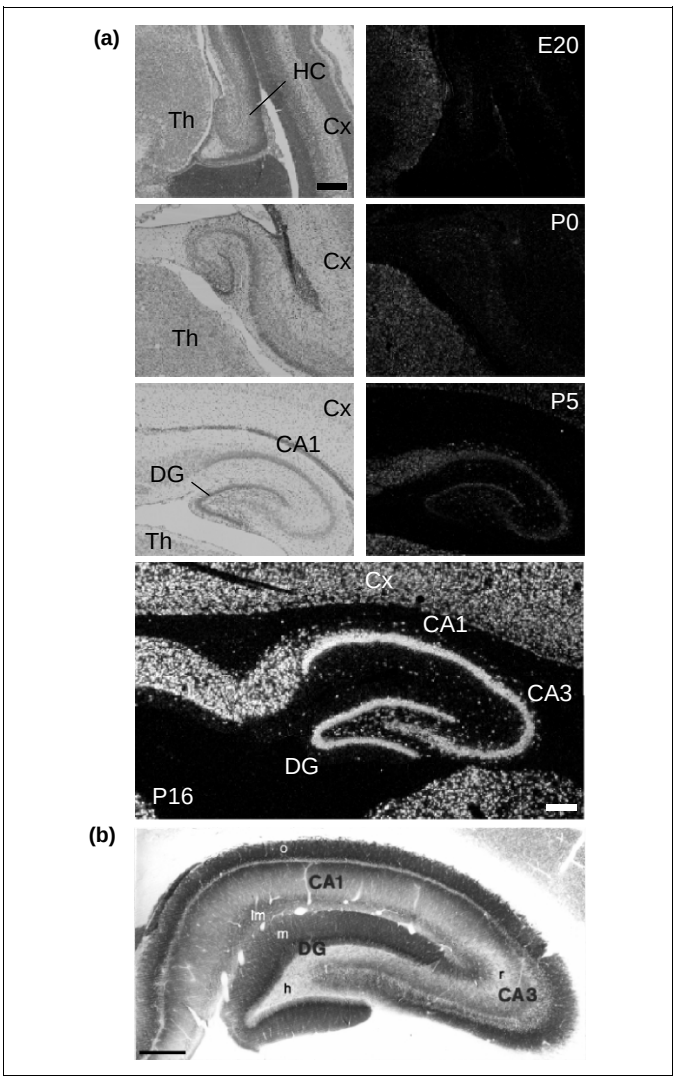

Fig. 4. Expression patterns of K^+–Cl^- co-transporter 2 (KCC2) in the rat hippocampus. (a) Developmental expression of KCC2 mRNA [28] as seen using *in situ* hybridization in sagittal sections, with bright-field images on the left and dark-field images on the right and in the lower panel. At the final embryonic stage, embryonic day (E) 20, KCC2 is seen in the thalamus (Th) but not in the hippocampus (HC) or neocortex (Cx). At birth [postnatal day (P) 0], weak mRNA labeling is evident in stratum pyramidale and subiculum of the hippocampus. The *in situ* hybridization signal is more intense at P5, when KCC2 is also expressed in the dentate gyrus (DG) and cortex. At P16, the pattern is similar to that in the adult. Scale bars, 200 μm for E20–P5; 400 μm for P16. (b) Distribution of KCC2 protein (immunoperoxidase staining) in the adult rat hippocampus [67]. Protein levels are highest in dendritic areas, as seen in stratum oriens (o) of the CA1 and CA3 areas as well as in stratum moleculare (m) of the dentate gyrus, with moderate staining in stratum lacunosum-moleculare (lm). The hilus (h) and CA3 stratum radiatum (r) show low levels of expression, and even less is seen in the soma layers. Scale bar, 600 μm. Reproduced, with permission, from Ref [28], © (1999) Nature Publishing Group (http://www.nature.com), and Ref. [67].

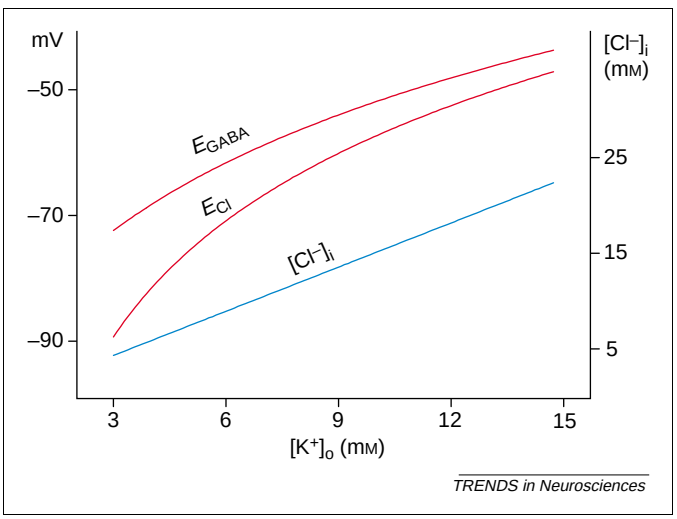

Fig. 5. K^+–Cl^- co-transporter 2 (KCC2) can mediate an effective coupling between extracellular K^+ and intracellular Cl^- concentrations {$[K^+]_o$ and $[Cl^-]_I$, respectively}. In a cell with constitutively active KCC2, an increase in $[K^+]_o$ from its physiological level (3 mM) is immediately seen as an increase in neuronal $[Cl^-]_i$. In the graph, $[Cl^-]_i$, the equilibrium potential of Cl^- (E_{Cl}), and the reversal potential of the $GABA_A$ receptor-mediated current (E_{GABA}) have been calculated assuming that KCC2 is at thermodynamic equilibrium: $[Cl^-]_i = [Cl^-]_o[K^+]_o/[K^+]_I$ (i.e. $E_{Cl} = E_K$). The difference between E_{GABA} and E_{Cl} is due to the bicarbonate permeability of $GABA_A$-receptor channels that makes E_{GABA} significantly more positive than E_{Cl}, especially at low levels of $[Cl^-]_i$ [14,15]. The data are based on the Nernst, Henderson–Hasselbalch and Goldman–Hodgkin–Katz voltage equations, with parameters for normal physiological conditions: $[Cl^-]_o$, 135 mM; $[K^+]_I$, 87 mM (corresponding to an E_K of −90 mV when $[K^+]_o$ is 3 mM); $[HCO_3^-]_I$, 16 mM; $[HCO_3^-]_o$, 25 mM (i.e. $pH_i = 7.2$ and $pH_o = 7.4$, 5% CO_2); temperature 37 °C. The relative permeability of $GABA_A$ receptors to HCO_3^- versus Cl^- is 0.3 [2].

investigate is whether the developmental shift in E_{GABA} is promoted by brain-derived neurotrophic factor (BDNF).

Circadian and neuroendocrine rhythms

The suprachiasmatic nuclei (SCN) in the hypothalamus represent the control point for circadian rhythms in mammals [79,80]. It has been shown that GABA reversibly increases the firing rate of SCN neurons during the day with the opposite action at night, indicating a diurnal oscillation of $[Cl^-]_i$ in SCN neurons [81–83]. These studies suggest that the 'clock' genes in SCN neurons control the expression of CCCs [82].

In a similar manner, steroid-mediated sexual differentiation of the mammalian brain also appears to be based on mechanisms involving excitatory versus inhibitory actions of GABA [84]. GABA exerts an excitatory action on gonadotropin-releasing hormone (GnRH)-secreting immortalized neurons (GT1–7 cells), which have a non-differentiated phenotype [85]. Although a switch to a hyperpolarizing response at puberty would require active Cl^- extrusion [86], most adult GnRH neurons appear to maintain a high $[Cl^-]_i$ and are excited by GABA [87]. It is tempting to predict that long-term endocrine rhythms (e.g. reproductive cycles and hibernation) are influenced by concurrent changes in CCC gene expression in neuroendocrine cells.

Epileptogenesis

There are various *in vitro* models of epilepsy where the key feature is the generation of $GABA_A$ receptor-dependent responses that are functionally excitatory and associated with elevated extracellular K^+ concentration $\{[K^+]_o\}$. Here, it is of particular interest that a moderate increase in the level of $[K^+]_o$ is known to trigger epileptiform events in hippocampal slices [88,89]. As KCC2 has a high transport affinity for external K^+, elevation of $[K^+]_o$ is followed by an influx of Cl^- via KCC2 [45,58,90], and the consequent positive shift in E_{GABA} (Fig. 5) can lead to compromised inhibition and to increased gross excitability. This would be consistent with the anticonvulsant properties of diuretic compounds [91,92] that block CCCs, including KCC2. However, under some conditions GABA-mediated $[K^+]_o$ transients excite cells by a direct depolarization rather than by a positive shift in E_{GABA} [93,94].

BDNF is known to play a central role in the genesis and establishment of epileptic activity [95], and a recent study has shown that BDNF mediates a downregulation of KCC2, leading to compromised neuronal Cl^- regulation in

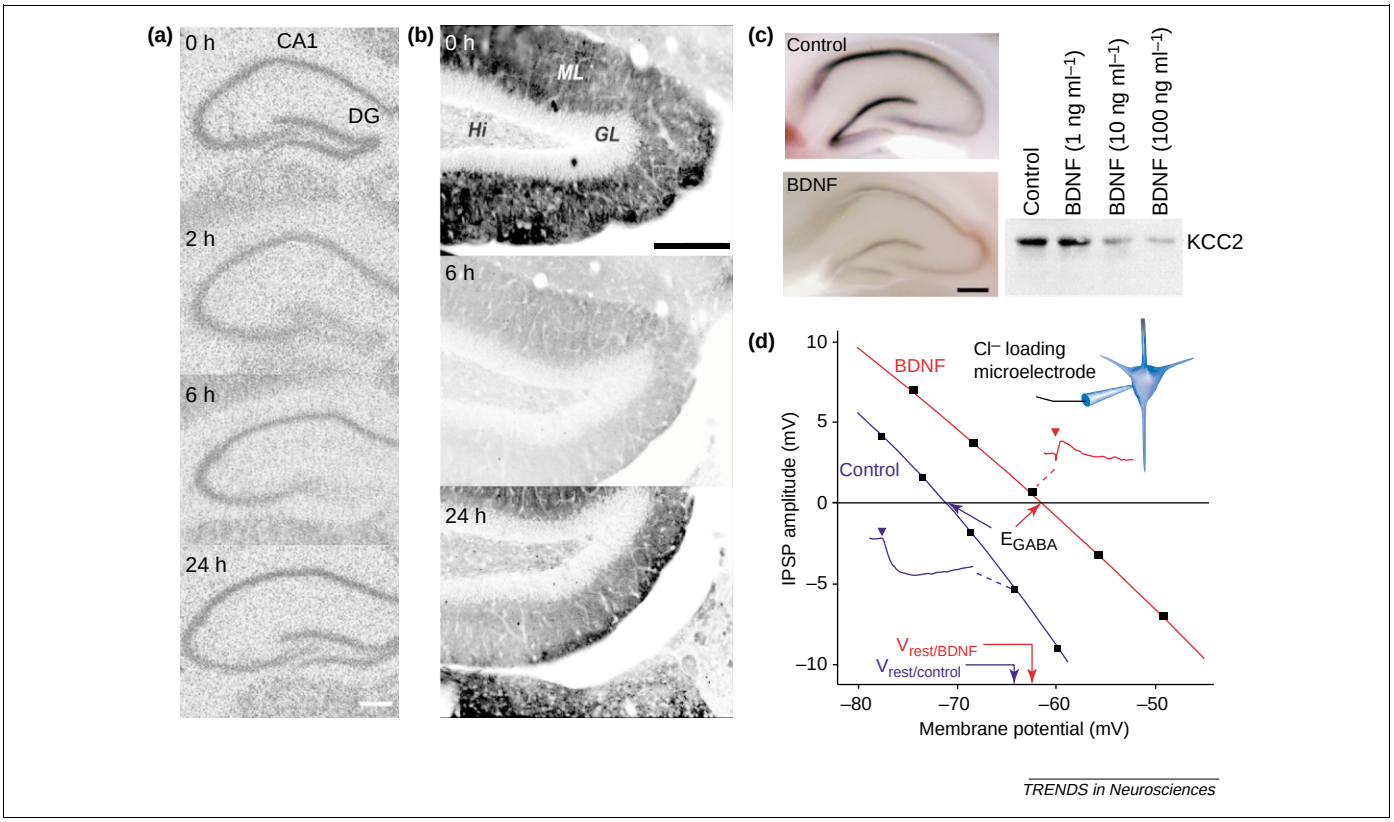

Fig. 6. Downregulation of K^+–Cl^- co-transporter 2 (KCC2) expression by epileptic activity and trkB receptor activation by brain-derived neurotrophic factor (BDNF). (a) Kindling-induced generalized seizures lead to a decrease in KCC2 mRNA, as seen in *in situ* hybridization of transverse sections of mice hippocampi made within 2 or 6 h of the last seizure. The decrease in KCC2 transcription is particularly conspicuous in the dentate gyrus (DG) and is paralleled by a decline in protein levels, as seen in the strong decrease in KCC2 immunoreactivity (b), especially in the molecular layer (*ML*) and hilus (*Hi*). A partial recovery of the KCC2 mRNA and protein levels is observed at 24 h. Abbreviation: *GL*, granule cell layer. Scale bars: 350 μm in (a), 100 μm in (b). (c) Exogenous BDNF decreases the expression of KCC2, as seen in immunostaining of acute rat hippocampal slices (left) and in western blots from organotypic slice cultures (right). Scale bar, 1 mm. (d) The BDNF-induced loss of KCC2 leads to an impairment of the neuronal Cl^- extrusion capacity (red). The inhibitory postsynaptic potential (IPSP) amplitudes were measured from CA1 cells (20–25 ms after stimulation) in acute rat hippocampal slices with a sharp microelectrode that contained 0.5 M Cl^- to impose a defined intracellular Cl^- load. Although the control CA1 cells are able to maintain an IPSP reversal potential ($E_{IPSP} = E_{GABA}$) that is more negative than the resting potential ($V_{rest/control}$) (blue), those exposed to BDNF have a significantly depolarized reversal level with respect to the resting potential ($V_{rest/BDNF}$). Modified, with permission, from Ref. [96], © The Rockefeller University Press.

hippocampi of kindled mice (Fig. 6) [96]. Interestingly, in human hippocampal slices from individuals with temporal lobe epilepsy, depolarizing GABA-mediated responses in a subgroup of pyramidal neurons in the subiculum were found to be involved in the generation of spontaneous interictal-like discharges [12,13].

Trauma

Traumatic insults to the brain (concussion, epileptic seizures or hypoxia–ischemia) cause excessive release of glutamate that leads to elevated $[Ca^{2+}]_i$, and to excitotoxic cellular damage and death [97]. Intriguingly, $GABA_A$ receptor-mediated responses after trauma have been shown to be depolarizing, which appears to result from an increase in $[Cl^-]_i$ [8–11]. A dramatic loss of KCC2 protein (60–80% of total) has been observed after either sustained interictal-like activity induced in hippocampal slices in the absence of Mg^{2+} [59] or after focal cerebral ischemia–excitotoxicity [98] and in an *in vivo* kindling model of epilepsy [96] (Fig. 6). It is noteworthy that no change in NKCC1 transcript or protein was observed in two trauma models [11,98] (but see Ref. [99]). Thus, the elevation in neuronal $[Cl^-]_i$ and depolarizing action of $GABA_A$ receptors that occur after trauma appear to be mainly caused by alterations in KCC2 expression. An elevated $[Cl^-]_i$ in dorsal motoneurons after axonal injury was also accompanied by a significant reduction in KCC2 transcript [11].

What is the functional significance of the depolarizing action of GABA after injury? Although it appears that a loss or reduction in hyperpolarizing inhibition would be detrimental and contribute to excitotoxicity, $GABA_A$ receptor-mediated depolarizations are ubiquitous in immature neurons. Thus, trauma might cause neurons to revert to a state where they have greater developmental flexibility, which is perhaps needed in sprouting and retargeting.

Concluding remarks

The CCCs are an integral part of the molecular machinery that underlies electrical signaling in the brain. While the study of brain CCCs is still in its infancy, the data reviewed here show that these ion transporters are intrinsically involved in mechanisms that control neuronal growth and maturation, synaptic development and plasticity, neuroendocrine functions, and the generation of network rhythms. The possibility of constructing genetically modified animals with mutations of individual transporters and with endogenous ion indicators for functional imaging [72] will provide exciting prospects for further studies of brain CCCs at molecular and systemic levels. In view of the key role of these transporters in various pathophysiological manifestations, such as epilepsy and trauma, this protein family holds much promise as a target for the development of novel drugs.

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